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| REGISTRATION REPORT  Part B  Section 5  Analytical Methods  Detailed summary of the risk assessment |
| Product code: IN233C1560  Product name(s): AVTAR  Chemical active substances:  Prothioconazole, 250 g/L Difenoconazole, 130 g/L |
| Central Zone  Zonal Rapporteur Member State: Poland |
| CORE ASSESSMENT  (Authorisation – Article 33) |
| Applicant: XXXX  Submission date: January 2022  Evaluation date: June 2023  zRMS Finalization date: February 2024 |

Version history

|  |  |
| --- | --- |
| When | What |
| January 2022 | First version of the document according to Article 33 of Regulation (EC) 1107/2009 |
| June 2023 | Version evaluated by zRMS PL |
| September 2023 | RR amended by zRMS after comments |
| November 2023 | Version updated following zRMS reply of the commenting table |
| February 2024 | Final RR amended by zRMS on residue methods. |

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# Analytical methods

## Conclusion and summary of assessment

Sufficiently sensitive and selective analytical methods are available for the active substances and relevant impurities in the plant protection product.

Noticed data gaps are:

* No data gap

Sufficiently sensitive and selective analytical methods are available for all analytes included in the residue definitions.

The dRR submitted was not rewritten by the zRMS. ThezRMS text/corrections is on grey background/grey boxes within the entire report.

~~The ILV studies should be finalized and completed in post registration~~. Confirmatory methods for MS/MS detection methods are not necessary.

Noticed data gaps are:

According to the current requirements (Regulation (EU) 284/2013) - ILV has to be provided by the applicant for difenoconazole and prothioconazole in drinking water asap in post registration.

According to the zRMS conclusion, the ILV for drinking water will be provided in the post registration

September 2023: the applicant informs that missing ILVs are ready for submission and zRMS proposes the post registration mode for it. The applicant informs about availability of the studies previously provided as drafts as well as not submitted [“*Validation of the Analytical Method for the Determination of Prothioconazole-desthio Residues in Aqueous Samples coming from the Ecotoxicological tests*” (Garagna D., 2022a – Report No. CH-0949/2022 - KCP 5.1.2/025) and “*Difenoconazole 130 g/L + Prothioconazole 250 g/L EC- IN233C1560: Validation of the Analytical Method for the Determination of Difenoconazole and Prothioconazole Residues in Pollen and Nectar from Ecotoxicological Study*” (Garagna D., 2022b – Report No. CH-0223/2022 - KCP 5.1.2/026)].

B5 was updated in line with comments in the commenting table received on 19 September 2023.

The missing ILVs for determining *Difenoconazole, Prothioconazole and TDMs Residues* in various plant matrices and honey were provided – thus the agreed gaps were filled, the data evaluated and accepted.

| Commodity/crop | Supported/ Not supported |
| --- | --- |
| Cereals | Supported |
| OSR | Supported |
| Honey | Supported |

## Methods used for the generation of pre-authorization data (KCP 5.1)

### Analysis of the plant protection product (KCP 5.1.1)

#### Determination of active substance and/or variant in the plant protection product (KCP 5.1.1)

An overview on the acceptable methods and possible data gaps for analysis of prothioconazole and difenoconazole in plant protection product is provided as follows:

|  |  |
| --- | --- |
| Comments of zRMS: | The presented below, HPLC with UV detection analytical method has been validated according to EU Guidance SANCO/3030/99 rev.5. The method is acceptable for the simultaneous determination of prothioconazole and difenoconazole in the formulation IN233C1560. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.1/01 |
| Report | Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Validation of the Analytical Method for the Determination of the Active Ingredients Content  Urbani, M.  2021a  Report No. : CH – 0324/2021  + Amendment report No.1 to final report (Amdm1\_KCP 5.1.1/01) |
| Guideline(s): | Yes : EEC guideline SANCO/3030/99 rev. 5 dated 22/03/2019 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

Principle of the methods

The determination of the active ingredients (a.i.) is performed by HPLC using an external standard and UV detector.

The quantification of the active ingredients is achieved by calculating its concentration in the final solutions in respect to a linear calibration obtained using the working standard solutions prepared starting from the reference material.

Materials and methods

***1. Materials***

***1.A. Determination of prothioconazole and difenoconazole as active ingredients***

|  |  |
| --- | --- |
| HPLC: | Agilent 1260 System |
| Column: | Luna Phenyl-Hexyl 100 Å, 5 μm, 250 x 4.6 mm i.d. (Phenomenex) |
| Detector | UV/Vis operating at 244 nm |
| Column temperature | 20 °C |
| Eluent flow | 1.0 mL/min |
| Volume of injection | 10 µL |
| Total analysis time | 20 minutes |
| Retention time | Prothioconazole : about 10.4 minutes  Difenoconazole: about 14.2 minutes |
| Mobile phase: | Eluant A: Water, HPLC grade  Eluant B: Acetonitrile, HPLC grade  Eluant D: Phosphoric acid 85%, reagent grade  Eluant (isocratic) : A:B:D 35:35:10 |
| Test item: | Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560  Batch number : IND\_F032\_0321\_1 |
| Reference materials: | Difenoconazol, analytical standard,  Pyrity : 95.5%  Prothioconazole, analytical standard  Pyrity : 99.6% |
| Test substances: | Prothioconazole TC  CAS No. : [178928-70-6](https://commonchemistry.cas.org/detail?cas_rn=178928-70-6)  Batch No. : BCBT2374  Purity : 99.6 %  Expiry date: February 1, 2023  Difenoconazole 96% TECH  CAS No. : 119446-68-3  Batch No. : BCCD4900  Purity : 95.5 %  Expiry date: February 1, 2023  Placebo Difenoconazole 130 g/L + Prothioconazole 250 g/L  EC – IN233C1560  Batch No. : IN\_F032\_0321\_1  Expiry date: March 1, 2023 |

***2. Methods***

***2.A. Determination of prothioconazole and difenoconazole as active ingredients***

The analytical methods for the determination of prothioconazole and difenoconazole as active ingredients were first validated so as to comply with the Guidance Document SANCO/3030/99 rev.5 (dated 22/03/2019). The methods were validated in terms of linearity, precision, accuracy and specificity.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results (5 independently weighed samples of the test item). The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | The test was performed by spiking two aliquots of the Placebo with the Difenoconazole and Prothioconazole test substances, corresponding to additions of 100 % of the nominal concentration of each active ingredient.  ~~It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value.~~ In addition, it is important to note that the total recovery is calculated. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |

Validation - Results and discussions

Table 5.2‑1: Methods suitable for the determination of active substances prothioconazole and difenoconazole in plant protection product IN233C1560

|  | Prothioconazole  Content in PPP  250g/L (21.84%w/w) | Difenoconazole  Content in PPP  130g/L (11.36%w/w) |
| --- | --- | --- |
| Author(s), year | Urbani, M.  2021a | Urbani, M.  2021a |
| Principle of method | HPLC/UV | HPLC/UV |
| Linearity  (linear between  mg/L / % range of the declared content)  (correlation coefficient, expressed as r)  n = 5 | The calibration curve :   * was considered as valid over 137.42 – 412.27 g/L.   injected range from 54.03 to 162.10 µg/mL  was considered as valid over 12.01–36.02%w/w  Range is >>±20% of nominal concentration  Equation :  y = 1.49 \* x – 10.57  Correlation coefficient:  r² = 0.99738 | The calibration curve :   * was considered as valid over 68.31 – 204.94 g/L.   injected range from 26.86 to 80.58 µg/mL   * was considered as valid over 5.97 – 17.91%w/w   Range is >>±20% of nominal concentration  Equation :  y = 1.67 \* x – 6.11  Correlation coefficient:  r² = 0.99456 |
| Precision – Repeatability Mean  n = 5 independently weighed samples of the test item  (%RSD) | Mean value = 21.3 % w/w  %RSD = 1.43  %RSDr = 1.69  The Horrat value = 0.84 was lower than 1 and therefore the precision of the analytical method is considered acceptable. | Mean value = 11.1 % w/w  %RSD = 1.40  %RSDr = 1.89  The Horrat value = 0.75 was lower than 1 and therefore the precision of the analytical method is considered acceptable. |
| Accuracy  Placebo spiked 2 times at a single fortification level corresponding to 100 % of the nominal active ingredients content.  n = 5  (% Recovery) | **At Spike A level:**  % Recovery value : 100.46  **At Spike B level:**  % Recovery value : 102.66  **Mean recovery** : 101.6 %  %RSD: 1.04  For the active ingredient content ≥ 10 % w/w, recovery in the correct range (97 to 103 %) for each spike and mean can be considered acceptable. | **At Spike A level:**  % Recovery value : 97.60  **At Spike B level:**  % Recovery value : 98.34  **Mean recovery** : 98.0 %  %RSD: 0.35  For the active ingredient content ≥ 10 % w/w, recovery in the correct range (97 to 103 %) for each spike and mean can be considered acceptable. |
| Interference/ Specificity | For a.s. interference not >3% of total peak area for target analyte.  Therefore, analytical method results to be specific for prohioconazole as active substance in test item samples.  By using the conditions stated in the method, interferences can be avoided and the prothioconazole active ingredient can be reliably determined in test item formulation samples. | For a.s. interference not >3% of total peak area for target analyte.  Therefore, analytical method results to be specific for difenoconazole as active substance in test item samples.  By using the conditions stated in the method, interferences can be avoided and the difenoconazole active ingredient can be reliably determined in test item formulation samples. |
| Comment | No comments: the results obtained for prothioconazole comply with the requirements laid down in the Guidance Document SANCO/3030/99 rev. 5 | No comments: the results obtained for difenoconazole comply with the requirements laid down in the Guidance Document SANCO/3030/99 rev. 5 |

Conclusion

The analytical methods for the determination of the active substances prothioconazole and difenoconazole in the plant protection product IN233C1560 have been validated according to the Guidance Document SANCO/3030/99 rev. 5 (dated 22/03/2019): “Technical Active Substance and Plant protection products: Guidance for generating and reporting methods of analysis in support of pre and post registration data requirements for Annex (Section 4) of Regulation (EU) No. 283/2013 and Annex (Section 5) of Regulation (EU) No 284/2013”. Therefore, the analytical methods can be considered as acceptable.

#### Description of analytical methods for the determination of relevant impurities (KCP 5.1.1)

An overview on the acceptable methods and possible data gaps for analysis of relevant impurities in plant protection product is provided as follows:

|  |  |
| --- | --- |
| Comments of zRMS: | Study 1  The presented, below, GC with FID detection analytical method has been validated according to EU Guidance SANCO/3030/99 rev.5. The method is acceptable for the determination of the relevant impurity toluene in the formulation IN233C1560 at LOQ=0.00557%. |

**Study 1**

|  |  |
| --- | --- |
| Reference: | KCP 5.1.1/02 |
| Report | Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Validation of the Analytical Method for the Determination of Toluene as Relevant Impurity Content  Urbani, M.  2021b  Report No. : CH-0325/2021  + Amendment report No.1 to final report (Amdm1\_KCP 5.1.1/02) |
| Guideline(s): | Yes : EEC guideline SANCO/3030/99 rev.5 (dated 22/03/2019). |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The determination of the Toluene relevant impurity is performed by GC using an external standard and FID detector.  The quantification of Toluene is achieved by calculating its concentration in the final solutions in respect to a linear calibration obtained using the working standard solutions prepared starting from the reference material. |

|  |  |
| --- | --- |
| Comments of zRMS: | Study 2  The presented, below, HPLC with MS/MS detection analytical method has been validated according to EU Guidance SANCO/3030/99 rev.5. The method is acceptable for the determination of relevant impurity prothioconazole-desthio in the formulation IN233C1560 at LOQ=0.00523%. |

Study 2

|  |  |
| --- | --- |
| Reference: | KCP 5.1.1/03 |
| Report | Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Validation of the Analytical Method for the Determination of Prothioconazole-desthio as Relevant Impurity Content  Urbani, M.  2021c  Report No. : CH-0326/2021  + Amendment report No.1 to final report (Amdm1\_KCP 5.1.1/03) |
| Guideline(s): | Yes : EEC guideline SANCO/3030/99 rev.5 (dated 22/03/2019). |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The determination of the Prothioconazole-desthio relevant impurity is performed by HPLC using an external standard and MS/MS detector ESI positive in the MRM mode.  The quantification of Prothioconazole-desthio is achieved by calculating its concentration in the final solutions in respect to a linear calibration obtained using the working standard solutions prepared starting from the reference material. |

Materials and methods

***1. Materials***

***1.A. Determination of toluene as a relevant impurity (KCP 5.1.1/02)***

Toluene was assessed by GC/FID instrument with quantification by external standard (primary test) but for the confirmatory test, toluene was assessed by GC/MS/FID.

For the primary test

|  |  |
| --- | --- |
| GC: | Agilent 7890A System |
| Column: | GC column, HP-5, 30 m x 0.32 mm i.d., film thickness 0.25 μm (Agilent) |
| Detector | Flame ionization detector (FID) |
| Mobile phase: | Acetone – isocratic mode  Pesticide residue analysis |
| Analytical standards: | Toluene  CAS No. : 108-88-3  Batch No. : BCCB7930  Purity : 99.9 %  Expiry date: June 1, 2022 |

For the confirmatory test

|  |  |
| --- | --- |
| GC: | Agilent 7890A System |
| Column: | ZB-5MS UI, 30 m x 0.25 mm i.d., film thickness 0.25 µm (Agilent) |
| Detector | FID and EI MSD |
| Flow rate: | 2.1 mL/min |
| Injection: | Injection temperature: 220 °C  Volume of injection: 2µL |
| Carrier gas: | Helium (He), 21.869 psi (at 40 °C) |
| Retention time: | About 3.8 minutes |
| Total analysis time | 25 minutes |
| FID conditions: | Detector temperature: 300 °C  H2 Flow: 40 mL/min  Air Flow: 400 mL/min  Makeup Flow: 25 mL/min |
| EI MSD conditions: | Acquisition mode: Scan (from 30.0 to 500.0)  Solvent delay : 2.00 min  EM Voltage : 1553  MS Source : 230 °C  MS Quadrupole : 150 °C  MS OFF : from 6.0 minutes to 25.00 minutes |
| Mobile phase: | Acetone – isocratic mode  Pesticide residue analysis |
| Analytical standards: | Toluene  CAS No. : 108-88-3  Batch No. : BCCB7930  Purity : 99.9 %  Expiry date: June 1, 2022 |

***1.B. Determination of prothioconazole-desthio as a relevant impurity (KCP 5.1.1/03)***

The identity of Prothioconazole-desthio relevant impurity in the test item is confirmed using a HPLC/MS/MS technique, ESI positive and monitoring reaction mode (MRM). Since the analysis by HPLC/MS/MS gave quantification and identification data, the confirmatory test using another instrumental technique was not necessary.

|  |  |
| --- | --- |
| HPLC: | Agilent 1200 System |
| Column: | Zorbax Eclipse Plus C18, 3.5 μm, 100 x 4.6 mm i.d. (Agilent) |
| Mobile phase: | Water, HPLC grade  Acetonitrile, HPLC grade  Formic acid, high purity for mass spectroscopy  Ammonium formate, high purity (>99%) for mass spectroscopy |
| Analytical standards: | Prothioconazole-desthio  CAS No. : 120983-64-4  Batch No. : G1043839  Purity : 99.55 %  Expiry date: November 21, 2025 |

***2. Methods***

***2.A. Determination of toluene as a relevant impurity (KCP 5.1.1/02)***

The analytical methods for the determination of toluene as a relevant impurity were first validated so as to comply with the Guidance Document SANCO/3030/99 rev.5 (dated 22/03/2019). The methods were validated in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results (5 independently weighed samples of the test item). The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | The recovery test was performed by spiking the test item five times at low fortification level and two times at high fortification levels.  ~~It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value.~~  In addition, it is important to note that the total recovery is calculated. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

***2.A. Determination of prothioconazole as a relevant impurity (KCP 5.1.1/02)***

The analytical methods for the determination of prothioconazole as a relevant impurity were first validated so as to comply with the Guidance Document SANCO/3030/99 rev.5 (dated 22/03/2019). The methods were validated in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results (5 independently weighed samples of the test item). The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  % RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | The recovery test was performed by spiking the test item five times at low fortification level and two times at high fortification levels.  ~~It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value.~~ |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |
| Confirmatory of identity: | The identity of toluene relevant impurity in the test item is confirmed using a GC/MS with full scan analysis technique.  The identity of toluene was conducted injecting, in the GC/MS adjusted chromatographic conditions, the samples (wash (acetone), toluene reference material, test item, test item fortified at low level, test item fortified at high level) comparing the chromatograms in order to check possible cross contaminations. These solutions’ concentrations were in the range of this method, but their exact values are not reported, since they were not used in calculations. |

Validation - Results and discussions

Table 5.2‑2: Methods suitable for the determination of the toluene and prothioconazole-desthio as relevant impurities in plant protection product (PPP) IN233C1560

|  | Toluene  Maximum content in the technical material  5 g/kg in prothioconazole  +  5 g/kg in difenoconazole  Maximum content in PPP  2.0624 g/L (0.1802% w/w)\*  (1.3520 g/L coming from prothioconazole and  0.7103 g/L coming from difenoconazole) | Prothioconazole-desthio  Maximum content in the technical material  0.5 g/kg  Maximum content in PPP  0.1352 g/L (0.0118% w/w)\* |
| --- | --- | --- |
| Author(s), year | Urbani, M.  2021b | Urbani, M.  2021c |
| Principle of method | ***Primary test :***  GC/FID instrument with quantification by external standard  ***Confirmatory test*** :  GC/MS/FID | HPLC/MS/MS |
| Linearity  (linear between  mg/L)  (correlation coefficient, expressed as r)  n = 5 | The calibration curve :   * was considered as valid over 27.85 – 2784.71 µg/g.   (nominal (actual) content in PPP is 76.5 µg/g)  injected range from 0.28 to 27.85 µg/mL  Range is >>±20% of nominal concentration  Equation :  y = 83461 \* x - 10768  Correlation coefficient:  r² = 0.99995 | The calibration curve :   * was considered as valid over 10.45 – 522.64 µg/g.   injected range from 10.45 to 522.64 ng/mL  Equation :  y = 241 \* x + 4039  Correlation coefficient:  r² = 0.99964 |
| Precision – Repeatability Mean  n = 5  (%RSD) | %RSD = 3.67  %RSDr = 5.58  The Horrat value = 0.66 was lower than 1 and therefore the precision of the analytical method is considered acceptable. | %RSD = 5.13  %RSDr = 6.18  The Horrat value = 0.83 was lower than 1 and therefore the precision of the analytical method is considered acceptable.  The Prothioconazole-desthio relevant impurity was not detectable in repeatability test, the precision was determined via the recovery test with the lowest forfication level. |
| Accuracy  n = 5 (at low level)  n = 2 (at high level)  (% Recovery) | **At low level:**  (Spiked amount : 55.69 µg/g)  % Recovery : 102.47 - 121.38  Mean recovery : 111.6 %  **At high level:**  (Spiked amount : 556.94 µg/g)  % Recovery : 88.24 – 91.76  Mean recovery : 90.0 %  At low and high recovery levels, the relative standard deviation was lower than 10 %.  For relevant impurity content < 0.01%w/w, recovery in the correct range (70 to 130%) for low and high forfications levels can be considered acceptable. | **At low level:**  (Spiked amount : 52.26 µg/g)  % Recovery : 83.20 – 84.38  Mean recovery : 83.91 %  **At high level:**  (Spiked amount : 261.32 µg/g)  % Recovery : 91.36 – 94.52  Mean recovery : 92.94 %  At low and high recovery levels, the relative standard deviation was lower than 10 %.  For relevant impurity content < 0.01%w/w, recovery in the correct range (70 to 130%) for low and high forfications levels can be considered acceptable. |
| Interference/ Specificity | The analytical method results to be specific for Toluene relevant impurity in test item samples.  Therefore, by using the conditions stated in the method, interferences can be avoided and the Toluene relevant impurity can be reliably determined in test item formulation samples. | The analytical method results to be specific for Prothioconazole-desthio relevant impurity in test item samples.  Therefore, by using the conditions stated in the method, interferences can be avoided and the Prothioconazole-desthio relevant impurity can be reliably determined in test item formulation samples. |
| LOQ | ~~50.00~~ 55.7µg/g (as lowest fortification level of recovery)  ~~(or 0.50 µg/mL injected)~~ | ~~50.00~~ 52.3µg/g (as lowest forification level of recovery)  ~~(or 50.00 ng/mL injected)~~ |
| LOD | 12.50 µg/g  (or 0.13 µg/mL injected) | 5.00 µg/g  (or 5.00 ng/mL injected) |
| Confirmatory | The identity of Toluene relevant impurity in the test item is confirmed using a GC/MS with full scan analysis techique. | The identity of Prothio-desthio relevant impurity in the test item is confirmed using HPLC/MS/MS technique, ESI positive and monitoring reaction mode (MRM).  The confirmatory test using another instrumental technique was not necessary. |
| Comment | No comment: The result obtained for Toluene relevant impurity comply with the requirements laid down in the Guidance Document. | No comment: The result obtained for Toluene relevant impurity comply with the requirements laid down in the Guidance Document. |

\* based on max. content of pure active substances in the product (according to FAO limits in table 1.4-1) – 265 g/L of pure prothioconazole and 137.8 g/L of pure difenoconazole (270.4 g/L of prothioconazole TC and 142.1 g/L of difenoconazole TC)

Conclusion

The analytical methods for the determination of toluene and prothioconazole-desthio as relevant impurities in the plant protection product IN233C1560 have been validated according to the Guidance Document SANCO/3030/99 rev.5 (22/03/2019): “Technical Active Substance and Plant protection products: Guidance for generating and reporting methods of analysis in support of pre and post registration data requirements for Annex (Section 4) of Regulation (EU) No. 283/2013 and Annex (Section 5) of Regulation (EU) No 284/2013”. Therefore, the analytical methods can be considered as acceptable.

#### Description of analytical methods for the determination of formulants (KCP 5.1.1)

There are no relevant formulants in IN233C1560. Therefore no methods are required.

#### Applicability of existing CIPAC methods (KCP 5.1.1)

**Prothioconazole:**

The CIPAC code No. 745 has been assigned to prothioconazole. Prothioconazole is determined by reversed phase high performance liquid chromatography using UV detection at 254 nm and external standard calibration. This analytical method is usable for EC formulation. Moreover, the substance is listed in the current CIPAC Handbook : the Handbook O.

**Difenoconazole:**

The CIPAC code No. 687 has been assigned to difenoconazole, nevertheless the applicant is not aware of any CIPAC method for difenoconazole in formulation IN233C1560. Moreover, the substance is not listed in the current CIPAC Handbook and no drafts or provisional methods are available.

CIPAC methods for the analysis of the active substances in plant protection products apply only to a single active ingredient being present in a formulation.

### Methods for the determination of residues (KCP 5.1.2)

An overview on the acceptable methods and possible data gaps for analysis of residues of prothioconazole and difenoconazole for the generation of pre-authorization data is given in the following tables. In addition, the triazole metabolites, triazole derivative metabolites (TDMs), also have a validation method for the generation of pre-authorization data. For the detailed evaluation of studies it is referred to Appendix 2.

The tables 5.2-3 – 5.2-6 can be considered acceptable, ~~however there are some method that will be provided because they are ongoing.~~ Currently, these methods are finalized and presented in more details in Appendix 2.

Table 5.2‑3: Validated methods for the generation of pre-authorization data for prothioconazole

| Component of residue definition:  **prothioconazole** | | | | |
| --- | --- | --- | --- | --- |
| Matrix type | Method type | Method LOQ | Principle of method  (i.e. GC-MS or HPLC-UV) | Author(s), year / missing / EU agreed |
| High oil content (Rapeseed seeds)  (Residues) | Primary | 0.01 mg/kg | HPLC-MS/MS | Longhi, D., 2021a. Report No. : GLP-study-21-31 / Not evaluated at EU level yet  KCP 5.1.2/02 |
| ILV | 0.01 mg/kg | HPLC-MS/MS | ~~Ongoing~~  ~~See study plan~~:  ~~Rigamonti, E~~. Nichetti, S. 2022~~a~~ c / Report No. CH-1083/2021  KCP 5.1.2/~~16~~ 18 |
| Confirmatory | 0.01 mg/kg | HPLC-MS/MS | Longhi, D., 2021a. Report No. : GLP-study-21-31 / Not evaluated at EU level yet  KCP 5.1.2/02 |
| High oil content (Rapeseed whole plant)  (Residues) | Primary | 0.01 mg/kg | HPLC-MS/MS | Longhi, D., 2021a. Report No. : GLP-study-21-31 / Not evaluated at EU level yet  KCP 5.1.2/02 |
| ILV | 0.01 mg/kg | HPLC-MS/MS | ~~Ongoing~~  ~~See study plan~~:  ~~Rigamonti, E~~. Nichetti, S 2022~~b~~ d / Report No. CH-1084/2021  KCP 5.1.2/~~17~~ 19 |
| Confirmatory | 0.02 mg/kg | HPLC-MS/MS | Longhi, D., 2021a. Report No. : GLP-study-21-31 / Not evaluated at EU level yet  KCP 5.1.2/02 |
| High starch content (Wheat grain)  (Residues) | Primary | * 1. mg/kg | HPLC-MS/MS | Longhi, D., 2021a. Report No. : GLP-study-21-31 / Not evaluated at EU level yet  KCP 5.1.2/02 |
| ILV | 0.01 mg/kg | HPLC-MS/MS | ~~Ongoing~~  ~~See study plan~~:  ~~Rigamonti, E~~. Nichetti, S. 2022~~c~~ b / Report No. CH-1082/2021  KCP 5.1.2/~~18~~ 17 |
| Confirmatory | 0.01 mg/kg | HPLC-MS/MS | Longhi, D., 2021a. Report No. : GLP-study-21-31 / Not evaluated at EU level yet  KCP 5.1.2/02 |
| High starch content (Wheat straw)  (Residues) | Primary | 0.01 mg/kg | HPLC-MS/MS | Longhi, D., 2021a. Report No. : GLP-study-21-31 / Not evaluated at EU level yet  KCP 5.1.2/02 |
| ILV | 0.01 mg/kg | HPLC-MS/MS | ~~Ongoing~~  ~~See study plan~~:  ~~Rigamonti, E~~. Nichetti, S. 2022~~d~~ a / Report No. CH-1081/2021  KCP 5.1.2/~~19~~ 16 |
| Confirmatory | 0.01 mg/kg | HPLC-MS/MS | Longhi, D., 2021a. Report No. : GLP-study-21-31 / Not evaluated at EU level yet  KCP 5.1.2/02 |
| Water (reconstituted water and Agal growth medium)\*  Water (Elendt M’ medium)\*\*  (Ecotoxicology) | Primary | 0.05 mg/L\*  0.01 mg/L\*\* | HPLC/MS/QQQ | Garagna, D. 2021b. Report No. : CH-0227/2021 /Not evaluated at EU level yet  KCP 5.1.2/03 |
| Confirmatory | Not required | Not required | Not required |
| Soil  (Ecotoxicology) | Primary | 0.23 mg/kg | HPLC/MS | Garagna, D. 2021a. Report No. : CH-0235/2021 / Not evaluated at EU level yet  KCP 5.1.2/01 |
| Confirmatory | Not required | Not required | Not required |
| *Daphnia magna*  (Ecotoxicology) | Primary | 11.6 µg/L | HPLC/MS/QQQ | Noè, F. 2021a. Report No. : CH-0229/2021 /Not evaluated at EU level yet  KCP 5.1.2/06 |
| Confirmatory | Not required | Not required | Not required |
| *Pseudokirchneriella subcapitata*    (Ecotoxicology) | Primary | 11 µg/L | HPLC/MS/QQQ | Noè, F. 2021b. Report No. : CH-0230/2021 /Not evaluated at EU level yet  KCP 5.1.2/07 |
| Confirmatory | Not required | Not required | Not required |
| *Brachydanio rerio*  (Ecotoxicology) | Primary | 11.6 µg/L | HPLC/MS/QQQ | Noè, F. 2021c. Report No. : CH-0228/2021 /Not evaluated at EU level yet  KCP 5.1.2/08 |
| Confirmatory | Not required | Not required | Not required |
| *Eisenia fetida*  (Ecotoxicology) | Primary | 50.8 µg/kg | HPLC/MS/QQQ | Dini, R. 2021a. Report No. : CH-0239/2021 /Not evaluated at EU level yet  KCP 5.1.2/09 |
| Confirmatory | Not required | Not required | Not required |
| Collembola  (Ecotoxicology) | Primary | 50.8 µg/kg | HPLC/MS/QQQ | Dini, R. 2021b. Report No. : CH-0240/2021 /Not evaluated at EU level yet  KCP 5.1.2/10 |
| Confirmatory | Not required | Not required | Not required |
| *Bombus terrestris*  (Ecotoxicology – oral test) | Primary | 6.75 mg/L | HPLC/MS-QQQ | Ponti, B. 2021a. Report No. : CH-0234/2021 / Not evaluated at EU level yet  KCP 5.1.2/11 |
| Confirmatory | Not required | Not required | Not required |
| *Bombus terrestris*  (Ecotoxicology – contact test) | Primary | 0.21 mg/L | HPLC/MS-QQQ | Ponti, B. 2021a. Report No. : CH-0234/2021 / Not evaluated at EU level yet  KCP 5.1.2/11 |
|  | Confirmatory | Not required | Not required | Not required |
| *Hypoaspis (Geolaepaps) aculeifer*  (Ecotoxicology) | Primary | 50.8 µg/kg | HPLC/MS-MS | Dini, R. 2021c. Report No. : CH-0241/2021 / Not evaluted at EU level yet  KCP 5.1.2/12 |
| Confirmatory | Not required | Not required | Not required |
| *Apis mellifera* L*.*  (Ecotoxicology) | Primary | 6.57 mg/L | HPLC/MS-QQQ | Ponti, B. 2021b. Report No. : CH-0669/2021 / Not evaluated at Eu level yet  KCP 5.1.2/13 |
| Confirmatory | Not required | Not required | Not required |
| Stock solutions  (Ecotoxicology) | Primary | 1 mg/L | HPLC/MS-QQQ | Garagna, D. 2021c. Report No. : CH-0232/2021 / Not evaluated at EU level yet  KCP 5.1.2/04 |
| Confirmatory | Not required | Not required | Not required |
| Feed solutions  (Ecotoxicology) | Primary | 30 mg/L | HPLC/MS-QQQ | Garagna, D. 2021d. Report No. : CH-0668/2021 / Not evaluated at EU level yet  KCP 5.1.2/05 |
| Confirmatory | Not required | Not required | Not required |
| Pollen  (Ecotoxicology ) | Primary | 55.5 µg/kg | HPLC/MS-QQQ | Garagna, D. 2022b. Report No.: CH-0223/2022 / Not evaluated at EU level yet  KCP 5.1.2/26 |
| Confirmatory | Not required | Not required | Not required |
| Nectar  (Ecotoxicology) | Primary | 55.5 µg/kg | HPLC/MS-QQQ | Garagna, D. 2022b. Report No.: CH-0223/2022 / Not evaluated at EU level yet  KCP 5.1.2/26 |
| Confirmatory | Not required | Not required | Not required |
| Pollen  (Ecotoxicology – Tunnel test) | Primary | 55.5 µg/kg | HPLC/MS-QQQ | Garagna, D. 2022c. Report No.: 168191033 : Not evaluated at EU level yet  KCP 5.1.2/27 |
| Confirmatory | Not required | Not required | Not required |
| Nectar  (Ecotoxicology– Tunnel test) | Primary | 55.5 µg/kg | HPLC/MS-QQQ | Garagna, D. 2022c. Report No.: 168191033 : Not evaluated at EU level yet  KCP 5.1.2/27 |
| Confirmatory | Not required | Not required | Not required |

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| Comments of zRMS: | The comments has been accepted. |

Table 5.2‑4: Validated methods for the generation of pre-authorization data for prothioconazole-desthio metabolites in cereal straw

| Component of residue definition:  **Prothioconazole-desthio metabolites** | | | | |
| --- | --- | --- | --- | --- |
| Matrix type | Method type | Method LOQ | Principle of method  (i.e. GC-MS or HPLC-UV) | Author(s), year / missing / EU agreed |
| High starch content (Cereal straw)  (Residues) | Primary | 0.01 mg/kg | HPLC-MS/MS | Longhi, D., 2021c. Report No. : GLP-study-21-120 / Not evaluated at EU level yet  KCP 5.1.2/15 |
| ILV | 0.01 mg/kg | HPLC-MS/MS | ~~Ongoing~~  ~~See study plan~~:  ~~Rigamonti, E~~. Nichetti, S. 2022e / Report No. CH-10~~8~~91/2021  KCP 5.1.2/20 |
| Confirmatory | 0.01 mg/kg | HPLC-MS/MS | Longhi, D., 2021c. Report No. : GLP-study-21-120 / Not evaluated at EU level yet  KCP 5.1.2/15 |
| Aqueous solution test  (Ecotoxicology) | Primary | 10.6 µg/L | HPLC/MS-QQQ | Garagna, D. 2022a. Report No.: CH-0949/2022 / Not evaluated at EU level yet  KCP 5.1.2/25 |
| Confirmatory | Not required | Not required | Not required |

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| Comments of zRMS: | The update has been accepted. |

Table 5.2‑5: Validated methods for the generation of pre-authorization data for difenoconazole

| Component of residue definition:  **Difenoconazole** | | | | |
| --- | --- | --- | --- | --- |
| Matrix type | Method type | Method LOQ | Principle of method  (i.e. GC-MS or HPLC-UV) | Author(s), year / missing / EU agreed |
| High oil content (Rapeseed seeds)  (Residues) | Primary | 0.01 mg/kg | HPLC-MS/MS | Longhi, D., 2021a. Report No. : GLP-study-21-31 / Not evaluated at EU level yet  KCP 5.1.2/02 |
| ILV | 0.01 mg/kg | HPLC-MS/MS | ~~Ongoing~~  ~~See study plan~~:  ~~Rigamonti, E~~. Nichetti, S. 2022~~a~~ c / Report No. CH-1083/2021  KCP 5.1.2/~~16~~ 18 |
| Confirmatory | 0.01 mg/kg | HPLC-MS/MS | Longhi, D., 2021a. Report No. : GLP-study-21-31 / Not evaluated at EU level yet  KCP 5.1.2/02 |
| High oil content (Rapeseed whole plant)  (Residues) | Primary | 0.01 mg/kg | HPLC-MS/MS | Longhi, D., 2021a. Report No. : GLP-study-21-31 / Not evaluated at EU level yet  KCP 5.1.2/02 |
| ILV | 0.01 mg/kg | HPLC-MS/MS | ~~Ongoing~~  ~~See study plan~~:  ~~Rigamonti, E~~. Nichetti, S 2022~~b~~ d / Report No. CH-1084/2021  KCP 5.1.2/~~17~~ 19 |
| Confirmatory | 0.01 mg/kg | HPLC-MS/MS | Longhi, D., 2021a. Report No. : GLP-study-21-31 / Not evaluated at EU level yet  KCP 5.1.2/02 |
| High starch content (Wheat grain)  (Residues) | Primary | mg/kg | HPLC-MS/MS | Longhi, D., 2021a. Report No. : GLP-study-21-31 / Not evaluated at EU level yet  KCP 5.1.2/02 |
| ILV | 0.01 mg/kg | HPLC-MS/MS | ~~Ongoing~~  ~~See study plan~~:  ~~Rigamonti, E~~. Nichetti, S. 2022~~c~~ b / Report No. CH-1082/2021  KCP 5.1.2/~~18~~ 17 |
| Confirmatory | 0.01 mg/kg | HPLC-MS/MS | Longhi, D., 2021a. Report No. : GLP-study-21-31 / Not evaluated at EU level yet  KCP 5.1.2/02 |
| High starch content (Wheat straw)  (Residues) | Primary | 0.01 mg/kg | HPLC-MS/MS | Longhi, D., 2021a. Report No. : GLP-study-21-31 / Not evaluated at EU level yet  KCP 5.1.2/02 |
| ILV | 0.01 mg/kg | HPLC-MS/MS | ~~Ongoing~~  ~~See study plan~~:  ~~Rigamonti, E~~. Nichetti, S. 2022~~d~~ a / Report No. CH-1081/2021  KCP 5.1.2/~~19~~ 16 |
| Confirmatory | 0.01 mg/kg | HPLC-MS/MS | Longhi, D., 2021a. Report No. : GLP-study-21-31 / Not evaluated at EU level yet  KCP 5.1.2/02 |
| Water (reconstituted water and Agal growth medium)\*  Water (Elendt M’ medium)\*\*  (Ecotoxicology) | Primary | 0.05 mg/L\*  0.01 mg/L\*\* | HPLC/MS/QQQ | Garagna, D. 2021b. Report No. : CH-0227/2021 /Not evaluated at EU level yet  KCP 5.1.2/03 |
| Confirmatory | Not required | Not required | Not required |
| Soil  (Ecotoxicology) | Primary | 0.23 mg/kg | HPLC/MS | Garagna, D. 2021a. Report No. : CH-0235/2021 / Not evaluated at EU level yet  KCP 5.1.2/01 |
| Confirmatory | Not required | Not required | Not required |
| *Daphnia magna*  (Ecotoxicology) | Primary | 6.3 µg/L | HPLC/MS/QQQ | Noè, F. 2021a. Report No. : CH-0229/2021 /Not evaluated at EU level yet  KCP 5.1.2/06 |
| Confirmatory | Not required | Not required | Not required |
| *Pseudokirchneriella subcapitata*    (Ecotoxicology) | Primary | 5.9 µg/L | HPLC/MS/QQQ | Noè, F. 2021b. Report No. : CH-0230/2021 /Not evaluated at EU level yet  KCP 5.1.2/07 |
| Confirmatory | Not required | Not required | Not required |
| *Brachydanio rerio*  (Ecotoxicology) | Primary | 6.3 µg/L | HPLC/MS/QQQ | xxx2021c. Report No. : CH-0228/2021 /Not evaluated at EU level yet  KCP 5.1.2/08 |
| Confirmatory | Not required | Not required | Not required |
| *Eisenia fetida*  (Ecotoxicology) | Primary | 27.5 µg/kg | HPLC/MS/QQQ | Dini, R. 2021a. Report No. : CH-0239/2021 /Not evaluated at EU level yet  KCP 5.1.2/09 |
| Confirmatory | Not required | Not required | Not required |
| Collembola  (Ecotoxicology) | Primary | 27.5 µg/kg | HPLC/MS/QQQ | Dini, R. 2021b. Report No. : CH-0240/2021 /Not evaluated at EU level yet  KCP 5.1.2/10 |
| Confirmatory | Not required | Not required | Not required |
| *Bombus terrestris*  (Ecotoxicology – oral test) | Primary | 3.56 mg/L | HPLC/MS-QQQ | Ponti, B. 2021a. Report No. : CH-0234/2021 / Not evaluated at EU level yet  KCP 5.1.2/11 |
| Confirmatory | Not required | Not required | Not required |
| *Bombus terrestris*  (Ecotoxicology- contact test) | Primary | 0.11 mg/L | HPLC/MS-QQQ | Ponti, B. 2021b. Report No. : CH-0234/2021 / Not evaluated at EU level yet  KCP 5.1.2/11 |
| Confirmatory | Not required | Not required | Not required |
| *Hypoaspis (Geolaepaps) aculeifer*  (Ecotoxicology) | Primary | 27.5 µg/kg | HPLC/MS-MS | Dini, R. 2021c. Report No. : CH-0241/2021 / Not evaluted at EU level yet  KCP 5.1.2/12 |
| Confirmatory | Not required | Not required | Not required |
| *Apis mellifera* L*.*  (Ecotoxicology) | Primary | 3.56 mg/L | HPLC/MS-QQQ | Ponti, B. 2021b. Report No. : CH-0669/2021 / Not evaluated at Eu level yet  KCP 5.1.2/13 |
| Confirmatory | Not required | Not required | Not required |
| Stock solutions  (Ecotoxicology) | Primary | 1 mg/L | HPLC/MS-QQQ | Garagna, D. 2021c. Report No. : CH-0232/2021 / Not evaluated at EU level yet  KCP 5.1.2/04 |
| Confirmatory | Not required | Not required | Not required |
| Feed solutions  (Ecotoxicology) | Primary | 30 mg/L | HPLC/MS-QQQ | Garagna, D. 2021d. Report No. : CH-0668/2021 / Not evaluated at EU level yet  KCP 5.1.2/05 |
| Confirmatory | Not required | Not required | Not required |
| Pollen  (Ecotoxicology ) | Primary | 55.5 µg/kg | HPLC/MS-QQQ | Garagna, D. 2022b. Report No.: CH-0223/2022 / Not evaluated at EU level yet  KCP 5.1.2/26 |
| Confirmatory | Not required | Not required | Not required |
| Nectar  (Ecotoxicology) | Primary | 55.5 µg/kg | HPLC/MS-QQQ | Garagna, D. 2022b. Report No.: CH-0223/2022 / Not evaluated at EU level yet  KCP 5.1.2/26 |
| Confirmatory | Not required | Not required | Not required |
| Pollen  (Ecotoxicology – Tunnel test) | Primary | 55.5 µg/kg | HPLC/MS-QQQ | Garagna, D. 2022c. Report No.: 168191033 : Not evaluated at EU level yet  KCP 5.1.2/27 |
| Confirmatory | Not required | Not required | Not required |
| Nectar  (Ecotoxicology– Tunnel test) | Primary | 55.5 µg/kg | HPLC/MS-QQQ | Garagna, D. 2022c. Report No.: 168191033 : Not evaluated at EU level yet  KCP 5.1.2/27 |
| Confirmatory | Not required | Not required | Not required |

|  |  |
| --- | --- |
| Comments of zRMS: | The updates has been accepted. |

Table 5.2‑6: Validated methods for the generation of pre-authorization data for triazole derivative metabolites (TDMs)

| Component of residue definition:  **Triazole Derivative Metabolites (TDMs)** | | | | |
| --- | --- | --- | --- | --- |
| Matrix type | Method type | Method LOQ | Principle of method  (i.e. GC-MS or HPLC-UV) | Author(s), year / missing / EU agreed |
| High oil content (Rapeseed seed)  (Residues) | Primary | 0.01 mg/kg | HPLC-MS/MS | Longhi, D., 2021b. Report No. : GLP-study-21-108 / Not evaluated at EU level yet  KCP 5.1.2/14 |
| ILV | 0.01 mg/kg | HPLC-MS/MS | ~~Ongoing~~  ~~See study plan~~:  ~~Rigamonti, E~~. Nichetti, S. 2022f / Report No. CH-1090/2021  KCP 5.1.2/21 |
| Confirmatory | 0.01 mg/kg | HPLC-MS/MS | Longhi, D., 2021b. Report No. : GLP-study-21-108 / Not evaluated at EU level yet  KCP 5.1.2/14 |
| High oil content (Rapeseed whole plant)  (Residues) | Primary | 0.01 mg/kg | HPLC-MS/MS | Longhi, D., 2021b. Report No. : GLP-study-21-108 / Not evaluated at EU level yet  KCP 5.1.2/14 |
| ILV | 0.01 mg/kg | HPLC-MS/MS | ~~Ongoing~~  ~~See study plan~~:  ~~Rigamonti, E~~. Nichetti, S.2022g/ Report No. CH-1085/2021  KCP 5.1.2/22 |
| Confirmatory | 0.01 mg/kg | HPLC-MS/MS | Longhi, D., 2021b. Report No. : GLP-study-21-108 / Not evaluated at EU level yet  KCP 5.1.2/14 |
| Dry commodities  (Wheat grain)  (Residues) | Primary | 0.01 mg/kg | HPLC-MS/MS | Longhi, D., 2021b. Report No. : GLP-study-21-108 / Not evaluated at EU level yet  KCP 5.1.2/14 |
| ILV | 0.01 mg/kg | HPLC-MS/MS | ~~Ongoing~~  ~~See study plan~~:  ~~Rigamonti, E~~. Nichetti, S. 2022h/ Report No. CH-1087/2021  KCP 5.1.2/23 |
| Confirmatory | 0.01 mg/kg | HPLC-MS/MS | Longhi, D., 2021b. Report No. : GLP-study-21-108 / Not evaluated at EU level yet  KCP 5.1.2/14 |
| Dry commodities  (Wheat straw)  (Residues) | Primary | 0.01 mg/kg | HPLC-MS/MS | Longhi, D., 2021b. Report No. : GLP-study-21-108 / Not evaluated at EU level yet  KCP 5.1.2/14 |
| ILV | 0.01 mg/kg | HPLC-MS/MS | ~~Ongoing~~  ~~See study plan~~:  ~~Rigamonti, E~~. Nichetti, S. 2022i/ Report No. CH-1086/2021  KCP 5.1.2/24 |
| Confirmatory | 0.01 mg/kg | HPLC-MS/MS | Longhi, D., 2021b. Report No. : GLP-study-21-108 / Not evaluated at EU level yet  KCP 5.1.2/14 |
| High water content  (Barley beer)  (Residues) | Primary | 0.01 mg/kg | HPLC-MS/MS | Longhi, D., 2021b. Report No. : GLP-study-21-108 / Not evaluated at EU level yet  KCP 5.1.2/14 |
| Confirmatory | 0.01 mg/kg | HPLC-MS/MS | Longhi, D., 2021b. Report No. : GLP-study-21-108 / Not evaluated at EU level yet  KCP 5.1.2/14 |

## Methods for post-authorization control and monitoring purposes (KCP 5.2)

### Analysis of the plant protection product (KCP 5.2)

Analytical methods for the determination of the active substances and relevant impurities in the plant protection product shall be submitted, unless the applicant shows that these methods already submitted in accordance with the requirements set out in point 5.2.1 can be applied.

### Description of analytical methods for the determination of residues of Prothioconazole (KCP 5.2)

The methods listed below in tables are accepted unless otherwise noted. ~~Some of them are still being ongoing, but this was also noted then.~~

#### Overview of residue definitions and levels for which compliance is required

Compared to the residue definition proposed in the Draft Assessment Report (incl. its addenda) the current legal residue definition is ~~not~~ identical.

~~For the monitoring, the residue definition is the prothioconazole-desthio in matrix “food of plant origin” and is the sum of prothioconazole-desthio and its glucuronide conjugate, expressed as prothioconazole-desthio in matrix “food of animal origin”. (United Kingdom (DAR), 2004 ; ESFA Scientific Report (2007) 1006, 1-98).~~

~~For the enforcement, the residue definition is proposed as prothioconazole-desthio (sum of isomers) only in matrix “food of animal origin” (EFSA Journal 2014;(5):3689).~~

~~For the enforcement, the residue definition is proposed as prothioconazole-desthio only in matrix “plant origin” (United Kingdom, (RAR), 2018).~~

The applicant: The residue definition for monitoring (enforcement) for prothioconazole will be updated following the correct definition: prothioconazole-desthio sum of isomers.

The relevant reference for plant and animal commodities of prothioconazole will be updated with the current reference (Reg. (EU) 2019/552).

As stipulated in the zRMS’s comments above, the current residue definition for monitoring (enforcement) are implemented in the Regulation Reg. (EU) 2019/552. This current regulation defined the prothioconazole-desthio sum of isomers as the residue definition for monitoring for prothioconazole.

Table 5.3‑1: Relevant residue definitions for monitoring/enforcement and levels for which compliance is required

| Matrix | Residue definition | MRL / limit | Reference for MRL/level Remarks |
| --- | --- | --- | --- |
| Plant, high water content | ~~Prothioconazole (JAU6476)~~  ~~Its metabolite Prothioconazole-desthio (JAU6476-desthio)~~  prothioconazole-desthio sum of isomers | 0.01 mg/kg | ~~EFSA Journal 2014; 12(5):3689~~  Reg. (EU) 2019/552 |
| Plant, high acid content | 0.02 mg/kg | ~~EFSA Journal 2014; 12(5):3689~~  Reg. (EU) 2019/552 |
| Plant, high protein/high starch content (dry commodities) | 0.02 mg/kg | ~~EFSA Journal 2014; 12(5):3689~~  Reg. (EU) 2019/552 |
| Plant, high oil content | 0.02 mg/kg | ~~EFSA Journal 2014; 12(5):3689~~  Reg. (EU) 2019/552 |
| Plant, difficult matrices (hops, spices, tea) | 0.02 mg/kg | ~~EFSA Journal 2014; 12(5):3689~~  Reg. (EU) 2019/552 |
| Muscle | ~~Prothioconazole (JAU6476)~~  ~~Its metabolite Prothioconazole-desthio (JAU6476-desthio)~~  prothioconazole-desthio sum of isomers | 0.01 mg/kg | ~~EFSA Journal 2014; 12(5):3689~~  Reg. (EU) 2019/552 |
| Milk | 0.005 mg/kg | ~~EFSA Journal 2014; 12(5):3689~~  Reg. (EU) 2019/552 |
| Eggs | 0.01 mg/kg | ~~EFSA Journal 2014; 12(5):3689~~  Reg. (EU) 2019/552 |
| Fat | 0.01 mg/kg | ~~EFSA Journal 2014; 12(5):3689~~  Reg. (EU) 2019/552 |
| Liver, kidney | 0.5 mg/kg | ~~EFSA Journal 2014; 12(5):3689~~  Reg. (EU) 2019/552 |
| Soil  (Ecotoxicology) | ~~Prothioconazole (JAU6476)\*~~  ~~Its metabolite Prothioconazole-desthio (JAU6476-desthio)\*\*~~  prothioconazole-desthio sum of isomers | 0.05 mg/kg\*  0.01 mg/kg\*\* | \* Current limit for soil  SANTE/2020/12830  \*\* EFSA Scientific Report (2007) 106 |
| Drinking water  (Human toxicology) | ~~Prothioconazole (JAU6476)\*~~  ~~Its metabolite Prothioconazole-desthio (JAU6476-desthio)\*\*~~  prothioconazole-desthio sum of isomers | * 1. ~~µg/L\*~~   0.05 µg/L~~\*\*~~ | EFSA Scientific Report (2007) 106 |
| Surface water  (Ecotoxicology) | ~~Prothioconazole(JAU6476)\*~~  ~~Its metabolite Prothioconazole-desthio (JAU6476-desthio)\*\*~~  prothioconazole-desthio sum of isomers | * 1. ~~µg/L\*~~   0.05 µg/L~~\*\*~~ | EFSA Scientific Report (2007) 106 |
| Air | ~~Prothioconazole(JAU6476)\*~~  ~~Its metabolite Prothioconazole-desthio (JAU6476-desthio)\*\*~~  prothioconazole-desthio sum of isomers | ~~0.015 µg/m~~~~3~~~~\*~~  0.0006 µg/m3~~\*\*~~ | AOEL sys : 0.02 mg/kg bw/d  SANTE/2020/12830  EFSA Scientific Report (2007) 106 |
| Tissue (meat or liver) | ~~Its metabolite Prothioconazole-desthio (JAU6476-desthio)~~  prothioconazole-desthio sum of isomers | 0.01 mg/L | Classified as T (Reproduction toxicity Cat 2) |
| Body fluids | 0.05 mg/L | Classified as T (Reproduction toxicity Cat 2) |

|  |  |
| --- | --- |
| Comments of zRMS: | The update has been accepted. |

#### Description of analytical methods for the determination of residues in plant matrices (KCP 5.2)

An overview on the acceptable methods and possible data gaps for analysis of prothioconazole in plant matrices is given in the following tables.

Table 5.3‑2: Validated methods for food and feed of plant origin (required for all matrix types, “difficult” matrix only when indicated by intended GAP)

| Component of residue definition: prothioconazole-desthio | | | | |
| --- | --- | --- | --- | --- |
| Matrix type | Method type | Method LOQ | Principle of method (i.e. GC-MS or HPLC-UV) | Author(s), year / missing / EU agreed |
| High oil content:  Rapeseed | Primary | * 1. ~~mg/kg~~   0.01 mg/kg | ~~GC/MS~~  HPLC-MS/MS | ~~Weeren & Pelz. 2000. Report No. : M-027637-01-1 / EU agreed, DAR, United Kingdom, 2004~~  Longhi, D., 2021a. Report No. : GLP-study-21-31 / Not evaluated at EU level yet  KCP 5.1.2/02 |
| ILV | * 1. ~~mg/kg~~   0.01 mg/kg | ~~GC/MS~~  HPLC-MS/MS | ~~Class, 2001. Report No. : M-033019-01-1 / EU agreed, DAR, United Kingdom, 2004~~  ~~Ongoing Rigamonti (2022)~~  Nichetti, S. 2022c / Report No. CH-1083/2021/ Not evaluated at EU level yet  KCP 5.1.2/18 |
| Confirmatory | ~~Not required~~  0.01 mg/kg | ~~Not required~~  HPLC-MS/MS | ~~Not required~~  Longhi, D., 2021a. Report No. : GLP-study-21-31 / Not evaluated at EU level yet  KCP 5.1.2/02 |
| High protein/high starch content (dry):  Wheat (grain)  Barley (grain) | Primary | 0.01 mg/kg | ~~GC/MS~~  HPLC-MS/MS | ~~Weeren & Pelz. 2000. Report No. : M-027637-01-1 / EU agreed, DAR, United Kingdom, 2004~~  Longhi, D., 2021a. Report No. : GLP-study-21-31 / Not evaluated at EU level yet  KCP 5.1.2/02 |
| ILV | 0.01 mg/kg | ~~GC/MS~~  HPLC-MS/MS | ~~Class, 2001. Report No. : M-033019-01-1 / EU agreed, DAR, United Kingdom, 2004~~  ~~Ongoing Rigamonti (2022)~~  Nichetti, S. 2022b / Report No. CH-1082/2021/ Not evaluated at EU level yet  KCP 5.1.2/17 |
| Confirmatory | ~~Not required~~  0.01 mg/kg | ~~Not required~~  HPLC-MS/MS | ~~Not required~~  Longhi, D., 2021a. Report No. : GLP-study-21-31 / Not evaluated at EU level yet  KCP 5.1.2/02 |
| Dry commodities:  Wheat (straw, forage)  Barley (straw, forage) | Primary | * 1. ~~mg/kg~~   0.01 mg/kg | ~~GC/MS~~  HPLC-MS/MS | ~~Weeren & Pelz. 2000. Report No. : M-027637-01-1 / EU agreed, DAR, United Kingdom, 2004~~  Longhi, D., 2021a. Report No. : GLP-study-21-31 / Not evaluated at EU level yet  KCP 5.1.2/02 |
| ILV | ~~0.05 mg/kg~~  0.01 mg/kg | ~~GC/MS~~  HPLC-MS/MS | ~~Class, 2001. Report No. : M-033019-01-1 / EU agreed, DAR, United Kingdom, 2004~~  ~~Ongoing Rigamonti (2022)~~  Nichetti, S. 2022a / Report No. CH-1081/2021 / Not evaluated at EU level yet  KCP 5.1.2/16 |
| Confirmatory | ~~Not required~~  0.01 mg/kg | ~~Not required~~  HPLC-MS/MS | ~~Not required~~  Longhi, D., 2021a. Report No. : GLP-study-21-31 / Not evaluated at EU level yet  KCP 5.1.2/02 |
| High water content:  ~~Tomato~~  Whole plant | Primary | ~~0.02mg/kg~~  0.01 mg/kg | ~~GC/MS~~  HPLC-MS/MS | ~~Weeren & Pelz. 2000. Report No. : M-027637-01-1 / EU agreed, DAR, United Kingdom, 2004~~  Longhi, D., 2021a. Report No. : GLP-study-21-31 / Not evaluated at EU level yet  KCP 5.1.2/02 |
| ILV | ~~0.02mg/kg~~  0.01 mg/kg | ~~GC/MS~~  HPLC-MS/MS | ~~Class, 2001. Report No. : M-033019-01-1 / EU agreed, DAR, United Kingdom, 2004~~  ~~Ongoing Rigamonti (2022)~~  Nichetti, S. 2022f / Report No. CH-1084/2021/ Not evaluated at EU level yet  KCP 5.1.2/29 |
| Confirmatory | ~~Not required~~  0.01 mg/kg | ~~Not required~~  HPLC-MS/MS | ~~Not required~~  Longhi, D., 2021a. Report No. : GLP-study-21-31 / Not evaluated at EU level yet  KCP 5.1.2/02 |
| High acid content:  Orange fruit | Primary | ~~0.02mg/kg~~  Not required | ~~GC/MS~~  Not required | ~~Weeren & Pelz. 2000. Report No. : M-027637-01-1 / EU agreed, DAR, United Kingdom, 2004~~  Not required according the SANTE/2020/12830, Rev.2\*. |
| ILV | ~~0.02mg/kg~~  Not required | ~~GC/MS~~  Not required | ~~Class, 2001. Report No. : M-033019-01-1 / EU agreed, DAR, United Kingdom, 2004~~  Not required |
| Confirmatory | Not required | Not required | Not required |

\* According the SANTE/2020/12830, Rev.2, as the high-water content are extracted at a controlled pH, a validation method for commodities with high acid content is not required.

|  |  |
| --- | --- |
| Comments of zRMS: | The update has been accepted. |

**Table 5.3‑3: Statement on extraction efficiency**

|  | Method for products of plant origin |
| --- | --- |
| Required, available from: | - |
| Not required, because: | High efficiency of extraction of prothioconazole-desthio from agricultural commodities is demonstrated from the finding of recovery experiments in ~~Weeren & Pelz (2000 - Report No. : M-027637-01-1) and Class (2001- Report No. : M-033019-01-1)~~ Longhi, D. (2021a. Report No. : GLP-study-21-31) within the guideline requirements (70-120% with RSD ≤20% according to SANTE/2020/12830, Rev.1). |

#### Description of analytical methods for the determination of residues in animal matrices (KCP 5.2)

The adequate MS/MS methods for milk, meat and offal (JAU6476-desthio, JAU6476- 3-hydroxy-desthio and JAU6476-4-hydroxy-desthio) from the DAR are presently not protected and suitable for the purpose of the present authorization. These methods with independent laboratory validation of milk, meat and offal are also proper for enforcement purposes. Due to the high selectivity of MS/MS based methods, further confirmatory techniques are not necessary.

**EFSA Journal 2012;10(11):2952:** An analytical method for the determination of residues in accordance with the current enforcement residue definition for food of animal origin (sum of prothioconazole-desthio and its glucuronide conjugate, expressed as prothioconazole-desthio)14 was not available in the dossier evaluated in the DAR and considered during the peer review under Directive 91/414/EEC (the United Kingdom, 2004, 2007; EFSA, 2007a). The methods available in the dossier are based on HPLC-MS-MS and allow quantification of prothioconazole-desthio and two metabolites (prothioconazole-3-hydroxy-desthio15 and prothioconazole-4-hydroxy-desthio16 at a limit of quantification of 0.01 mg/kg (meat, liver, kidney, fat) or 0.004 mg/kg (milk). These methods were not stereo selective. A method is not available to monitor the glucuronide conjugate in products of animal origin (EFSA; 2009).

Moreover:

*Will be covered by data that would be available/generated/finalised at the time of the renewal of the a.s.*

According to the commenting table, an overview on the acceptable methods and possible data gaps for analysis of prothioconazole in animal matrices is given in the following tables.

Furthermore, regarding method for the monitoring residue in animal commodities, this was a data GAP of the active ingredient and it is currently under evaluation in the renewal process. This data point will be suitably addressed by the Applicant during the product renewal (Art 43). No additional data need to be provided by the applicant in the framework of the authorisation of the product.

Table 5.3.2‑1: Validated methods for food and feed of animal origin

| Component of residue definition: prothioconazole-desthio | | | | |
| --- | --- | --- | --- | --- |
| Matrix type | Method type | Method LOQ | Principle of method (*i.e.* GC-MS or HPLC-UV) | Author(s), year / missing |
| Milk | Primary | 0.004 mg/kg | HPLC-MS/MS | Heinemann, 2001c. EU agreed, DAR, United Kingdom, 2004. |
| ILV | 0.004 mg/kg | HPLC-MS/MS | Dubey, 2001. EU agreed, DAR, United Kingdom, 2004. |
| Confirmatory | Not required | Not required | Not required |
| Muscle | Primary | 0.01 mg/kg | HPLC-MS/MS | Heinemann, 2001a. EU agreed, DAR, United Kingdom, 2004. |
| ILV | 0.01 mg/kg | HPLC-MS/MS | Dubey, 2001. EU agreed, DAR, United Kingdom, 2004. |
| Confirmatory | Not required | Not required | Not required |
| ILV | 0.01 mg/kg | HPLC-MS/MS | Dubey, 2001. EU agreed, DAR, United Kingdom, 2004. |
| Confirmatory | Not required | Not required | Not required |
| Kidney, liver | Primary | 0.01 mg/kg | HPLC-MS/MS | Heinemann, 2001a. EU agreed, DAR, United Kingdom, 2004 |
| ILV | 0.01 mg/kg | HPLC-MS/MS | Dubey, 2001. EU agreed, DAR, United Kingdom, 2004. |
| Confirmatory | Not required | Not required | Not required |

| prothioconazole-3-hydroxy-desthio | | | | |
| --- | --- | --- | --- | --- |
| Matrix type | Method type | Method LOQ | Principle of method (*i.e.* GC-MS or HPLC-UV) | Author(s), year / missing |
| Milk | Primary | 0.004 mg/kg | HPLC-MS/MS | Heinemann, 2001c. EU agreed, DAR, United Kingdom, 2004. |
| ILV | 0.004 mg/kg | HPLC-MS/MS | Dubey, 2001. EU agreed, DAR, United Kingdom, 2004. |
| Confirmatory | Not required | Not required | Not required |
| Muscle | Primary | 0.01 mg/kg | HPLC-MS/MS | Heinemann, 2001a. EU agreed, DAR, United Kingdom, 2004 |
| ILV | 0.01 mg/kg | HPLC-MS/MS | Dubey, 2001. EU agreed, DAR, United Kingdom, 2004. |
| Confirmatory | Not required | Not required | Not required |
| Kidney, liver | Primary | 0.01 mg/kg | HPLC-MS/MS | Heinemann, 2001a. EU agreed, DAR, United Kingdom, 2004 |
| ILV | 0.01 mg/kg | HPLC-MS/MS | Dubey, 2001. EU agreed, DAR, United Kingdom, 2004. |
| Confirmatory | Not required | Not required | Not required |

| prothioconazole-4-hydroxy-desthio | | | | |
| --- | --- | --- | --- | --- |
| Matrix type | Method type | Method LOQ | Principle of method (*i.e.* GC-MS or HPLC-UV) | Author(s), year / missing |
| Milk | Primary | 0.004 mg/kg | HPLC-MS/MS | Heinemann, 2001c. EU agreed, DAR, United Kingdom, 2004. |
| ILV | 0.004 mg/kg | HPLC-MS/MS | Dubey, 2001. EU agreed, DAR, United Kingdom, 2004. |
| Confirmatory | Not required | Not required | Not required |
| Muscle | Primary | 0.01 mg/kg | HPLC-MS/MS | Heinemann, 2001a. EU agreed, DAR, United Kingdom, 2004 |
| ILV | 0.01 mg/kg | HPLC-MS/MS | Dubey, 2001. EU agreed, DAR, United Kingdom, 2004. |
| Confirmatory | Not required | Not required | Not required |
| Kidney, liver | Primary | 0.01 mg/kg | HPLC-MS/MS | Heinemann, 2001a. EU agreed, DAR, United Kingdom, 2004 |
| ILV | 0.01 mg/kg | HPLC-MS/MS | Dubey, 2001. EU agreed, DAR, United Kingdom, 2004. |
| Confirmatory | Not required | Not required | Not required |

|  |  |
| --- | --- |
| Comments of zRMS: | The update has been accepted. |

#### Description of methods for the analysis of soil (KCP 5.2)

An overview on the acceptable methods and possible data gaps for analysis of prothioconazole in soil is given in the following tables.

Table 5.3‑4: Validated methods for soil

| Component of residue definition: prothioconazole-desthio (M04) | | | |
| --- | --- | --- | --- |
| Method type | Method LOQ | Principle of method  (*i.e.* GC-MS or HPLC-UV) | Author(s), year / missing |
| Primary | 0.006 mg/kg | HPLC/MS/MS | Schramel, O., 2006. Report No.: M-041798-01-1 / EU agreed, DAR, United Kingdom, 2004. |
| Confirmatory | Not required | Not required | Not required |

| Component of residue definition: prothioconazole-S-methyl (M01) | | | |
| --- | --- | --- | --- |
| Method type | Method LOQ | Principle of method  (*i.e.* GC-MS or HPLC-UV) | Author(s), year / missing |
| Primary | 0.01 mg/kg | GC-MS | Steinhauer, S. 2001. Report No. : M-067970-01-1 / EU agreed, DAR, United Kingdom, 2004. |
| Confirmatory | Not required | Not required | Not required |

#### Description of methods for the analysis of water (KCP 5.2)

An overview on the acceptable methods and possible data gaps for analysis of prothioconazole in surface and drinking water is given in the following tables.

Table 5.3‑5: Validated methods for water

| Component of residue definition: prothioconazole | | | | |
| --- | --- | --- | --- | --- |
| Matrix type | Method type | Method LOQ | Principle of method (i.e. GC-MS or HPLC-UV) | Author(s), year / missing |
| Drinking water | Primary | * 1. µg/L | HPLC-MS/MS | Sommer, H., 2001. Report No.: M-079449-01-1 / EU agreed, DAR, United Kingdom, 2004. |
| Confirmatory | Not required | Not required | Not required |
| Surface water | Primary | 0.05 µg/L | HPLC-MS/MS | Sommer, H., 2001. Report No.: M-079449-01-1 / EU agreed, DAR, United Kingdom, 2004. |
| Confirmatory | Not required | Not required | Not required |

| Component of residue definition: prothioconazole-desthio (M04) | | | | |
| --- | --- | --- | --- | --- |
| Matrix type | Method type | Method LOQ | Principle of method (i.e. GC-MS or HPLC-UV) | Author(s), year / missing |
| Drinking water | Primary | * 1. µg/L | HPLC-MS/MS | Sommer, H., 2001. Report No.: M-079449-01-1 / EU agreed, DAR, United Kingdom, 2004. |
| Confirmatory | Not required | Not required | Not required |
| Surface water | Primary | 0.05 µg/L | HPLC-MS/MS | Sommer, H., 2001. Report No.: M-079449-01-1 / EU agreed, DAR, United Kingdom, 2004. |
| Confirmatory | Not required | Not required | Not required |

According to Regulation (EU) 284/2013 an ILV has to be provided for drinking water also for product authorization. Therefore, this lack have to be set as the data gap to fulfil in post registration.

According to the zRMS conclusion, the ILV for drinking water will be provided in the post registration as explained in the applicant’s comment during the commenting table:

“ *Regarding method for the monitoring residue in water, this was a data GAP of the active ingredient and it is currently under evaluation in the renewal process. This data point will be suitably addressed by the Applicant during the product renewal (Art 43). No additional data need to be provided by the applicant in the framework of the authorisation of the product*.”

|  |  |
| --- | --- |
| Comments of zRMS: | Agreed |

#### Description of methods for the analysis of air (KCP 5.2)

Laboratory route and rate soil studies indicated that volatilisation of prothioconazole and prothioconazole-desthio is unlikely to take place because no volatiles were detected at levels above 0.1% AR. Therefore, a method of analysis for air is not necessarily required.

Table 5.3‑6: Validated methods for air

| Component of residue definition: prothioconazole | | | | |
| --- | --- | --- | --- | --- |
| Matrix type | Method type | Method LOQ | Principle of method (i.e. GC-MS or HPLC-UV) | Author(s), year / missing |
| Air | Primary | 0.015 mg/m³ | HPLC-MS/MS | Maasfeld, W. 2002. Report No. : M-032554-01-1 / EU agreed, DAR, United Kingdom, 2004. |
| Confirmatory | Not required | Not required | Not required |

| Component of residue definition: prothioconazole-desthio (M04) | | | | |
| --- | --- | --- | --- | --- |
| Matrix type | Method type | Method LOQ | Principle of method (i.e. GC-MS or HPLC-UV) | Author(s), year / missing |
| Air | Primary | 0.006 mg/m³ | HPLC-MS/MS | Sommer, H., 2001. Report No.: M-079449-01-1 / EU agreed, DAR, United Kingdom, 2004. |
| Confirmatory | 0.0003 mgm³ | HPLC-MS/MS | ~~Anft & Bardel, 2005. Report No.: M-242870-01-1 / EU agreed, RAR, United Kingdom, 2018.~~ |

#### Description of methods for the analysis of body fluids and tissues (KCP 5.2)

As the requirement according to Regulation (EU) 284/2013 that monitoring methods for body fluids and tissues have to be provided also for such product authorization is later than the generating the data for the DAR in zRMS opinion the fulfilment of this gap can be postponed for the renewal.

However this data MUST be covered as is declared below.

*Will be covered by data that would be available/generated/finalised at the time of the renewal of the a.s.*

To argue the italic sentence, the applicant commented this data point during the commenting table. So, the applicant’s comment is:

“*A fully validated method for the determination of residues of difenoconazole in animal tissue is available at EU level and it is considered suitable for body tissues as well.*

*A method for the determination of residues of Difenoconazole in body fluids is currently handled at EU level in the process of renewal of the active substance This data point will be suitably addressed by the Applicant during the product renewal (Art 43).*

*No additional data need to be provided by the applicant in the framework of the authorisation of the product*.”

|  |  |
| --- | --- |
| Comments of zRMS: | Agreed. |

#### Other studies/ information

~~Not required.~~

**5.3.2.8.1 Honey**

A honey residue trials for difenoconazole, prothioconazole and its metabolite prothioconazole-desthio in honey, ~~are ongoing according~~ to the guidance document SANTE/11956/2016 rev. 9. The study is conducted according to Good Laboratory Practice (GLP) and is composed of two phases: the field phase and the analytical phase.

Therefore, the analytical phase is conducted according to the guidance document SANTE/2020/12830, Rev.2 (14/02/2023). The analytical determination is carried out using a HPLC-MS/MS method. The final study report ~~will~~ ~~be~~ is available ~~in November 2023~~: “*Validation of an analytical method for the quantification of Difenoconazole, Prothioconazole and Prothioconazole-desthio in honey*” (Longhi, D. 2023a – Report No.: LBN-0092-2023).

For details on the validation method for honey, please see the Appendix 2.

Table 5.3.2.8.1-1: Validated methods for honey

| Component of residue definition: prothioconazole | | | | |
| --- | --- | --- | --- | --- |
| Matrix type | Method type | Method LOQ | Principle of method (i.e. GC-MS or HPLC-UV) | Author(s), year / missing |
| Honey | Primary | 0.01 mg/kg | HPLC-MS/MS | Longhi, D. 2023a. Report No.: LBN-0092-2023. KCP 5.3.2.8/01.  Not yet evaluated at EU level |
| ILV | 0.01 mg/kg | HPLC-MS/MS | Mattioli, B. Report No.: CH-0859-2023. KCP 5.3.2.8/03  Not yet evaluated at EU level |
| Confirmatory | 0.01 mg/kg | HPLC-MS/MS | Longhi, D. 2023a. Report No.: LBN-0092-2023. KCP 5.3.2.8/01.  Not yet evaluated at EU level |

| Component of residue definition: prothioconazole-desthio | | | | |
| --- | --- | --- | --- | --- |
| Matrix type | Method type | Method LOQ | Principle of method (i.e. GC-MS or HPLC-UV) | Author(s), year / missing |
| Honey | Primary | 0.01 mg/kg | HPLC-MS/MS | Longhi, D. 2023a. Report No.: LBN-0092-2023. KCP 5.3.2.8/01.  Not yet evaluated at EU level |
| ILV | 0.01 mg/kg | HPLC-MS/MS | Mattioli, B. Report No.: CH-0859-2023. KCP 5.3.2.8/03  Not yet evaluated at EU level |
| Confirmatory | 0.01 mg/kg | HPLC-MS/MS | Longhi, D. 2023a. Report No.: LBN-0092-2023. KCP 5.3.2.8/01.  Not yet evaluated at EU level |

For details on the analytical part of the honey residue trials, please see the Appendix 2.

Table 5.3.2.8.1-2: Analytical method for honey residue trial

| Component of residue definition: prothioconazole | | | | |
| --- | --- | --- | --- | --- |
| Matrix type | Method type | Method LOQ | Principle of method (i.e. GC-MS or HPLC-UV) | Author(s), year / missing |
| Honey | Primary | 0.01 mg/kg | HPLC-MS/MS | Rovetto, I. 2023. Report No.: 1111.4F.SAG23. KCP 5.3.2.8/04.  Not yet evaluated at EU level |
| Confirmatory | Not required | Not required | Not required |

|  |  |
| --- | --- |
| Comments of zRMS: | The update has been accepted. |

**5.3.2.8.2 TDMs**

A honey residue trials for the triazole-derivative metabolites (TDMs): triazole-alanine (TA), 1,2,4-triazole (1,2,4-T), triazole lactic acid (TLA), triazole acetic acid (TAA) in honey, ~~are ongoing~~ according to the guidance document SANTE/11956/2016 rev. 9.

The study is conducted according to Good Laboratory Practice (GLP) and is composed of two phases: the field phase and the analytical phase.

Therefore, the analytical phase is conducted according to the guidance document SANTE/2020/12830, Rev.2 (14/02/2023). The analytical determination is carried out using a HPLC-MS/MS method. The final study report ~~will be~~ is available ~~in November 2023~~: “*Validation of an analytical method for the quantification of Triazole Derivative Metabolites (TDMs) in honey*” (Longhi, D. 2023b – Report No.: LBN-0093-2023).

For details on the validation method for honey, please see the Appendix 2.

Table 5.3.2.8-2: Validated methods for honey

| Component of residue definition: 1,2,4-triazole (TRZ) | | | | |
| --- | --- | --- | --- | --- |
| Matrix type | Method type | Method LOQ | Principle of method (i.e. GC-MS or HPLC-UV) | Author(s), year / missing |
| Honey | Primary | 0.01 mg/kg | HPLC-MS/MS | Longhi, D. 2023b. Report No.: LBN-0093-2023. KCP 5.3.2.8/02.  Not yet evaluated at EU level |
| ILV | 0.01 mg/kg | HPLC-MS/MS | Mattioli, B. Report No.: CH-0859-2023. KCP 5.3.2.8/03  Not yet evaluated at EU level |
| Confirmatory | 0.01 mg/kg | HPLC-MS/MS | Longhi, D. 2023b. Report No.: LBN-0093-2023. KCP 5.3.2.8/02.  Not yet evaluated at EU level |

| Component of residue definition: triazole alanine (TA) | | | | |
| --- | --- | --- | --- | --- |
| Matrix type | Method type | Method LOQ | Principle of method (i.e. GC-MS or HPLC-UV) | Author(s), year / missing |
| Honey | Primary | 0.01 mg/kg | HPLC-MS/MS | Longhi, D. 2023b. Report No.: LBN-0093-2023. KCP 5.3.2.8/02.  Not yet evaluated at EU level |
| ILV | 0.01 mg/kg | HPLC-MS/MS | Mattioli, B. Report No.: CH-0859-2023. KCP 5.3.2.8/03  Not yet evaluated at EU level |
| Confirmatory | 0.01 mg/kg | HPLC-MS/MS | Longhi, D. 2023b. Report No.: LBN-0093-2023. KCP 5.3.2.8/02.  Not yet evaluated at EU level |

| Component of residue definition: triazole lactic acid (TLA) | | | | |
| --- | --- | --- | --- | --- |
| Matrix type | Method type | Method LOQ | Principle of method (i.e. GC-MS or HPLC-UV) | Author(s), year / missing |
| Honey | Primary | 0.01 mg/kg | HPLC-MS/MS | Longhi, D. 2023b. Report No.: LBN-0093-2023. KCP 5.3.2.8/02.  Not yet evaluated at EU level |
| ILV | 0.01 mg/kg | HPLC-MS/MS | Mattioli, B. Report No.: CH-0859-2023. KCP 5.3.2.8/03  Not yet evaluated at EU level |
| Confirmatory | 0.01 mg/kg | HPLC-MS/MS | Longhi, D. 2023b. Report No.: LBN-0093-2023. KCP 5.3.2.8/02.  Not yet evaluated at EU level |

| Component of residue definition: triazole acetic acid (TAA) | | | | |
| --- | --- | --- | --- | --- |
| Matrix type | Method type | Method LOQ | Principle of method (i.e. GC-MS or HPLC-UV) | Author(s), year / missing |
| Honey | Primary | 0.01 mg/kg | HPLC-MS/MS | Longhi, D. 2023b. Report No.: LBN-0093-2023. KCP 5.3.2.8/02.  Not yet evaluated at EU level |
| ILV | 0.01 mg/kg | HPLC-MS/MS | Mattioli, B. Report No.: CH-0859-2023. KCP 5.3.2.8/03  Not yet evaluated at EU level |
| Confirmatory | 0.01 mg/kg | HPLC-MS/MS | Longhi, D. 2023b. Report No.: LBN-0093-2023. KCP 5.3.2.8/02.  Not yet evaluated at EU level |

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| --- | --- |
| Comments of zRMS: | The update has been accepted. |

### Description of analytical methods for the determination of residues of Difenoconazole (KCP 5.2)

The methods listed below in tables are accepted unless otherwise noted. ~~Some of them are still being ongoing, but this was also noted then.~~

#### Overview of residue definitions and levels for which compliance is required

Compared to the residue definition proposed in the Draft Assessment Report (incl. its addenda) the current legal residue definition is identical.

The residue definition for monitoring (enforcement) for difenoconazole will be updated following the correct definition: difenoconazole.

The relevant reference for plant and animal commodities of difenoconazole will be updated with the current reference (Reg. (EU) 2019/552).

As stipulated in the zRMS’s comments above, the current residue definition for monitoring (enforcement) and its Regulation Reg. (EU) 2019/552 are implemented in the following table.

Table 5.3‑7: Relevant residue definitions for monitoring/enforcement and levels for which compliance is required

| Matrix | Residue definition | MRL / limit | Reference for MRL/level Remarks |
| --- | --- | --- | --- |
| Plant, high water content | ~~Difenoconazole and its metabolite toluene~~  Difenoconazole | 0.02 mg/kg | ~~EFSA Journal 2011; 9(1):1967~~  Reg. (EU) 2019/552 |
| Plant, high acid content | 0.6 mg/kg | Commission Regulation (EU) 2019/552 of 4 April 2019 |
| Plant, high protein/high starch content (dry commodities) | 0.05 mg/kg | ~~EFSA Journal 2011; 9(1):1967~~  Reg. (EU) 2019/552 |
| Plant, high oil content | 0.05 mg/kg | ~~EFSA Journal 2011; 9(1):1967~~  Reg. (EU) 2019/552 |
| Plant, difficult matrices (hops, spices, tea) | 0.05 mg/kg | ~~EFSA Journal 2011; 9(1):1967~~  Reg. (EU) 2019/552 |
| Muscle | ~~Difenoconazole and its metabolite toluene~~  Difenoconazole | 0.05 mg/kg | Commission Regulation (EU) 2019/552 of 4 April 2019 |
| Milk | 0.005 mg/kg | ~~EFSA Journal 2011; 9(1):1967~~  Reg. (EU) 2019/552 |
| Eggs | 0.05 mg/kg | Commission Regulation (EU) 2019/552 of 4 April 2019 |
| Fat | 0.05 mg/kg | Commission Regulation (EU) 2019/552 of 4 April 2019 |
| Liver, kidney | 0.2 mg/kg | Commission Regulation (EU) 2019/552 of 4 April 2019 |
| Soil  (Ecotoxicology) | ~~Difenoconazole and its metabolite toluene~~  Difenoconazole | 0.01 mg/kg | EFSA Journal 2011; 9(1):1967 |
| Drinking water  (Human toxicology) | Difenoconazole | 0.05 µg/L | EFSA Journal 2011; 9(1):1967 |
| Surface water  (Ecotoxicology) | Difenoconazole | * 1. µg/L | EFSA Journal 2011; 9(1):1967 |
| Air | Difenoconazole | 0.99 ng/L | AOEL sys: 0.16 mg/kg bw/d  EFSA Journal 2011; 9(1):1967 |
| Tissue (meat or liver) | Not required | Not required | Not classified as T / T+ |
| Body fluids | Not required | Not classified as T / T+ |

#### Description of analytical methods for the determination of residues in plant matrices (KCP 5.2)

An overview on the acceptable methods and possible data gaps for analysis of difenoconazole in plant matrices is given in the following tables.

Table 5.3‑8: Validated methods for food and feed of plant origin (required for all matrix types, “difficult” matrix only when indicated by intended GAP)

| Component of residue definition: difenoconazole | | | | |
| --- | --- | --- | --- | --- |
| Matrix type | Method type | Method LOQ | Principle of method (i.e. GC-MS or HPLC-UV) | Author(s), year / missing / EU agreed |
| High oil content:  Rapeseed oil | Primary | 0.05 mg/kg | HPLC-MS/MS | EFSA Journal 2011; 9(1):1967 |
| ILV | 0.01 mg/kg | LC-MS/MS | EFSA Journal 2011; 9(1):1967 |
| Confirmatory | Not required | Not required | Not required |
| High protein/high starch content (dry):  Wheat grain | Primary | 0.01 mg/kg | HPLC-MS/MS | EFSA Journal 2011; 9(1):1967 |
| ILV | 0.01 mg/kg | LC-MS/MS | EFSA Journal 2011; 9(1):1967 |
| Confirmatory | Not required | Not required | Not required |
| High water content :  Apple; Lettuce | Primary | 0.02 mg/kg | HPLC-MS/MS | EFSA Journal 2011; 9(1):1967 |
| ILV | 0.01 mg/kg | LC-MS/MS | EFSA Journal 2011; 9(1):1967 |
| Confirmatory | Not required | Not required | Not required |

Table 5.3‑9: Statement on extraction efficiency

|  | Method for products of plant origin |
| --- | --- |
| Required, available from: | - |
| Not required, because: | High efficiency of extraction of prothioconazole-desthio from agricultural commodities is demonstrated from the finding of recovery experiments within the guideline requirements (70-120% with RSD ≤20% according to SANTE/2020/12830, Rev.1). |

#### Description of analytical methods for the determination of residues in animal matrices (KCP 5.2)

The information from the DAR for the purpose of the formal requirement of the approval.

**

Moreover:

*Will be covered by data that would be available/generated/finalised at the time of the renewal of the a.s.*

As stipulated in the commenting table, an overview on the acceptable methods and possible data gaps for analysis of Difenoconazole in animal matrices is given in the following tables. No new/additional studies presented. Indeed, regarding method for the monitoring residue in animal commodities, this was a data GAP of the active ingredient and it is currently under evaluation in the renewal process.

A fully validated method for the determination of residues of difenoconazole in animal tissue is available at EU level and it is considered suitable for body tissues as well.

A method for the determination of residues of Difenoconazole in body fluids is required and this issue is currently handled at EU level in the process of renewal of the a.i.

No additional data need to be provided by the applicant in the framework of the authorisation of the product.

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| Comments of zRMS: | The update has been accepted. |

Table 5.3.3-1: Validated methods for food and feed of animal origin (if appropriate)

| Component of residue definition: Difenoconazole | | | | |
| --- | --- | --- | --- | --- |
| Matrix type | Method type | Method LOQ | Principle of method (*i.e.* GC-MS or HPLC-UV) | Authors, year / missing |
| Bovine liver, kidney, muscle, fat, milk, blood and hen eggs | Primary | 0.01 mg/kg  Bovine liver, kidney, muscle, fat, milk and hen eggs | GC-NPD | Crook S.J. 2004  Ryan J. 2004b  Benazeraf L. 2004  EU agreed |
| Primary | 0.01 mg/kg Milk  0.05 mg/kg  Bovine liver, kidney, muscle, fat and hen eggs | LC-MS/MS | Wurx R.E.M. 1994  EU agreed |
| Primary | 0.01 mg/kg Bovine liver, kidney, muscle, fat  10 µg/l blood  5 µg/l Milk | LC-MS/MS | Tribolet, R. 2000  EU agreed |

**Table 5.3.3‑2: Validated methods for food and feed of animal origin (if appropriate)**

| **Component of residue definition: Metabolite CGA 205375** | | | | |
| --- | --- | --- | --- | --- |
| **Matrix type** | **Method type** | **Method LOQ** | **Principle of method (*i.e.* GC-MS or HPLC-UV)** | **Authors, year / missing** |
| Bovine liver, kidney, muscle, fat, milk, blood and hen eggs | Primary | 0.01 mg/kg  Bovine liver, kidney, muscle, fat, milk and hen eggs | LC-MS/MS | Crook S.J. 2004  Ryan J. 2004b  Benazeraf L. 2004  EU agreed |
| Primary | 0.01 mg/kg Bovine liver, kidney, muscle, fat  10 µg/l blood  5 µg/l Milk | LC-MS/MS | Tribolet, R. 2000  EU agreed |

Table 5.3.3‑3: Statement on extraction efficiency

|  | Method for products of animal origin |
| --- | --- |
| Not required, because: | Multiresidue methods are available for the extraction of this active substance. In compliance with SANCO/825/00 rev. 8.1, it is not necessary to address extraction efficiency since there aren’t matrix groups for which residues are ≥LOQ. |

#### Description of methods for the analysis of soil (KCP 5.2)

An overview on the acceptable methods and possible data gaps for analysis of residues in soil is given in the following table.

Table 5.3‑10: Validated methods for soil

| Component of residue definition: difenoconazole (and its metabolite CGA205375) | | | |
| --- | --- | --- | --- |
| Method type | Method LOQ | Principle of method  (*i.e.* GC-MS or HPLC-UV) | Author(s), year / missing |
| Primary  Sandy loam and silty clay laom | 0.01 mg/kg | LC-MS/MS | Tummon, O.J. 2004a. Report No. : RJ3459B / EU agreed, DAR, Sweden, 2006. |
| Confirmatory | Not required | Not required | Not required |

#### Description of methods for the analysis of water (KCP 5.2)

*Will be covered by data that would be available/generated/finalised at the time of the renewal of the a.s.*

As stipulated in the commenting table, an overview on the acceptable methods and possible data gaps for analysis of Difenoconazole in surface and drinking water is given in the following tables. No new/additional studies presented. Indeed, regarding method for the monitoring residue in water, this was a data GAP of the active ingredient and it is currently under evaluation in the renewal process.

No additional data need to be provided by the applicant in the framework of the authorisation of the product.

Table 5.3.3.5-1: Validated methods for water

| Component of residue definition: Difenoconazole | | | | |
| --- | --- | --- | --- | --- |
| Matrix type | Method type | Method LOQ | Principle of method (i.e. GC-MS or HPLC-UV) | Authors, year / missing |
| Drinking water and Surface water | Primary | 0.05-0.1 µg/l surface water and drinking water (potable) | GC-ECD | Tribolet, R. 1999a  Tribolet, R. 1999b  EU agreed |
| Confirmatory | 0.05-0.1 µg/l surface water and drinking water (potable) | HPLC-UV | Tribolet, R. 1999a  Tribolet, R. 1999b  EU agreed |
| Primary | 0.1 µg/l HPLC-grade and drinking water (potable) | GC-ECD | Tribolet, R. 1990  EU agreed |
| Confirmatory | 0.1 µg/l HPLC-grade and drinking water (potable) | HPLC-UV | Tribolet, R. 1990  EU agreed |

|  |  |
| --- | --- |
| Comments of zRMS: | The update has been accepted. |

#### Description of methods for the analysis of air (KCP 5.2)

~~A method of analysis for air is not required because the active substance is not a volatile substance.~~

According to Regulation (EU) 284/2013 a monitoring method for air has to be provided (regardless of the volatility) also for product authorization.

**The DAR:** Acceptable methods of analysis were available for the active substance as manufactured (GC-FID) and for difenoconazole in the representative formulations (Score 250 EC: GC-FID; Dividend: 030 FS: HPLC-UV). Moreover, **acceptable monitoring methods were available for analysis of residues of difenoconazole** in food/feed of plant origin and animal origin (HPLC-MS/MS), soil (HPLC-MS/MS, GC-ECD, GC-AFID or GC-NPD), **drinking and surface water** (GC-ECD) **and air (HPLC-MS/MS).**

As explained in the zRMS’s comments above, acceptable methods of analysis were available in the DAR. So, an overview on the acceptable methods and possible data gaps for analysis of Difenoconazole in air is given in the following tables. No new/additional study presented.

Table 5.3.3.6-1: Validated methods for air

| Component of residue definition: Difenoconazole | | | |
| --- | --- | --- | --- |
| Method type | Method LOQ | Principle of method  (i.e. GC-MS or HPLC-UV) | Author(s), year / missing |
| Primary | 0.99 ng/l | LC-MS/MS | Tummon O.J. 2004b  EU agreed |
| Primary | 0.1 µg/l | GC-ECD | Tribolet, R. 1992  Tribolet, R. 1996  EU agreed |

#### Description of methods for the analysis of body fluids and tissues (KCP 5.2)

~~A method of analysis for body fluids and tissues is not required because the active substance is not classified as toxic or very toxic.~~

As the requirement according to Regulation (EU) 284/2013 that monitoring methods for body fluids and tissues have to be provided also for such product authorization is later than the generating the data for the DAR in zRMS opinion the fulfilment of this gap can be postponed for the renewal.

A method for the determination of residues of Difenoconazole in body fluids is currently handled at EU level in the process of renewal of the active substance This data point will be suitably addressed by the Applicant during the product renewal (Art 43).

#### Other studies/ information

~~Not required.~~

**5.3.3.8.1 Honey**

A honey residue trials for difenoconazole, prothioconazole and its metabolite prothioconazole-desthio in honey, ~~are ongoing~~ according to the guidance document SANTE/11956/2016 rev. 9. The study is conducted according to Good Laboratory Practice (GLP) and is composed of two phases: the field phase and the analytical phase.

Therefore, the analytical phase is conducted according to the guidance document SANTE/2020/12830, Rev.2 (14/02/2023). The analytical determination is carried out using a HPLC-MS/MS method. The final study report is ~~will be~~ available ~~in November 2023~~: “*Validation of an analytical method for the quantification of Difenoconazole, Prothioconazole and Prothioconazole-desthio in honey*” (Longhi, D. 2023a – Report No.: LBN-0092-2023).

For details on the validation method for honey, please see the Appendix 2.

Table 5.3.3.8-1: Validated methods for honey

| Component of residue definition: difenoconazole | | | | |
| --- | --- | --- | --- | --- |
| Matrix type | Method type | Method LOQ | Principle of method (i.e. GC-MS or HPLC-UV) | Author(s), year / missing |
| Honey | Primary | 0.01 mg/kg | HPLC-MS/MS | Longhi, D. 2023a. Report No.: LBN-0092-2023a. KCP 5.3.2.8/01.  Not yet evaluated at EU level |
| ILV | 0.01 mg/kg | HPLC-MS/MS | Mattioli, B. Report No.: CH-0859-2023. KCP 5.3.2.8/03  Not yet evaluated at EU level |
| Confirmatory | 0.01 mg/kg | HPLC-MS/MS | Longhi, D. 2023. Report No.: LBN-0092-2023. KCP 5.3.2.8/01.  Not yet evaluated at EU level |

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| --- | --- |
| Comments of zRMS: | The update has been accepted. |

**5.3.3.8.2 TDMs**

A honey residue trials for the triazole-derivative metabolites (TDMs): triazole-alanine (TA), 1,2,4-triazole (1,2,4-T), triazole lactic acid (TLA), triazole acetic acid (TAA) in honey, ~~are ongoing~~ according to the guidance document SANTE/11956/2016 rev. 9.

The study is conducted according to Good Laboratory Practice (GLP) and is composed of two phases: the field phase and the analytical phase.

Therefore, the analytical phase is conducted according to the guidance document SANTE/2020/12830, Rev.2 (14/02/2023). The analytical determination is carried out using a HPLC-MS/MS method. The final study report is ~~will be~~ available ~~in November 2023~~: “*Validation of an analytical method for the quantification of Triazole Derivative Metabolites (TDMs) in honey*” (Longhi, D. 2023b – Report No.: LBN-0093-2023).

For the overview of validation method of TDMs for honey, please refer to the datapoint 5.3.2.8.

For details on the validation method for honey, please see the Appendix 2.

1. Lists of data considered in support of the evaluation

List of data submitted by the applicant and relied on

| Data point | Author(s) | Year | Title Company Report No.  Source (where different from company) GLP or GEP status Published or not | Vertebrate study  Y/N | Owner |
| --- | --- | --- | --- | --- | --- |
| KCP 5.1.1/01 | Urbani, M. | 2021a | Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Validation of the Analytical Method for the Determination of Active Ingredients Content  Report No. : CH – 0324/2021  + Amendment report No.1 to final report (Amdm1\_KCP 5.1.1/01)  ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy  GLP : Yes  Unpublished | N | INDOFIL Industries (Netherlands) B.V. |
| KCP 5.1.1/02 | Urbani, M. | 2021b | Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Validation of the Analytical Method for the Determination of Toluene as Relevant Impurity Content  Report No. : CH – 0325/2021  + Amendment report No.1 to final report (Amdm1\_KCP 5.1.1/02)  ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy  GLP : Yes  Unpublished | N | INDOFIL Industries (Netherlands) B.V. |
| KCP 5.1.1/03 | Urbani, M. | 2021c | Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Validation of the Analytical Method for the Determination of Prothioconazole-desthio as Relevant Impurity Content  Report No. : CH – 0326/2021  + Amendment report No.1 to final report (Amdm1\_KCP 5.1.1/03)  ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy  GLP : Yes  Unpublished | N | INDOFIL Industries (Netherlands) B.V. |
| KCP 5.1.2/01 | Garagna, D. | 2021a | Validation of the Analytical Method for the Determination of Difenoconazole and Prothioconazole residues in soil samples of Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560 coming from the Ecotoxicological tests  Report No. : CH – 0235/2021  + Amendment report No.1 to final report (Amdm1\_KCP 5.1.2/01)  ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy  GLP : Yes  Unpublished | N | INDOFIL Industries (Netherlands) B.V. |
| KCP 5.1.2/02 | Longhi, D. | 2021a | Validation of an analytical method for the quantification of Difenoconazole and Prothioconazole-desthio in wheat, barley, oilseed rape and processed commodities  Report No. : 21-31  LabAnalysis s.r.l., Casanova Lonati – Italy  GLP : Yes  Unpublished | N | INDOFIL Industries (Netherlands) B.V. |
| KCP 5.1.2/03 | Garagna, D. | 2021b | Validation of the Analytical Method for the Determination of Difenoconazole and Prothioconazole residues in aqueous samples of Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560 coming from the Ecotoxicological tests  Report No. : CH – 0227/2021  + Amendment report No.1 to final report (Amdm1\_KCP 5.1.2/03)  ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy  GLP : Yes  Unpublished | N | INDOFIL Industries (Netherlands) B.V. |
| KCP 5.1.2/04 | Garagna, D. | 2021c | Validation of the Analytical Method for the Determination of Difenoconazole and Prothioconazole content in stock solutions of Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560 coming from the Ecotoxicological tests  Report No. : CH – 0232/2021  + Amendment report No.1 to final report (Amdm1\_KCP 5.1.2/04)  ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy  GLP : Yes  Unpublished | N | INDOFIL Industries (Netherlands) B.V. |
| KCP 5.1.2/05 | Garagna, D. | 2021d | Validation of the Analytical Method for the Determination of Difenoconazole and Prothioconazole content in feeding solutions of Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560 coming from the Ecotoxicological tests  Report No. : CH – 0668/2021  + Amendment report No.1 to final report (Amdm1\_KCP 5.1.2/05)  ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy  GLP : Yes  Unpublished | N | INDOFIL Industries (Netherlands) B.V. |
| KCP 5.1.2/06 | Noè, F. | 2021a | Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Acute Toxicity to *Daphnia magna* in a 48-hour Immobilization Test under Semi-Static Exposure  Report No. : CH-0229/2021  + Amendment report No.1 to final report (Amdm1\_KCP 5. 1.2/06)  ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy  GLP : Yes  Unpublished | N | INDOFIL Industries (Netherlands) B.V. |
| KCP 5.1.2/07 | Noè, F. | 2021b | Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Toxicity to Green Algae *Pseudokirchneriella subcapitata* in a Growth Inhibition Study  Report No. : CH-0230/2021  + Amendment report No.1 to final report (Amdm1\_KCP 5.1.2/07)  ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy  GLP : Yes  Unpublished | N | INDOFIL Industries (Netherlands) B.V. |
| KCP 5.1.2/08 | xxx. | 2021c | Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Acute Toxicity to Zebrafish (*Brachydanio rerio*) in a 96-hour Study under Semi-Static Exposure  Report No. : CH-0228/2021  + Amendment report No.1 to final report (Amdm1\_KCP 5.1.2/08)  xxxx  GLP : Yes  Unpublished | Y | INDOFIL Industries (Netherlands) B.V. |
| KCP 5.1.2/09 | Dini, R. | 2021a | Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Effects on Reproduction of Earthworm *Eisenia fetida* in an Artificial Soil Study  Report No. : CH-0239/2021  ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy  GLP : Yes  Unpublished | N | INDOFIL Industries (Netherlands) B.V. |
| KCP 5.1.2/10 | Dini, R. | 2021b | Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Effects on Collembolan Reproduction in an Artificial Soil Study  Report No. : CH-0240/2021  ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy  GLP : Yes  Unpublished | N | INDOFIL Industries (Netherlands) B.V. |
| KCP 5.1.2/11 | Ponti, B. | 2021a | Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Acute Oral and Contact Toxicity to adult worker bumblebees *Bombus terrestris* L.  Report No. : CH-0234/2021  ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy  GLP : Yes  Unpublished | N | INDOFIL Industries (Netherlands) B.V. |
| KCP 5.1.2/12 | Dini, R. | 2021c | Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Effects on *Hypoaspis (Geolaelaps) aculeifer* Reproduction in an Artificial Soil Study  Report No. : CH-0241/2021  ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy  GLP : Yes  Unpublished | N | INDOFIL Industries (Netherlands) B.V. |
| KCP 5.1.2/13 | Ponti, B. | 2021b | Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Chronic Oral Toxicity to adult worker honeybees *Apis mellifera* L. (10-day feeding)  Report No. : CH-0669/2021  ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy  GLP : Yes  Unpublished | N | INDOFIL Industries (Netherlands) B.V. |
| KCP 5.1.2/14 | Longhi, D. | 2021b | Validation of an analytical method for the quantification of Triazole Derivative Metabolites (TDMs) in wheat, barley, oilseed rape and processed commodities  Report No. : 21-108  LabAnalysis s.r.l., Casanova Lonati – Italy  GLP : Yes  Unpublished | N | INDOFIL Industries (Netherlands) B.V. |
| KCP 5.1.2/15 | Longhi, D. | 2021c | Validation of an analytical method for the quantification of prothioconazole-desthio-3-hydroxy, prothioconazole-desthio-4-hydroxy, prothioconazole-desthio-5-hydroxy, prothioconazole-desthio-6-hydroxy and prothioconazole-desthio-alpha-hydroxy in cereal straw  Report No. : 21-120  + Amendment report No.1 to stduy plan (Amdm1\_KCP 5.1.2/15)  LabAnalysis s.r.l., Casanova Lonati – Italy  GLP : Yes  Unpublished | N | INDOFIL Industries (Netherlands) B.V. |
| ~~KCP 5.1.2/16~~ | ~~Rigamonti, E.~~ | ~~2022a~~ | ~~Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of Difenoconazole and Prothio-desthio in Rapeseed seeds~~  ~~Study plan No. : CH-1083/2021~~  ~~ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy~~  ~~GLP : Yes~~  ~~Unpublished~~ | ~~N~~ | ~~INDOFIL Industries (Netherlands) B.V.~~ |
| ~~KCP 5.1.2/17~~ | ~~Rigamonti, E.~~ | ~~2022b~~ | ~~Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of Difenoconazole and Prothio-desthio in Whole Plant (Rapeseed)~~  ~~Study plan No. : CH-1084/2021~~  ~~ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy~~  ~~GLP : Yes~~  ~~Unpublished~~ | ~~N~~ | ~~INDOFIL Industries (Netherlands) B.V.~~ |
| ~~KCP 5.1.2/18~~ | ~~Rigamonti, E.~~ | ~~2022c~~ | ~~Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of Difenoconazole and Prothio-desthio in Grain (Wheat)~~  ~~Study plan No. : CH-1082/2021~~  ~~ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy~~  ~~GLP : Yes~~  ~~Unpublished~~ | ~~N~~ | ~~INDOFIL Industries (Netherlands) B.V.~~ |
| ~~KCP 5.1.2/19~~ | ~~Rigamonti, E.~~ | ~~2022d~~ | ~~Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of Difenoconazole and Prothio-desthio in Straw (wheat)~~  ~~Study plan No. : CH-1081/2021~~  ~~ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy~~  ~~GLP : Yes~~  ~~Unpublished~~ | ~~N~~ | ~~INDOFIL Industries (Netherlands) B.V.~~ |
| KCP 5.1.2/16 | Nichetti, S. | 2022a | Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of Difenoconazole and Prothio-desthio in Straw (wheat)  Report No. : CH-1081/2021  ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy  GLP : Yes  Unpublished | N | INDOFIL Industries (Netherlands) B.V. |
| KCP 5.1.2/17 | Nichetti, S. | 2022b | Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of Difenoconazole and Prothio-desthio in Grain (Wheat)  Report No.: CH-1082/2021  ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy  GLP : Yes  Unpublished | N | INDOFIL Industries (Netherlands) B.V. |
| KCP 5.1.2/18 | Nichetti, S. | 2022c | Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of Difenoconazole and Prothio-desthio in Rapeseed seeds  Report No.: CH-1083/2021  ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy  GLP : Yes  Unpublished | N | INDOFIL Industries (Netherlands) B.V. |
| KCP 5.1.2/19 | Nichetti, S. | 2022d | Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of Difenoconazole and Prothio-desthio in Whole Plant (Rapeseed)  Report No.: CH-1084/2021  ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy  GLP : Yes  Unpublished | N | INDOFIL Industries (Netherlands) B.V. |
| KCP 5.1.2/20 | ~~Rigamonti, E.~~  Nichetti, S. | 2022e | Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of Prothio-desthio metabolites in Cereal straw  ~~Study plan~~ Report No. : CH-1091/2021  ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy  GLP : Yes  Unpublished | N | INDOFIL Industries (Netherlands) B.V. |
| KCP 5.1.2/21 | ~~Rigamonti, E.~~  Nichetti, S. | 2022f | Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of TDM in Rapeseed seeds  ~~Study plan~~ Report No. : CH-1090/2021  ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy  GLP : Yes  Unpublished | N | INDOFIL Industries (Netherlands) B.V. |
| KCP 5.1.2/22 | ~~Rigamonti, E.~~  Nichetti, S. | 2022g | Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of TDM in Whole Plant (Rapeseed)  ~~Study plan~~ Report No. : CH-1085/2021  ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy  GLP : Yes  Unpublished | N | INDOFIL Industries (Netherlands) B.V. |
| KCP 5.1.2/23 | ~~Rigamonti, E.~~  Nichetti, S. | 2022h | Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of TDM in Grain (wheat)  ~~Study plan~~ Report No. : CH-1087/2021  ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy  GLP : Yes  Unpublished | N | INDOFIL Industries (Netherlands) B.V. |
| KCP 5.1.2/24 | ~~Rigamonti, E.~~  Nichetti, S. | 2022i | Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of TDM in Straw (wheat)  ~~Study plan~~ Report No. : CH-1086/2021  ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy  GLP : Yes  Unpublished | N | INDOFIL Industries (Netherlands) B.V. |
| KCP 5.1.2/25 | Garagna, D. | 2022a | Validation of the Analytical Method for the Determination of Prothioconazole-desthio Residues in Aqueous Samples coming from the Ecotoxicological tests  Report No.: CH-0949/2021  ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy  GLP : Yes  Unpublished | N | INDOFIL Industries (Netherlands) B.V. |
| KCP 5.1.2/26 | Garagna, D. | 2022b | Difenconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560 Validation of the Analytical Method for the Determination of Difenoconazole and Prothioconazole Residues in Pollen and Nectar from Ecotoxicological Study  Report No.: CH-0223/2022  ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy  GLP : Yes  Unpublished | N | INDOFIL Industries (Netherlands) B.V. |
| KCP 5.1.2/27 | Garagna, D. | 2022c | Difenconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Effects on Honey Bee Brood (Apis mellifera L.) under Semi-field Conditions – Tunnel Test (Analytical Phase)  Report No.: 168191033  Report No test site study.: CH-0695/2022  Ibacom gmBH, Rossdorf - Germany  GLP : Yes  Unpublished | N | INDOFIL Industries (Netherlands) B.V. |
| KCP 5.3.2.8/01 | Longhi, D. | 2023a | Validation of an analytical method for the quantification of Difenoconazole, Prothioconazole and Prothioconazole-desthio in honey  Report No.: LBN-0092-2023  LabAnalysis s.r.l., Casanova Lonati – Italy  GLP : Yes  Unpublished | N | INDOFIL Industries (Netherlands) B.V. |
| KCP 5.3.2.8/02 | Longhi, D. | 2023b | Validation of an analytical method for the quantification of Triazole Derivative Metabolites (TDMs) in honey  Report No.: LBN-0093-2023  LabAnalysis s.r.l., Casanova Lonati – Italy  GLP : Yes  Unpublished | N | INDOFIL Industries (Netherlands) B.V. |
| KCP 5.3.2.8/03 | Mattioli, B. | 2023 | Independent Laboratory Validation (ILV) of the Analytical Method fo the Determination of Difenoconazole, Prothioconazole, Prothioconazole-desthio and Triazole Derivatives Metabolites (TDMs) residue in Honey  Report No.: CH-0859-2023  ChemService S.r.l. Controlli e Ricerche, Novate Milanese (MI) – Italy  GLP: Yes  Unpublished | N | INDOFIL Industries (Netherlands) B.V. |
| KCP 5.3.2.8/04 | Rovetto, I. | 2023 | Analytical phase report - Magnitude of the residue of difenoconazole, prothioconazole, prothioconazole-desthio and triazole-derivative-metabolites (TDMs) in honey after one application of IN233C1560 380 EC on Phacelia crop under semi field conditions in four trials in Northern Europe and Southern Europe – 2023  Multisite study: 1111.4F.SAG23  Report No.: LBN-0108-2023  LabAnalysis s.r.l., Casanova Lonati – Italy  GLP : Yes  Unpublished | N | INDOFIL Industries (Netherlands) B.V. |

List of data submitted or referred to by the applicant and relied on, but already evaluated at EU peer review for prothioconazole

| Data point | Author(s) | Year | Title Company Report No.  Source (where different from company) GLP or GEP status Published or not | Vertebrate study  Y/N | Owner |
| --- | --- | --- | --- | --- | --- |
| ~~CP 5.2~~ | ~~Anft & Bardel~~ | ~~2005~~ | ~~Modification M001 of method 00731 for the determination of residues of JAU 6476-desthio (SXX 0665) in air by HPLCMS/ MS~~  ~~Bayer CropScience,~~  ~~Report No.: 00731/M001~~  ~~GLP~~  ~~Unpublished~~ | ~~N~~ | ~~Bayer CropScience~~  ~~AG~~ |
| CP 5.2 | Scharmel, O. | 2006 | Report No.: M-041798-01-1  EU agreed, DAR, United Kingdom, 2004.  GLP  Unpublished | N | Bayer CropScience  AG |
| CP 5.2 | Steinhauer, S. | 2001 | Report No. : M-067970-01-1  EU agreed, DAR, United Kingdom, 2004.  GLP  Unpublished | N | Bayer CropScience  AG |
| CP 5.2 | Sommer, H | 2001 | Report No.: M-079449-01-1  EU agreed, DAR, United Kingdom, 2004.  GLP  Unpublished | N | Bayer CropScience  AG |
| CP 5.2 | Massfeld | 2002 | Report No. : M-032554-01-1  EU agreed, DAR, United Kingdom, 2004.  GLP  Unpublished | N | Bayer CropScience  AG |
| ~~CP 5.2~~ | ~~Weeren & Pelz~~ | ~~2000~~ | ~~Report No. : M-027637-01-1~~  ~~EU agreed, DAR, United Kingdom, 2004~~  ~~GLP~~  ~~Unpublished~~ | ~~N~~ | ~~Bayer CropScience~~  ~~AG~~ |
| ~~CP 5.2~~ | ~~Class, T.~~ | ~~2001~~ | ~~Report No. : M-033019-01-1~~  ~~EU agreed, DAR, United Kingdom, 2004~~  ~~GLP~~  ~~Unpublished~~ | ~~N~~ | ~~Bayer CropScience~~  ~~AG~~ |
| CP 5.2 | Heinemann, O. | 2001a | Analytical determination of residues of JAU6476-3-hydroxy-desthio,JAU6476-4-hydroxy-desthio, and JAU6476-desthio in/on matrices of animal origin by HPLC-MS/MS  Bayer AG,  Report No.: 00655  GLP  Unpublished  EU agreed, DAR, United Kingdom, 2004 | N | Bayer CropScience  AG |
| CP 5.2 | Heinemann, O. | 2001c | Analytical determination of residues of JAU6476-3-hydroxy-desthio, JAU6476-4-hydroxy-desthio, and JAU6476-desthio in milk by HPLCMS/MS (00655/M001)  Bayer AG,  Report No.: 00655/M001  GLP  Unpublished  EU agreed, DAR, United Kingdom, 2004 | N | Bayer CropScience  AG |
| CP 5.2 | Dubey, L. | 2001 | Independent laboratory validation ofbayer methods 00655 and 00655/M001 for the determination of residues of JAU6476-3-hydroxydesthio,JAU6476-4-hydroxy-desthio, and JAU6476-desthio in/on matreces of animal origin by HPLC-MS/MS  Battelle, Geneva Research Centres, Carouge/Geneva, SwitzerlandBayer AG,  Report No.: A-14-01-01  GLP  Unpublished  EU agreed, DAR, United Kingdom, 2004 | N | Bayer CropScience  AG |

List of data submitted or referred to by the applicant and relied on, but already evaluated at EU peer review for difenoconazole

| Data point | Author(s) | Year | Title Company Report No.  Source (where different from company) GLP or GEP status Published or not | Vertebrate study  Y/N | Owner |
| --- | --- | --- | --- | --- | --- |
| CP 5.2 | Tummon, O.J. | 2004a | Difenoconazole. Validation of an Analytical Method for the Determination of Residues of Difenoconazole and CGA205375 in Soil  Syngenta Crop Protection AG, Basel, Switzerland  Report No RJ3459B  GLP : Yes  Unpublished | N | Syngenta |
| CP 5.2 | Crook, S. | 2004 | Residue Method for the Determination of Difenoconazole (CGA169374) in Various Crops and Processed Crop Fractions. Final Determination by LC-MS/MS  Syngenta Crop Protection AG, Basel, Switzerland  Syngenta, Jealott’s Hill, United Kingdom,  Report No.: REM147.08  Not GLP  not published | N | Syngenta |
| CP 5.2 | Benazeraf, L. | 2004 | EU agreed, DAR, Sweden, 2006.  Unpublished | N | Syngenta |
| CP 5.2 | Wurrx R.E.M. | 1994 | EU agreed, DAR, Sweden, 2006.  Unpublished | N | Syngenta |
| CP 5.2 | Tribolet, R. | 2000 | EU agreed, DAR, Sweden, 2006.  Unpublished | N | Syngenta |
| CP 5.2 | Tribolet, R. | 1999a | EU agreed, DAR, Sweden, 2006.  Unpublished | N | Syngenta |
| CP 5.2 | Tribolet, R. | 1999a | EU agreed, DAR, Sweden, 2006.  Unpublished | N | Syngenta |
| CP 5.2 | Tribolet, R. | 1992 | Sampling of air and determination of residues of parent compound by gas chromatography  Novartis Crop Protection AG, Basel, Switzerland  Ciba-Geigy Ltd., Basel, Switzerland,  Report No REM-147-02  Not GLP  Not Published | N | Syngenta |
| CP 5.2 | Tribolet, R. | 1996 | Report on Special Study 102/96. Validation of method REM 147.02 in air, Validation by analysis of fortified specimens and determination of recoveries  Novartis Crop Protection AG, Basel, Switzerland  Ciba-Geigy Ltd., Basel, Switzerland,  Report No 102/96  GLP  Not Published | N | Syngenta |

1. Detailed evaluation of submitted analytical methods
   1. Analytical methods for prothioconazole
      1. Methods used for the generation of pre-authorization data (KCP 5.1)
         * 1. Analytical method 1

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  In method HPLC-MS/MS detection was used.  The specificity of the method was assured by MS/MS detection. All validation parameters are in the currently required range.  The method is suitable for the intended purposes. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/01 |
| Report | Validation of the Analytical Method for the Determination of Difenoconazole and Prothioconazole residues in soil samples of Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560 coming from the Ecotoxicological tests  Garagna, D.  2021a  ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy  Report No. : CH – 0235/2021  + Amendment report No.1 to final report (Amdm1\_KCP 5.1.2/01) |
| Guideline(s): | Yes : SANTE/2020/12830, rev. 1 dated 24/02/21 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The determination of Prothioconazole residues is performed by HPLC using an external standard and MS triple quadrupole detector.  Transitions (MS/MS).  Its quantification is achieved using the calibration curve obtained by plotting standard solutions concentrations and the corresponding peak area. |

Materials and methods

***1. Materials***

***1.A. Determination of prothioconazole residues in soil samples***

The determination of prothioconazole residues in soil samples was assessed by HLPC/MS-QQQ.

|  |  |
| --- | --- |
| HPLC: | Shimadzu Technologies 8050 System |
| Column: | Zorbax Eclipse Plus C18, 3.5 µm, 100 x 4.6 mm i.d. |
| Detector | MS Triple quadrupole (Scan in MRM mode) |
| Flow rate | 0.7 mL/min |
| Volume of injection | 10 µL |
| Retention time | Approximatively 7.6 minutes for prothioconazole |
| Total analysis time | 20 minutes |
| Mobile phase: | Eluent A : Water, HLPC grade  Eluent A : Demineralised water  Eluent A : Formic acid, high purity for mass spectroscopy  Eluent A : Ammonium formate, for HPLC  Eluent B : Acetonitrile, for HPLC grade |
| Mixture | |  |  |  | | --- | --- | --- | | % A | % B | Time (min) | | 45 | 55 | 12 | | 10 | 90 | 14 | | 45 | 55 | 16 | | 45 | 55 | 20 | |
| Analytical standards: | Prothioconazole PESTANAL ®  CAS No. : 178928-70-6  Batch No. : BCBT2374  Purity : 99.6 %  Expiry date: November 1, 2021 |

***2. Methods***

***2.A. Preparation of soil samples***

Soil samples were prepared according to the guidelines :

* OECD 222
* OECD 226
* OECD 232

Soil samples were extracted by solvent using the following extraction procedure :

1. Weigh in 50 mL plastic tube approximately 5 g of soil
2. Add 10 mL of Acetonitrile
3. Shake for 1 hour
4. Centrifugation at 5000 rpm for 5 minutes
5. Filter with PTFE 0.45 µm
6. If necessary, dilute with “soil extracted solvent”
7. Inject

***2.B. Determination of prothioconazole residues in soil samples***

The analytical methods for the determination of prothioconazole residues in soil samples were first validated so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were validated in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A 7.1: Recovery results from method validation of prothioconazole using the analytical method

| Matrix effect | Analyte | Fortification level  (n = x) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| 33.5 % | Prothioconazole | Low level:  45.2 µg/kg (mean found)  N = 5 | 89.0 | 14 % | No comments |
| 33.5% | Prothioconazole | High level:  172.13 µg/kg (mean found)  N = 5 | 79.9 | 7 % | No comments |

Table A 8: Characteristics for the analytical method used for validation of prothioconazole and difenoconazole residues for purposes of soil ecotoxicological tests

|  | Prothioconazole |
| --- | --- |
| Specificity | Untreated blank < 30 % LOQ  Result : 0.0 %  This % ratio (Blank vs LOQ) demontrates that there was no interference of the blank matrix at the retention time of the analyte. |
| Linearity / Calibration | ***Quantifier transition***  ***m/z 344 🡪 m/z 326 :***  The calibration curve (µg/kg):   * was considered as valid over 14.9 – 137.3 µg/kg.   Equation (µg/kg)  y = 345 \* x – 749  Correlation coefficient:  r² = 99.773  The calibration curve (µg/L):   * was considered as valid over 7.5 – 74.7 µg/L.   Equation (µg/L)  y = 628 \* x – 234  Correlation coefficient:  r² = 99.777  ***Quantifier 1 transition***  ***m/z 344 🡪 m/z 154 :***  The calibration curve (µg/kg):   * was considered as valid over 14.9 – 137.3 µg/kg.   Equation (µg/kg)  y = 90 \* x – 338  Correlation coefficient:  r² = 99.966  The calibration curve (µg/L):   * was considered as valid over 7.5 – 74.7 µg/L.   Equation (µg/L)  y = 163 \* x – 204  Correlation coefficient:  r² = 99.967  ***Quantifier 2 transition***  ***m/z 344 🡪 m/z 189 :***  The calibration curve (µg/kg):   * was considered as valid over 14.9 – 137.3 µg/kg.   Equation (µg/kg)  y = 88 \* x + 773  Correlation coefficient:  r² = 99.330  The calibration curve (µg/L):   * was considered as valid over 7.5 – 74.7 µg/L.   Equation (µg/L)  y = 160 \* x + 905  Correlation coefficient:  r² = 99.324 |
| Limit of determination (LOD) | LOD = 14.9 µg/kg  Lowest calibration level |
| Limit of quantification (LOQ) | LOQ = 50.8 µg/kg  Lowest fortified level |
| Stability of final extract (7 days) | % recovery = 81.3 %  (mean value of 5 replicates)  % RSD = 5 %  Range of recovery:  75.8 – 86.5 % |
| Stability of standard (3 days) | Difference = -50.6 %  Not stable, the standards are prepared always freshly. |

Conclusion

The analytical method for the quantification of prothioconazole residues in soil samples was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability for prothioconazole.

* + - * 1. Analytical method 2

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  In method HPLC-MS/MS detection was used.  The specificity of the method was assured by MS/MS detection. All validation parameters are in the currently required range.  See also into the present section B7 where this validated method was employed to generation of the data in paragraphs of Appendix 2: A 2.1.3.1.1,2,3; A 2.1.5.2.1,2,3; A 2.2.3.1.1,2,3; A 2.2.5.2.1,2,3.  The extraction efficiency was proven also. See on next pages A 2.1.1.1.2.3-Extraction efficiency for described details.  Independent laboratory validation ~~is ongoing and~~ is included in study plans submitted (CH-1083/2021, CH-1084/2021, CH-1082/2021, CH-1081/2021)  The studies have been submitted – relevant paragraph for evaluation details. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/02 |
| Report | Validation of an analytical method for the quantification of Difenoconazole and Prothioconazole-desthio in wheat, barley, oilseed rape and processed commodities  Longhi, D.  2021a  ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy  Report No. : 21-31 |
| Guideline(s): | Yes : SANTE/2020/12830 rev. 1 (dated 24/02/2021) ;  SANTE2017/10632 rev. 3 (dated 22/11/2017) ;  OECD ENV/JM/MONO(2007)17 ;  CEN EN 15662:2018 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The analytical method for the determination of Prothioconazole-desthio in the tested matrices (AM-GLP-STUDY-21-31) was based on the QuEChERS method (EN 15662\_2018). The instrumental determination was carried out using a HPLC-MS/MS system (high-performance liquid chromatography + triple quadrupole mass spectrometry). |

Materials and methods

***1. Materials***

***1.A. Quantification of Prothioconazole-desthio in wheat, barley, oilseed rape and processed commodities***

The quantification of Prothioconazole-desthio in wheat, barley, oilseed rape and processed commodities was assessed by HLPC/MS/MS.

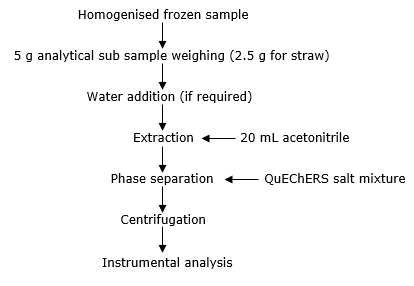
|  |  |
| --- | --- |
| HPLC: | Agilent 1290 Infinity II |
| Column: | Phenomenex Kinetix C18, 1.7 µm, 2.1 x 50 mm |
| Detector: | Agilent MS spectrometer 6470A Triple Quad |
| Column temperature: | 40 °C |
| Flow rate: | 0.6 mL/min |
| Volume of injection: | 2.5 µL |
| Retention time: | Approximatively 2.0 minutes for prothioconazole-desthio |
| Total analysis time: | 5 minutes for each run + 1 minute of post time \* 5 standard solutions |
| Divert valve: | 0 minute to waste  1.5 minutes to MS  3 minutes to waste |
| Gas temperature: | 350 °C |
| Gas flow: | 5 L/min |
| Nebulizer | 40 psi |
| Sheath gas heater: | 400 °C |
| Sheath gas flow | 12 L/min |
| Capillary: | Positive mode 3500 V  Negative mode 3000 V |
| Mobile phase: | Eluent A : Water, LC-MS grade  Eluent A : Ammonium formate  Eluent A : Formic acid  Eluent B : Methanol, LC-MS grade  Eluent B : Ammonium formate  Eluent B : Formic acid |
| Mixture | |  |  |  | | --- | --- | --- | | % A | % B | Time (min) | | 50 | 50 | 0 | | 50 | 50 | 0.5 | | 0 | 100 | 3 | |
| Analytical standards: | Prothioconazole-desthio  CAS No. : 120983-64-4  Batch No. : G1043839  Purity : 99.55 % w/w |

***2. Methods***

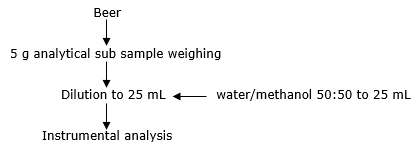
The analytical method for the quantification of prothioconazole-desthio in the tested matrices was based on the QuEChERS method (EN 15662-2018).

***2.A. Schematic diagram of the analytical method***

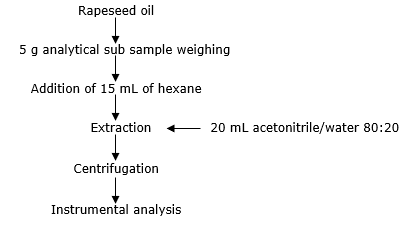
Plant matrices and processed commodities (whole plant, rapeseed seeds, wheat grain, white bread, straw)



Beer



Rapeseed oil



***2.B. Quantification of prothioconazole-desthio in wheat, barley, oilseed rape and processed commodities***

The analytical methods for the quantification of prothioconazole-desthio in wheat, barley, oilseed rape and processed commodities were first validated so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were validated in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |

Results and discussions

Method validation data is summarised in Table A.1 and A2.1 to A2.7. There is a primary test for each matrix. In view of the similar results between the primary and confirmatory tests, an independent laboratory validation (ILV) study is not required.

Table A 1: Recovery results from method validation of prothioconazole-desthio using the analytical method

| Matrix | Analyte | Fortification level | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Whole Plant (Rapeseed) | Prothioconazole-desthio | At low level :  0.01 mg/kg (LOQ) | ***Primary transition***:  85.4 % | ***Primary transition***:  8.5 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | ***Primary transition***:  82.7 % | ***Primary transition***:  8.1 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Rapeseed seeds | Prothioconazole-desthio | At low level :  0.01 mg/kg (LOQ) | ***Primary transition***:  89.3 % | ***Primary transition***:  5.1 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | ***Primary transition***:  81.5 % | ***Primary transition***:  6.5 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Grain (wheat) | Prothioconazole-desthio | At low level :  0.01 mg/kg (LOQ) | ***Primary transition***:  87.4 % | ***Primary transition***:  4.5 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | ***Primary transition***:  86.7 % | ***Primary transition***:  6.4 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Rapeseed oil | Prothioconazole-desthio | At low level :  0.01 mg/kg (LOQ) | ***Primary transition***:  79.5 % | ***Primary transition***:  6.7 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | ***Primary transition***:  77.9 % | ***Primary transition***:  1.4 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| White bread (wheat) | Prothioconazole-desthio | At low level :  0.01 mg/kg (LOQ) | ***Primary transition***:  97.7 % | ***Primary transition***:  1.8 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | ***Primary transition***:  96.0 % | ***Primary transition***:  0.42 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Beer (barley) | Prothioconazole-desthio | At low level :  0.01 mg/kg (LOQ) | ***Primary transition***:  87.3 % | ***Primary transition***:  10.8 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | ***Primary transition***:  80.4 % | ***Primary transition***:  2.3 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Straw (wheat) | Prothioconazole-desthio | At low level :  0.01 mg/kg (LOQ) | ***Primary transition***:  80.1 % | ***Primary transition***:  3.6 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | ***Primary transition***:  86.9 % | ***Primary transition***:  6.3 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

Table A 2.1: Characteristics for the analytical method used for validation of prothioconazole-desthio residues in whole plant (rapeseed)

|  | Prothioconazole-desthio |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Primarity transition:***  % interference mean = 1.5 |
| Calibration (type, number of data points) | ***Primarity transition:***  Equation : Y = 1240.335143 \* x + 41.225438  Coefficient of correlation: r² = 99.891110 |
| Calibration range | Accepted calibration range in concentration units 0.5 – 50.0 µg/L (from 20 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 8.5 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability (3 days) | Δ% = -13.4 |

Table A 2.2: Characteristics for the analytical method used for validation of prothioconazole-desthio residues in rapeseed seeds

|  | Prothioconazole-desthio |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Primarity transition:***  % interference mean = 1 |
| Calibration (type, number of data points) | ***Primarity transition:***  Equation : Y = 1033.676304 \* x – 7.425110  Coefficient of correlation: r² = 99.978284 |
| Calibration range | Accepted calibration range in concentration units 0.5 – 50.0 µg/L (from 20 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 16.4 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability (3 days) | Δ% = 0.5 |

Table A 2.3: Characteristics for the analytical method used for validation of prothioconazole-desthio residues in grain (wheat)

|  | Prothioconazole-desthio |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Primarity transition:***  % interference mean = 0.2 |
| Calibration (type, number of data points) | ***Primarity transition:***  Equation : Y = 1086.126498 \* x + 28.886023  Coefficient of correlation: r² = 99.932386 |
| Calibration range | Accepted calibration range in concentration units 0.5 – 50.0 µg/L (from 20 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 16.7 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability (3 days) | Δ% = 3.8 |

Table A 2.4: Characteristics for the analytical method used for validation of prothioconazole-desthio residues in rapeseed oil

|  | Prothioconazole-desthio |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Primarity transition:***  % interference mean = 1.5 |
| Calibration (type, number of data points) | ***Primarity transition:***  Equation : Y = 1033.676304 \* x – 7.425110  Coefficient of correlation: r² = 99.942577 |
| Calibration range | Accepted calibration range in concentration units 0.5 – 50.0 µg/L (from 20 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 13.0 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability (3 days) | Δ% = 4.8 |

Table A 2.5: Characteristics for the analytical method used for validation of prothioconazole-desthio residues in white bread (wheat)

|  | Prothioconazole-desthio |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Primarity transition:***  % interference mean = 0.25 |
| Calibration (type, number of data points) | ***Primarity transition:***  Equation : Y = 1242.277359 \* x + 138.386250  Coefficient of correlation: r² = 99.947981 |
| Calibration range | Accepted calibration range in concentration units 0.5 – 50.0 µg/L (from 20 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 10.5 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability (3 days) | Δ% = -7.5 |

Table A 2.6: Characteristics for the analytical method used for validation of prothioconazole-desthio residues in beer (barley)

|  | Prothioconazole-desthio |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Primarity transition:***  % interference mean = 0.3 |
| Calibration (type, number of data points) | ***Primarity transition:***  Equation : Y = 1096.261065 \* x + 153.749773  Coefficient of correlation: r² = 99.952307 |
| Calibration range | Accepted calibration range in concentration units 0.5 – 50.0 µg/L (from 25 % of LOQ to 150 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.0025 – 0.250 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 3.7 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability (3 days) | Δ% = 2.3 |

Table A 2.7: Characteristics for the analytical method used for validation of prothioconazole-desthio residues in straw (wheat)

|  | Prothioconazole-desthio |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Primarity transition:***  % interference mean = 11 |
| Calibration (type, number of data points) | ***Primarity transition:***  Equation : Y = 1318.462397 \* x + 97.326629  Coefficient of correlation: r² = 99.697112 |
| Calibration range | Accepted calibration range in concentration units 0.3 – 25.0 µg/L (from 24 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.0024 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 55.6 %  Significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.3 µg/L  (0.00240 mg/kg) |
| Stability (3 days) | Δ% = -6.7 |

Conclusion

The analytical method for the quantification of prothioconazole-desthio in wheat, barley, oilseed rape and processed commodities was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the linearity, accuracy, precision, limits of determination and quantification and stability for prothioconazole-desthio.

Independent laboratory validation

~~Not required.~~

~~The proposed ILV Rigamonti, E. 2022 / Report No. CH-1083/2021 is ongoing and wiil be submitted.~~

Determination of Prothio-desthio in straw (wheat)

|  |  |
| --- | --- |
| Comments of zRMS: | The submitted ILV of the analytical method adjusted and validated in GLP study Code GLP-STUDY-21-31 by LabAnalysis s.r.l. for the determination of Difenoconazole and Prothioconazole-desthio residues in Straw (wheat) has been accepted.  The analytical method, using the HPLC/MS/MS in MRM mode is highly specific and gave both quantification and identification data, so the confirmatory method is not necessary.  All recovery values for each analyte at both fortification levels (L.O.Q and 10 x LOQ) resulted to be in the range 70 to 120 %, with an RSD% lower than 20% therefore the analytical method can be considered suitable to quantify Difenoconazole and Prothioconazole-desthio in Straw (wheat) samples with an established L.O.Q of 0.010 mg/kg. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/16 |
| Report | Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of Difenoconazole and Prothio-desthio in Straw (wheat)  Report No.: CH-1081/2021  Nichetti, S. (2022a)  ChemService S.r.l Controlli e Ricerche - Novate Milanese (MI) - Italy |
| Guideline(s): | Yes  SANTE/2020/12830 rev. 1 dated 24/02/21: Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes. |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

Materials and methods

|  |  |
| --- | --- |
| **Principle of the method** | The Test Facility conducted an Independent Laboratory Validation (ILV) of the analytical method adjusted and validated in GLP study Code GLP-STUDY-21-31 by LabAnalysis s.r.l. for the determination of Prothioconazole-desthio residues in Straw (wheat). |

**Materials:**

|  |  |
| --- | --- |
| UHPLC/MS-QQQ: | Agilent mod. 1290, equipped with binary pump, autosampler coupled with an Agilent Jet Stream (AJS) ESI |
| Column: | Kinetex C18 100 Å, 1.7 μm, 50 x 2.1 mm i.d. |
| Detector: | MS Triple quadrupole |
| Ionisation type: | Electrospray (ESI) |
| Polarity: | Positive ion mode |
| Scan type: | MS/MS, multiple reaction monitoring |
| Drying gas temperature: | 350 °C |
| Drying gas flow: | 5 L/min |
| Nebulizer pressure: | 40 psi |
| Capillary voltage: | 3500 V |
|  |  |
| Analytical standards: | Prothioconazole-desthio  Batch No. : G1043839  Purity : 99.55 %  Expiry date: November 1, 2025 |
|  |  |
| Reagents: | Water, HPLC grade, obtained by the Lab Water Purification System  Methanol, HPLC grade  Acetonitrile, HPLC grade  Ammonium formate, high purity (>99%) for mass spectroscopy  Formic acid, high purity for mass spectroscopy |
|  |  |
| Matrix: | Straw (wheat)  Stored at -20°C in the dark |

**Methods:**

The analytical methods for the determination of prothioconazole-desthio in straw (wheat) were confirmed so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were confirmed in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A 11: Recovery results from independent laboratory validation of prothioconazole-desthio using the analytical method

| Matrix | Analyte | Fortification level (mg/kg) (n = 5) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Straw (wheat) | Prothioconazole-desthio  (product ion: 69.8 m/z)\* | At low level :  0.01 mg/kg (LOQ) | 86.3 | 12.60 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 83.4 | 3.16 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Prothioconazole-desthio  (product ion: 125.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 87.5 | 15.81 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 84.4 | 1.12 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

\* For the prothioconazole-desthio, it was not possible to find both products ions with m/z > 100, as required by SANTE/2020/12830 rev.1. This analyte fragmented only in two products ions, one of them with m/z < 100.

Table A 12: Characteristics for the analytical method used for independent laboratory validation of prothioconazole-desthio residues in straw (wheat)

|  | Prothioconazole-desthio |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Product ion (m/z = 69.8):***  % interference mean = 0.0  ***Product ion (m/z = 125):***  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Product ion (m/z = 69.8):***  Equation : Y = 66 \* x + 64  Coefficient of correlation: r² = 99.787  ***Product ion (m/z = 125):***  Equation : Y = 46 \* x + 38  Coefficient of correlation: r² = 99.819 |
| Calibration range | Accepted calibration range in concentration units 0.34 – 28.12 µg/L  Corresponding calibration range in mass ratio units for the sample (0.0024 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 33 %  Significant matrix effects for Prothioconazole-desthio residues in Straw (wheat) matrix were found (> ± 20%).  Therefore, the quantification should be performed using working standard solutions prepared in Straw (wheat) (matrix matched calibration). |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.30 µg/L  (0.0024 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-31 |

Conclusion

The independent laboratory validation for the quantification of prothioconazole-desthio in straw (wheat) commodities was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability.

Determination of Prothio-desthio in grain (wheat)

|  |  |
| --- | --- |
| Comments of zRMS: | The submitted ILV of the analytical method adjusted and validated in GLP study Code GLP-STUDY-21-31 by LabAnalysis s.r.l. for the determination of Difenoconazole and Prothioconazole-desthio residues in Grain (wheat) has been accepted.  The analytical method, using the HPLC/MS/MS in MRM mode is highly specific and gave both quantification and identification data, so the confirmatory method is not necessary.  All recovery values for each analyte at both fortification levels (L.O.Q and 10 x LOQ) resulted to be in the range 70 to 120 %, with an RSD% lower than 20% therefore the analytical method can be considered suitable to quantify Difenoconazole and Prothioconazole-desthio in Grain (wheat) samples with an established L.O.Q of 0.010 mg/kg. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/17 |
| Report | Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of Difenoconazole and Prothio-desthio in Straw (wheat)  Report No.: CH-1082/2021  Nichetti, S. (2022b)  ChemService S.r.l Controlli e Ricerche - Novate Milanese (MI) - Italy |
| Guideline(s): | Yes  SANTE/2020/12830 rev. 1 dated 24/02/21: Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes. |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

Materials and methods

|  |  |
| --- | --- |
| **Principle of the method** | The Test Facility conducted an Independent Laboratory Validation (ILV) of the analytical method adjusted and validated in GLP study Code GLP-STUDY-21-31 by LabAnalysis s.r.l. for the determination of Prothioconazole-desthio residues in grain (wheat). |

**Materials:**

|  |  |
| --- | --- |
| UHPLC/MS-QQQ: | Agilent mod. 1290, equipped with binary pump, autosampler coupled with an Agilent Jet Stream (AJS) ESI |
| Column: | Kinetex C18 100 Å, 1.7 μm, 50 x 2.1 mm i.d. |
| Detector: | MS Triple quadrupole |
| Ionisation type: | Electrospray (ESI) |
| Polarity: | Positive ion mode |
| Scan type: | MS/MS, multiple reaction monitoring |
| Drying gas temperature: | 350 °C |
| Drying gas flow: | 5 L/min |
| Nebulizer pressure: | 40 psi |
| Capillary voltage: | 3500 V |
|  |  |
| Analytical standards: | Prothioconazole-desthio  Batch No. : G1043839  Purity : 99.55 %  Expiry date: November 1, 2025 |
|  |  |
| Reagents: | Water, HPLC grade, obtained by the Lab Water Purification System  Methanol, HPLC grade  Acetonitrile, HPLC grade  Ammonium formate, high purity (>99%) for mass spectroscopy  Formic acid, high purity for mass spectroscopy |
|  |  |
| Matrix: | Grain (wheat)  Stored at -20°C in the dark |

**Methods:**

The analytical methods for the determination of prothioconazole-desthio in grain (wheat) were confirmed so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were confirmed in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A 13: Recovery results from independent laboratory validation of prothioconazole-desthio using the analytical method

| Matrix | Analyte | Fortification level (mg/kg) (n = 5) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Grain (wheat) | Prothioconazole-desthio  (product ion: 69.8 m/z)\* | At low level :  0.01 mg/kg (LOQ) | 100.5 | 2.00 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 100.1 | 2.23 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Prothioconazole-desthio  (product ion: 125.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 97.2 | 4.13 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 100.3 | 2.64 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

\* For the prothioconazole-desthio, it was not possible to find both products ions with m/z > 100, as required by SANTE/2020/12830 rev.1. This analyte fragmented only in two products ions, one of them with m/z < 100.

Table A 14: Characteristics for the analytical method used for independent laboratory validation of prothioconazole-desthio residues in grain (wheat)

|  | Prothioconazole-desthio |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Product ion (m/z = 69.8):***  % interference mean = 0.0  ***Product ion (m/z = 125):***  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Product ion (m/z = 69.8):***  Equation : Y = 95 \* x - 21  Coefficient of correlation: r² = 99.844  ***Product ion (m/z = 125):***  Equation : Y = 63 \* x - 18  Coefficient of correlation: r² = 99.790 |
| Calibration range | Accepted calibration range in concentration units 0.54 – 54.25 µg/L  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 18 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-31 |

Conclusion

The independent laboratory validation for the quantification of prothioconazole-desthio in grain (wheat) was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability.

Determination of Prothio-desthio in rapeseed seeds

|  |  |
| --- | --- |
| Comments of zRMS: | The submitted ILV of the analytical method adjusted and validated in GLP study Code GLP-STUDY-21-31 by LabAnalysis s.r.l. for the determination of Difenoconazole and Prothioconazole-desthio residues in Rapeseed Seeds has been accepted.  The analytical method, using the HPLC/MS/MS in MRM mode is highly specific and gave both quantification and identification data, so the confirmatory method is not necessary.  All recovery values for each analyte at both fortification levels (L.O.Q and 10 x LOQ) resulted to be in the range 70 to 120 %, with an RSD% lower than 20% therefore the analytical method can be considered suitable to quantify Difenoconazole and Prothioconazole-desthio in Rapeseed Seeds samples with an established LOQ of 0.010 mg/kg. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/18 |
| Report | Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of Difenoconazole and Prothio-desthio in Rapeseed seeds  Report No.: CH-1083/2021  Nichetti, S. (2022c)  ChemService S.r.l Controlli e Ricerche - Novate Milanese (MI) - Italy |
| Guideline(s): | Yes  SANTE/2020/12830 rev. 1 dated 24/02/21: Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes. |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

Materials and methods

|  |  |
| --- | --- |
| **Principle of the method** | The Test Facility conducted an Independent Laboratory Validation (ILV) of the analytical method adjusted and validated in GLP study Code GLP-STUDY-21-31 by LabAnalysis s.r.l. for the determination of Prothioconazole-desthio residues in rapeseed seeds. |

**Materials:**

|  |  |
| --- | --- |
| UHPLC/MS-QQQ: | Agilent mod. 1290, equipped with binary pump, autosampler coupled with an Agilent Jet Stream (AJS) ESI |
| Column: | Kinetex C18 100 Å, 1.7 μm, 50 x 2.1 mm i.d. |
| Detector: | MS Triple quadrupole |
| Ionisation type: | Electrospray (ESI) |
| Polarity: | Positive ion mode |
| Scan type: | MS/MS, multiple reaction monitoring |
| Drying gas temperature: | 350 °C |
| Drying gas flow: | 5 L/min |
| Nebulizer pressure: | 40 psi |
| Capillary voltage: | 3500 V |
|  |  |
| Analytical standards: | Prothioconazole-desthio  Batch No. : G1043839  Purity : 99.55 %  Expiry date: November 1, 2025 |
|  |  |
| Reagents: | Water, HPLC grade, obtained by the Lab Water Purification System  Methanol, HPLC grade  Acetonitrile, HPLC grade  Ammonium formate, high purity (>99%) for mass spectroscopy  Formic acid, high purity for mass spectroscopy |
|  |  |
| Matrix: | Rapeseed seeds  Stored at -20°C in the dark |

**Methods:**

The analytical methods for the determination of prothioconazole-desthio in rapeseed seeds were confirmed so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were confirmed in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A 15: Recovery results from independent laboratory validation of prothioconazole-desthio using the analytical method

| Matrix | Analyte | Fortification level (mg/kg) (n = 5) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Rapeseed seeds | Prothioconazole-desthio  (product ion: 69.8 m/z)\* | At low level :  0.01 mg/kg (LOQ) | 89.4 | 16.04 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 97.9 | 1.46 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Prothioconazole-desthio  (product ion: 125.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 98.2 | 16.33 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 98.7 | 2.53 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

\* For the prothioconazole-desthio, it was not possible to find both products ions with m/z > 100, as required by SANTE/2020/12830 rev.1. This analyte fragmented only in two products ions, one of them with m/z < 100.

Table A 16: Characteristics for the analytical method used for independent laboratory validation of prothioconazole-desthio residues in rapeseed seeds

|  | Prothioconazole-desthio |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Product ion (m/z = 69.8):***  % interference mean = 0.0  ***Product ion (m/z = 125):***  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Product ion (m/z = 69.8):***  Equation : Y = 78 \* x + 1  Coefficient of correlation: r² = 99.996  ***Product ion (m/z = 125):***  Equation : Y = 52 \* x + 8  Coefficient of correlation: r² = 99.989 |
| Calibration range | Accepted calibration range in concentration units 0.56 – 55.75 µg/L  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 17 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-31 |

Conclusion

The independent laboratory validation for the quantification of prothioconazole-desthio in rapeseed seeds was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability.

Determination of Prothio-desthio in Whole Plant (rapeseed)

|  |  |
| --- | --- |
| Comments of zRMS: | The submitted ILV of the analytical method adjusted and validated in GLP study Code GLP-STUDY-21-31 by LabAnalysis s.r.l. for the determination of Difenoconazole and Prothioconazole-desthio residues in Whole Plant (Rapeseed) has been accepted.  The analytical method, using the HPLC/MS/MS in MRM mode is highly specific and gave both quantification and identification data, so the confirmatory method is not necessary.  All recovery values for each analyte at both fortification levels (L.O.Q and 10 x LOQ) resulted to be in the range 70 to 120 %, with an RSD% lower than 20% therefore the analytical method can be considered suitable to quantify Difenoconazole and Prothioconazole-desthio in Whole Plant (Rapeseed) samples with an established LOQ of 0.010 mg/kg. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/19 |
| Report | Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of Difenoconazole and Prothio-desthio in Whole Plant (Rapeseed)  Report No.: CH-1084/2021  Nichetti, S. (2022d)  ChemService S.r.l Controlli e Ricerche - Novate Milanese (MI) - Italy |
| Guideline(s): | Yes  SANTE/2020/12830 rev. 1 dated 24/02/21: Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes. |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

Materials and methods

|  |  |
| --- | --- |
| **Principle of the method** | The Test Facility conducted an Independent Laboratory Validation (ILV) of the analytical method adjusted and validated in GLP study Code GLP-STUDY-21-31 by LabAnalysis s.r.l. for the determination of Prothioconazole-desthio residues in whole plant (rapeseed). |

**Materials:**

|  |  |
| --- | --- |
| UHPLC/MS-QQQ: | Agilent mod. 1290, equipped with binary pump, autosampler coupled with an Agilent Jet Stream (AJS) ESI |
| Column: | Kinetex C18 100 Å, 1.7 μm, 50 x 2.1 mm i.d. |
| Detector: | MS Triple quadrupole |
| Ionisation type: | Electrospray (ESI) |
| Polarity: | Positive ion mode |
| Scan type: | MS/MS, multiple reaction monitoring |
| Drying gas temperature: | 350 °C |
| Drying gas flow: | 5 L/min |
| Nebulizer pressure: | 40 psi |
| Capillary voltage: | 3500 V |
|  |  |
| Analytical standards: | Prothioconazole-desthio  Batch No. : G1043839  Purity : 99.55 %  Expiry date: November 1, 2025 |
|  |  |
| Reagents: | Water, HPLC grade, obtained by the Lab Water Purification System  Methanol, HPLC grade  Acetonitrile, HPLC grade  Ammonium formate, high purity (>99%) for mass spectroscopy  Formic acid, high purity for mass spectroscopy |
|  |  |
| Matrix: | Whole plant (rapeseed)  Stored at -20°C in the dark |

**Methods:**

The analytical methods for the determination of prothioconazole-desthio in whole plant (rapeseed) were confirmed so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were confirmed in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A 17: Recovery results from independent laboratory validation of prothioconazole-desthio using the analytical method

| Matrix | Analyte | Fortification level (mg/kg) (n = 5) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Whole plant (Rapeseed) | Prothioconazole-desthio  (product ion: 69.8 m/z)\* | At low level :  0.01 mg/kg (LOQ) | 105.2 | 5.58 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 100.3 | 0.95 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Prothioconazole-desthio  (product ion: 125.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 107.8 | 8.37 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 101.0 | 1.17 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

\* For the prothioconazole-desthio, it was not possible to find both products ions with m/z > 100, as required by SANTE/2020/12830 rev.1. This analyte fragmented only in two products ions, one of them with m/z < 100.

Table A 18: Characteristics for the analytical method used for independent laboratory validation of prothioconazole-desthio residues in whole plant (rapeseed)

|  | Prothioconazole-desthio |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Product ion (m/z = 69.8):***  % interference mean = 0.0  ***Product ion (m/z = 125):***  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Product ion (m/z = 69.8):***  Equation : Y = 82 \* x + 7  Coefficient of correlation: r² = 99.982  ***Product ion (m/z = 125):***  Equation : Y = 53 \* x + 25  Coefficient of correlation: r² = 99.952 |
| Calibration range | Accepted calibration range in concentration units 0.55 – 54.75 µg/L  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 17 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-31 |

Conclusion

The independent laboratory validation for the quantification of prothioconazole-desthio in whole plant (rapeseed) was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability.

Confirmatory method

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  In method HPLC-MS/MS detection was used. A simultaneous confirmation to the primary detection was used using the HPLC-MS/MS, monitoring additional SRM transitions.  The specificity of the method was assured by MS/MS detection. All validation parameters are in the currently required range.  See also into the present section B7 where this validated method was employed to generation of the data in paragraphs of Appendix 2: A 2.1.3.1.1,2,3; A 2.1.5.2.1,2,3; A 2.2.3.1.1,2,3; A 2.2.5.2.1,2,3.  Independent laboratory validation ~~is ongoing and~~ is included in study plans submitted (CH-1083/2021, CH-1084/2021, CH-1082/2021, CH-1081/2021).  The studies have been submitted – see relevant paragraph for evaluation details. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/02 |
| Report | Validation of an analytical method for the quantification of Difenoconazole and Prothioconazole-desthio in wheat, barley, oilseed rape and processed commodities  Longhi, D.  2021a  ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy  Report No. : 21-31 |
| Guideline(s): | Yes : SANTE/2020/12830 rev. 1 (dated 24/02/2021) ;  SANTE2017/10632 rev. 3 (dated 22/11/2017) ;  OECD ENV/JM/MONO(2007)17 ;  CEN EN 15662:2018 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The analytical method for the determination of Prothioconazole-desthio in the tested matrices (AM-GLP-STUDY-21-31) was based on the QuEChERS method (EN 15662\_2018). The instrumental determination was carried out using a HPLC-MS/MS system (high-performance liquid chromatography + triple quadrupole mass spectrometry). |

Materials and methods

***1. Materials***

***1.A. Quantification of Prothioconazole-desthio in wheat, barley, oilseed rape and processed commodities***

The quantification of Prothioconazole-desthio in wheat, barley, oilseed rape and processed commodities was assessed by HLPC/MS/MS.

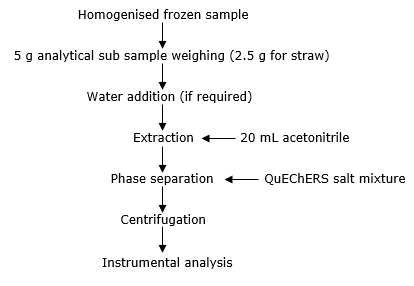
|  |  |
| --- | --- |
| HPLC: | Agilent 1290 Infinity II |
| Column: | Phenomenex Kinetix C18, 1.7 µm, 2.1 x 50 mm |
| Detector: | Agilent MS spectrometer 6470A Triple Quad |
| Column temperature: | 40 °C |
| Flow rate: | 0.6 mL/min |
| Volume of injection: | 2.5 µL |
| Retention time: | Approximatively 2.0 minutes for prothioconazole-desthio |
| Total analysis time: | 5 minutes for each run + 1 minute of post time \* 5 standard solutions |
| Divert valve: | 0 minute to waste  1.5 minutes to MS  3 minutes to waste |
| Gas temperature: | 350 °C |
| Gas flow: | 5 L/min |
| Nebulizer | 40 psi |
| Sheath gas heater: | 400 °C |
| Sheath gas flow | 12 L/min |
| Capillary: | Positive mode 3500 V  Negative mode 3000 V |
| Mobile phase: | Eluent A : Water, LC-MS grade  Eluent A : Ammonium formate  Eluent A : Formic acid  Eluent B : Methanol, LC-MS grade  Eluent B : Ammonium formate  Eluent B : Formic acid |
| Mixture | |  |  |  | | --- | --- | --- | | % A | % B | Time (min) | | 50 | 50 | 0 | | 50 | 50 | 0.5 | | 0 | 100 | 3 | |
| Analytical standards: | Prothioconazole-desthio  CAS No. : 120983-64-4  Batch No. : G1043839  Purity : 99.55 % w/w |

***2. Methods***

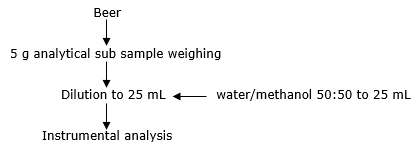
The confirmatory method for the quantification of prothioconazole-desthio in the tested matrices was based on the QuEChERS method (EN 15662-2018).

***2.A. Schematic diagram of the analytical method***

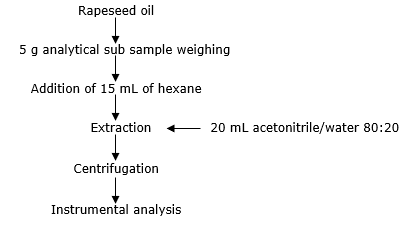
Plant matrices and processed commodities (whole plant, rapeseed seeds, wheat grain, white bread, straw)



Beer



Rapeseed oil



***2.B. Quantification of prothioconazole-desthio in wheat, barley, oilseed rape and processed commodities***

The confirmatory methods for the quantification of prothioconazole-desthio in wheat, barley, oilseed rape and processed commodities were first validated so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were validated in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |

Results and discussions

Confirmatory method validation data can be summarised in tables below ~~Table A.5 and A6.1 to A6.7~~. There are for each matrix a confirmatory test.

Table A~~5~~ 19: Recovery results from confirmatory method validation of prothioconazole-desthio using the analytical method

| Matrix | Analyte | Fortification level | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Whole Plant (Rapeseed) | Prothioconazole-desthio | At low level :  0.01 mg/kg (LOQ) | ***Confirmatory transition:***  *86.5 %* | ***Confirmatory transition:***  *8.0 %* | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | ***Confirmatory transition:***  *84.9 %* | ***Confirmatory transition:***  *8.1 %* | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Rapeseed seeds | Prothioconazole-desthio | At low level :  0.01 mg/kg (LOQ) | ***Confirmatory transition:***  *87.3 %* | ***Confirmatory transition:***  *5.0 %* | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | ***Confirmatory transition:***  *81.2 %* | ***Confirmatory transition:***  *7.8 %* | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Grain (wheat) | Prothioconazole-desthio | At low level :  0.01 mg/kg (LOQ) | ***Confirmatory transition:***  *86.7 %* | ***Confirmatory transition:***  *4.8 %* | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | ***Confirmatory transition:***  *88.6 %* | ***Confirmatory transition:***  *6.0 %* | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Rapeseed oil | Prothioconazole-desthio | At low level :  0.01 mg/kg (LOQ) | ***Confirmatory transition:***  *80.7 %* | ***Confirmatory transition:****7.2 %* | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | ***Confirmatory transition:***  *78.5 %* | ***Confirmatory transition:***  *1.1 %* | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| White bread (wheat) | Prothioconazole-desthio | At low level :  0.01 mg/kg (LOQ) | ***Confirmatory transition:***  *98.0 %* | ***Confirmatory transition:***  *2.5 %* | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | ***Confirmatory transition:***  *95.9 %* | ***Confirmatory transition:***  *0.47 %* | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Beer (barley) | Prothioconazole-desthio | At low level :  0.01 mg/kg (LOQ) | ***Confirmatory transition:***  *87.2 %* | ***Confirmatory transition:***  *8.8 %* | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | ***Confirmatory transition:***  *81.4 %* | ***Confirmatory transition:***  *2.9 %* | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Straw (wheat) | Prothioconazole-desthio | At low level :  0.01 mg/kg (LOQ) | ***Confirmatory transition:***  *82.9 %* | ***Confirmatory transition:***  *6.8 %* | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | ***Confirmatory transition:***  *86.4 %* | ***Confirmatory transition:***  *6.2 %* | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

Table A~~6.1~~ 20: Characteristics for the confirmatory method used for validation of prothioconazole-desthio residues in whole plant (rapeseed)

|  | Prothioconazole-desthio |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Confirmatory transition:***  % interference mean = 1 |
| Calibration (type, number of data points) | ***Confirmatory transition:***  Equation : Y = 735.122624 \* x + 38.281778  Coefficient of correlation: r² = 99.945879 |
| Calibration range | Accepted calibration range in concentration units 0.5 – 50.0 µg/L (from 20 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 8.5 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability (3 days) | Δ% = -13.4 |

Table A~~6.2~~ 21: Characteristics for the confirmatory method used for validation of prothioconazole-desthio residues in rapeseed seeds

|  | Prothioconazole-desthio |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Confirmatory transition:***  % interference mean = 3.5 |
| Calibration (type, number of data points) | ***Confirmatory transition:***  Equation : Y = 615.252494 \* x + 19.738163  Coefficient of correlation: r² = 99.982327 |
| Calibration range | Accepted calibration range in concentration units 0.5 – 50.0 µg/L (from 20 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 16.4 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability (3 days) | Δ% = 0.5 |

Table A~~6.3~~ 22:: Characteristics for the confirmatory method used for validation of prothioconazole-desthio residues in grain (wheat)

|  | Prothioconazole-desthio |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Confirmatory transition:***  % interference mean = 0.4 |
| Calibration (type, number of data points) | ***Confirmatory transition:***  Equation : Y = 631.657801 \* x + 37.522084  Coefficient of correlation: r² = 99.944797 |
| Calibration range | Accepted calibration range in concentration units 0.5 – 50.0 µg/L (from 20 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 16.7 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability (3 days) | Δ% = 3.8 |

Table A~~6.4~~ 23: Characteristics for the confirmatory method used for validation of prothioconazole-desthio residues in rapeseed oil

|  | Prothioconazole-desthio |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Confirmatory transition:***  % interference mean = 0.9 |
| Calibration (type, number of data points) | ***Confirmatory transition:***  Equation : Y = 1033.676304 \* x – 7.425110  Coefficient of correlation: r² = 99.874270 |
| Calibration range | Accepted calibration range in concentration units 0.5 – 50.0 µg/L (from 20 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 13.0 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability (3 days) | Δ% = 4.8 |

Table A~~6.5~~ 24: Characteristics for the confirmatory method used for validation of prothioconazole-desthio residues in white bread (wheat)

|  | Prothioconazole-desthio |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Confirmatory transition:***  % interference mean = 0.2 |
| Calibration (type, number of data points) | ***Confirmatory transition:***  Equation : Y = 750.062337\* x + 61.994929  Coefficient of correlation: r² = 99.903985 |
| Calibration range | Accepted calibration range in concentration units 0.5 – 50.0 µg/L (from 20 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 10.5 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability (3 days) | Δ% = -7.5 |

Table A~~6.6~~ 25: Characteristics for the confirmatory method used for validation of prothioconazole-desthio residues in beer (barley)

|  | Prothioconazole-desthio |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Confirmatory transition:***  % interference mean = 0.8 |
| Calibration (type, number of data points) | ***Confirmatory transition:***  Equation : Y = 650.556791 \* x + 95.678549  Coefficient of correlation: r² = 99.956099 |
| Calibration range | Accepted calibration range in concentration units 0.5 – 50.0 µg/L (from 25 % of LOQ to 150 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.0025 – 0.250 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 3.7 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability (3 days) | Δ% = 2.3 |

Table A~~6.7~~ 26: Characteristics for the confirmatory method used for validation of prothioconazole-desthio residues in straw (wheat)

|  | Prothioconazole-desthio |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Confirmatory transition:***  % interference mean = 16 |
| Calibration (type, number of data points) | ***Confirmatory transition:***  Equation : Y = 919.531136 \* x + 78.23008  Coefficient of correlation: r² = 99.900859 |
| Calibration range | Accepted calibration range in concentration units 0.3 – 25.0 µg/L (from 24 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.0024 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 55.6 %  Significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.3 µg/L  (0.00240 mg/kg) |
| Stability (3 days) | Δ% = -6.7 |

Conclusion

The confirmatory method for the quantification of prothioconazole-desthio in wheat, barley, oilseed rape and processed commodities was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the linearity, accuracy, precision, limits of determination and quantification and stability for prothioconazole-desthio.

Extraction efficiency

Extraction efficiency is guided by:

* European Commission (2017): SANTE 2017/10632 rev. 3, dated 22 November 2017: Technical Guideline on the Evaluation of Extraction Efficiency of Residue Analytical Methods.
* “European Committee for Standardization (CEN) EN 15662:2018. Foods of plant origin - Multimethod for the determination of pesticide residues using GC- and LC-based analysis following acetonitrile extraction/partitioning and clean-up by dispersive SPE - Modular QuEChERS-method”.

Aliquots of 5 g of specimen (2.5 for straw) were taken from the homogenized frozen samples and put in a 50 mL screw capped centrifuge PE test tube followed by the addition of the following amounts of LC-MS grade water:

|  |  |
| --- | --- |
| **Matrix** | **Water added (mL)** |
| Whole Plant (rapeseed) | 0 |
| Rapeseed seeds | 10 |
| Wheat (grain) | 10 |
| Wheat (straw) | 10 |
| Wheat (white bread) | 10 |

Then, 20 mL of acetonitrile were added and the obtained mixture was vigorously shaken for one minute. After that, a packet of QuEChERS extraction salt (4.0 g MgSO4, 1.0 g NaCl, 1.0 g trisodium citrate dehydrate, 0.5 g disodium hydrogen citrate sesquihydrate) was added and the mixture shaken again. The separation of the organic phase was achieved by centrifugation at 4500 rpm for 5 minutes. An aliquot of about 1 mL the organic supernatant was taken, transferred in a 2 mL HPLC glass vial and analyzed with a HPLC-MS/MS system.

**Beer**

Beer was analyzed after a 5-fold dilution in a mixture of water/methanol 50:50 (about 5 g to 25 mL) and directly analyzed with a HPLC-MS/MS system.

**Rapeseed oil**

An aliquot of about 5 g of rapeseed oil was put in a 50 mL screw capped centrifuge PE test tube. Then, 15 mL of hexane were added, followed by 20 mL of a mixture of acetonitrile/water 80:20. The mixture was vigorously shaken for about one minute and then centrifuged at 4500 rpm for 5 minutes, in order to obtain 2 phases. An aliquot of the lower organic phase (acetonitrile) was taken and transferred to a 2 mL glass HPLC vial for final determination with a HPLC-MS/MS system.

* + - * 1. Analytical method 3

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  In method HPLC-MS/MS detection was used. Two transitions were monitored.  The specificity of the method was assured by MS/MS detection. All validation parameters are in the currently required range.  The method is suitable for the intended purposes. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/03 |
| Report | Validation of the Analytical Method for the Determination of Difenoconazole and Prothioconazole residues in aqueous samples of Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560 coming from the Ecotoxicological tests  Garagna, D.  2021b  ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy  Report No. : CH – 0227/2021  + Amendment report No.1 to final report (Amdm1\_KCP 5.1.2/03) |
| Guideline(s): | Yes : SANTE/2020/12830, rev. 1 dated 24/02/21 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The determination of Prothioconazole residues is performed by HPLC using an external standard and MS triple quadrupole detector.  Its quantification is achieved using the calibration curve obtained by plotting standard solutions concentrations and the corresponding peak area. |

Materials and methods

***1. Materials***

***1.A. Determination of prothioconazole residues in aqueous samples (KCP 5.2/03)***

The determination of prothioconazole residues in aqueous samples was assessed by HLPC/MS-QQQ.

|  |  |
| --- | --- |
| HPLC: | Agilent Technologies 1200 System |
| Column: | Zorbax Eclipse Plus C18, 3.5 µm, 100 x 4.6 mm i.d. |
| Detector | MS Triple quadrupole (Scan in MRM mode) |
| Mobile phase: | Water, HPLC grade  Formic acid  Ammonium formate, for HPLC  Acetonitrile, HPLC grade  QuEChERS Extract Pouch |
| Analytical standards: | Prothioconazole  CAS No. : 178928-70-6  Batch No. : BCBT2374  Purity : 99.6 %  Expiry date: November 1, 2021 |

***2. Methods***

***2.A. Preparation of aqueous samples***

**Alga growth medium:**

It was prepared as described in Annex 3 of OECD 201, 2011 (Freshwater Alga and Cyanobacteria, Growth Inhibition Test).

The composition is as follows:

In de-ionized water (conductivity < 5 μS/cm), analytical grade, the following salts are added to a defined final nominal concentration:

* Macro-nutrients:

|  |  |
| --- | --- |
| NaHCO3 | 50.0 mg/L |
| CaCl2 x 2 H2O | 18.0 mg/L |
| NH4Cl | 15.0 mg/L |
| MgSO4 x 7 H2O | 15.0 mg/L |
| MgCl2 x 6 H2O | 12.0 mg/L |
| KH2PO4 | 1.6 mg/L |

* Trace elements:

|  |  |
| --- | --- |
| Na2EDTA x 2 H2O | 100.0 μg/L |
| FeCl3 x 6 H2O | 64.0 μg/L |
| MnCl2 x 4 H2O | 415.0 μg/L |
| H3BO3 | 185.0 μg/L |
| Na2MoO4 x 2 H2O | 7.0 μg/L |
| ZnCl2 | 3.0 μg/L |
| CoCl2 x 6 H2O | 1.5 μg/L |
| CuCl2 x 2 H2O | 0.01 μg/L |

**Reconstituted water:**

It was prepared as described in Annex 3 of OECD No. 202, 2004 (*Daphnia* sp., Acute Immobilization Test”).

The composition is as follows:

In de-ionized water (conductivity < 5 μS/cm), analytical grade, the following salts are added to a defined final nominal concentration:

|  |  |
| --- | --- |
| CaCl2 x 2 H2O | 2.0 mmol/L (= 294.0 mg/L) |
| MgSO4 x 7 H2O | 0.5 mmol/L (= 123.3 mg/L) |
| NaHCO3 | 0.771 mmol/L (= 64.8 mg/L) |
| KCl | 0.078 mmol/L (= 5.8 mg/L) |

**Elends M4:**

It was prepared as described in Annex 2 of OECD No. 211, 2012 (Daphnia magna, Reproduction Test). The composition is as follows:

In de-ionized water (conductivity < 5 μS/cm), analytical grade, the following salts are added to a defined final nominal concentration:

* Macro-nutrients:

|  |  |
| --- | --- |
| CaCl2 x 2 H2O | 293.8 mg/L |
| MgSO4 x 7 H2O | 123.3 mg/L |
| KCl | 5.8 mg/L |
| NaHCO3 | 64.8 mg/L |
| Na2SiO3 x 9 H2O | 10 mg/L |
| NaNO3 | 0.274 mg/L |
| KH2PO4 | 0.143 mg/L |
| K2HPO4 | 0.184 mg/L |
| Combined vitamine stock | - |

* Trace elements:

|  |  |
| --- | --- |
| H3BO3 | 2.860 mg/L |
| MnCl2 x 4 H2O | 0.361 mg/L |
| LiCl | 0.306 mg/L |
| RbCl | 0.071 mg/L |
| SrCl2 x 6 H2O | 0.152 mg/L |
| NaBr | 0.016 mg/L |
| MoNa2O4 x 2 H2O | 0.063 mg/L |
| CuCl2 x 2 H2O | 0.017 mg/L |
| ZnCl | 0.013 mg/L |
| CoCl2 x 6 H2O | 0.010 mg/L |
| KI | 0.003 mg/L |
| Na2SeO3 | 0.002 mg/L |
| NH4VO3 | 0.001 mg/L |
| Na2EDTA x 2 H2O | 2.500 mg/L |
| FeSO4 x 7 H2O | 0.996 mg/L |

***2.B. Determination of prothioconazole residues in aqueous samples (KCP 5.2/03)***

The analytical methods for the determination of prothioconazole residues in aqueous samples were first validated so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were validated in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A~~7.1~~ 27: Matric effects results from method validation of prothioconazole using the analytical method

| Matrix effect | Analyte | Slope of Algal growth medium | Slope of Reconstituted water | Slope of Elendt M4 | Comments |
| --- | --- | --- | --- | --- | --- |
| Result  < ± 20 % | Prothioconazole | 4.2 | -9.2 | 52.9 | No comments |

Table A~~7.2~~ 28: Recovery results from method validation of prothioconazole using the analytical method

| Matrix | Analyte | Fortification level  (n = x) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Reconstituted water | Prothioconazole | Low level:  10.6 µg/L  (mean found)  N = 5 | 91.8 | 11 | No comments |
| High level:  3.07 mg/L (mean found)  N = 5 | 88.5 | 12 | No comments |
| Alga growth medium | Prothioconazole | Low level:  11.4 µg/L  (mean found)  N = 5 | 103.7 | 6 | No comments |
| High level:  15.91 mg/L (mean found)  N = 5 | 103.2 | 1 | No comments |
| Elendt M4 | Prothioconazole | Low level:  2.13 µg/L  (mean found)  N = 5 | 97.1 | 6 | No comments |
| High level:  341.11 µg/L (mean found)  N = 5 | 104.3 | 10 | No comments |

Table A~~8~~ 29: Characteristics for the analytical method used for validation of prothioconazole residues for purposes of aqueous ecotoxicological tests

|  | Prothioconazole |
| --- | --- |
| Specificity | Untreated blank < 30 % LOQ  Result : 0.0 % for Alga growth medium  Result : 0.0 % for Reconstituted water  Result : 0.0 % for Elendt M4  These % ratio (Blank vs LOQ) demontrate that there was no interference of the blank matrix at the retention time of the analyte. |
| Linearity / Calibration | **Reconstituted water :**  ***Quantifier transition***  ***m/z 344 🡪 m/z 326 :***  The calibration curve (µg/L):   * was considered as valid over 2.0 – 199.2 µg/L.   Equation (µg/L)  y = 112 \* x – 144  Correlation coefficient:  r² = 99.991  ***Quantifier1 transition***  ***m/z 344 🡪 m/z 189.1 :***  The calibration curve (µg/L):   * was considered as valid over 2.0 – 199.2 µg/L.   Equation (µg/L)  y = 26 \* x – 11  Correlation coefficient:  r² = 99.962  ***Quantifier 2 transition***  ***m/z 344 🡪 m/z 125 :***  The calibration curve (µg/L):   * was considered as valid over 2.0 – 199.2 µg/L.   Equation (µg/L)  y = 18 \* x + 2  Correlation coefficient:  r² = 99.947  **Alga growth medium**  ***Quantifier transition***  ***m/z 344 🡪 m/z 326 :***  The calibration curve (µg/L):   * was considered as valid over 2.0 – 199.2 µg/L.   Equation (µg/L)  y = 120 \* x – 258  Correlation coefficient:  r² = 99.954  ***Quantifier1 transition***  ***m/z 344 🡪 m/z 189.1 :***  The calibration curve (µg/L):   * was considered as valid over 2.0 – 199.2 µg/L.   Equation (µg/L)  y = 28 \* x – 44  Correlation coefficient:  r² = 99.980  ***Quantifier 2 transition***  ***m/z 344 🡪 m/z 125 :***  The calibration curve (µg/L):   * was considered as valid over 2.0 – 199.2 µg/L.   Equation (µg/L)  y = 19 \* x - 8  Correlation coefficient:  r² = 99.959  **Elendt M4**  ***Quantifier transition***  ***m/z 344 🡪 m/z 326 :***  The calibration curve (µg/L):   * was considered as valid over 0.38 – 23.90 µg/L.   Equation (µg/L)  y = 1855 \* x – 7212  Correlation coefficient:  r² = 99.808  ***Quantifier1 transition***  ***m/z 344 🡪 m/z 189.1 :***  The calibration curve (µg/L):   * was considered as valid over 0.38 – 23.90 µg/L.   Equation (µg/L)  y = 461 \* x – 1691  Correlation coefficient:  r² = 99.679  ***Quantifier 2 transition***  ***m/z 344 🡪 m/z 125 :***  The calibration curve (µg/L):   * was considered as valid over 0.38 – 23.90 µg/L.   Equation (µg/L)  y = 281 \* x - 508  Correlation coefficient:  r² = 99.872 |
| Limit of determination (LOD) | **Reconstituted water :**  LOD = 2.0 µg/L  Lowest calibration level  **Alga growth medium**  LOD = 2.0 µg/L  Lowest calibration level  **Elendt M4**  LOD = 0.38 µg/L  Lowest calibration level |
| Limit of quantification (LOQ) | **Reconstituted water :**  LOD = 11.6 µg/L  Lowest fortified level  **Alga growth medium**  LOD = 11.0 µg/L  Lowest fortified level  **Elendt M4**  LOD = 2.19 µg/L  Lowest fortified level |

Conclusion

The analytical method for the quantification of prothioconazole residues in aqueous samples was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability for prothioconazole.

* + - * 1. Analytical method 4

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  In method HPLC-MS/MS detection was used. Two transitions were monitored.  The specificity of the method was assured by MS/MS detection. All validation parameters are in the currently required range.  The method is suitable for the intended purposes. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/04 |
| Report | Validation of the Analytical Method for the Determination of Difenoconazole and Prothioconazole content in stock solutions of Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560 coming from the Ecotoxicological tests  Garagna, D.  2021c  ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy  Report No. : CH – 0232/2021  + Amendment report No.1 to final report (Amdm1\_KCP 5.1.2/04) |
| Guideline(s): | Yes : SANTE/2020/12830, rev. 1 dated 24/02/21 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The determination of Prothioconazole residues is performed by HPLC using an external standard and MS triple quadrupole detector.  Its quantification is achieved using the calibration curve obtained by plotting standard solutions concentrations and the corresponding peak area. |

Materials and methods

***1. Materials***

***1.A. Determination of prothioconazole content in stock solutions***

The determination of prothioconazole content in stock solutions was assessed by HLPC/MS-QQQ.

|  |  |
| --- | --- |
| HPLC: | Shimadzu Technologies 8050 System |
| Column: | Zorbax Eclipse Plus C18, 3.5 µm, 100 x 4.6 mm i.d. |
| Detector | ESI interfaced Triple quadrupole Mass Detector |
| Mobile phase: | Water, HPLC grade  Demineralised water  Formic acid, high purity for mass spectroscopy  Ammonium formate, for HPLC  Acetonitrile, HPLC grade |
| Analytical standards: | Prothioconazole  CAS No. : 178928-70-6  Batch No. : BCBT2374  Purity : 99.6 %  Expiry date: November 1, 2021 |

***2. Methods***

***2.A. Determination of prothioconazole content in stock solutions***

The analytical methods for the determination of prothioconazole content in stock solutions were first validated so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were validated in terms of linearity, precision, accuracy, specificity, stability and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A~~7.1~~ 30: Recovery results from method validation of prothioconazole content in stock solutions using the analytical method

| Matrix effect | Analyte | Fortification level  (n = x) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| -9.1 % | Prothioconazole | Low level:  0.16 mg/L  (mean found)  N = 5 | 77.4 | 2 | No comments |
| -9.1% | Prothioconazole | High level:  10551.97 mg/L (mean found)  N = 5 | 80.4 | 5 | No comments |
| -9.1% | Prothioconazole | Ultra-high level:  89979.51 mg/L (mean found)  N = 5 | 91.5 | 18 | No comments |

Table A~~8~~ 31: Characteristics for the analytical method used for validation of prothioconazole content in stock solutions for purposes of ecotoxicological tests

|  | Prothioconazole |
| --- | --- |
| Specificity | Untreated blank < 30 % LOQ  Result : 0.0 %  This % ratio (Blank vs LOQ) demontrates that there was no interference of the blank matrix at the retention time of the analyte. |
| Linearity / Calibration | ***Quantifier transition***  ***m/z 344 🡪 m/z 326 :***  The calibration curve (mg/L):   * was considered as valid over 0.02 – 2.03 mg/L.   Equation (mg/L)  y = 797737 \* x – 50758  Correlation coefficient:  r² = 99.852  ***Quantifier 1 transition***  ***m/z 344 🡪 m/z 154 :***  The calibration curve (mg/L):   * was considered as valid over 0.02 – 2.03 mg/L.   Equation (mg/L)  y = 206129 \* x – 14441  Correlation coefficient:  r² = 99.741  ***Quantifier 2 transition***  ***m/z 344 🡪 m/z 189 :***  The calibration curve (mg/L):   * was considered as valid over 0.02 – 2.03 mg/L.   Equation (mg/L)  y = 214765 \* x - 13907  Correlation coefficient:  r² = 99.779 |
| Limit of determination (LOD) | LOD = 0.02 mg/L  Lowest calibration level |
| Limit of quantification (LOQ) | LOQ = 0.21 mg/L  Lowest fortified level |
| Stability of final extract (5 days) | % recovery = 71.5 %  (mean value of 5 replicates)  % RSD = 2 %  Range of recovery:  70.2 – 73.4 % |
| Stability of standard (5 days) | Difference = -153.6 %  Not stable, the standards are prepared always freshly. |

Conclusion

The analytical method for the prothioconazole content in stock solutions was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, stability and limits of determination and quantification for prothioconazole.

* + - * 1. Analytical method 5

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  In method HPLC-MS/MS detection was used. Two transitions were monitored.  The specificity of the method was assured by MS/MS detection. All validation parameters are in the currently required range.  The method is suitable for the intended purposes.  (the method submitted as draft report in word format)  The final report of the “*Validation of the Analytical Method for the Determination of Difenoconazole and Prothioconazole content in feeding solutions of Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560 coming from the Ecotoxicological tests*” (Garagna D., 2021d – Report No. CH-0668/2021 - KCP 5.1.2/05) is available (in pdf format). |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/05 |
| Report | Validation of the Analytical Method for the Determination of Difenoconazole and Prothioconazole content in feeding solutions of Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560 coming from the Ecotoxicological tests  Garagna, D.  2021d  ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy  Report No. : CH – 0668/2021  + Amendment report No.1 to final report (Amdm1\_KCP 5.1.2/05) |
| Guideline(s): | Yes : SANTE/2020/12830, rev. 1 dated 24/02/21 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The determination of Prothioconazole residues is performed by HPLC using an external standard and MS triple quadrupole detector.  Its quantification is achieved using the calibration curve obtained by plotting standard solutions concentrations and the corresponding peak area. |

Materials and methods

***1. Materials***

***1.A. Determination of prothioconazole content in feeding solutions***

The determination of prothioconazole content in feeding solutions was assessed by HLPC/MS-QQQ.

|  |  |
| --- | --- |
| HPLC: | Shimadzu Technologies 8050 System |
| Column: | Zorbax Eclipse Plus C18, 3.5 µm, 100 x 4.6 mm i.d. |
| Detector | ESI interfaced Triple quadrupole Mass Detector |
| Mobile phase: | Water, HPLC grade  Demineralised water  Formic acid, high purity for mass spectroscopy  Ammonium formate, for HPLC  Acetonitrile, HLPC grade |
| Analytical standards: | Prothioconazole  CAS No. : 178928-70-6  Batch No. : BCBT2374  Purity : 99.6 %  Expiry date: November 1, 2021 |

***2. Methods***

***2.A. Determination of prothioconazole content in feeding solutions***

The analytical methods for the determination of prothioconazole content in feeding solutions were first validated so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were validated in terms of linearity, precision, accuracy, specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A~~7.1~~ 32: Recovery results from method validation of prothioconazole content in feeding solutions using the analytical method

| Matrix effect | Analyte | Fortification level  (n = x) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| 14.0 % | Prothioconazole | Low level:  5.63 mg/L  (mean found)  N = 5 | 85.7 | 2 | No comments |
| 14.0 % | Prothioconazole | High level:  5749.32 mg/L (mean found)  N = 5 | 87.8 | 2 | No comments |

Table A~~8~~ 33: Characteristics for the analytical method used for validation of prothioconazole content in feeding solutions for purposes of ecotoxicological tests

|  | Prothioconazole |
| --- | --- |
| Specificity | Untreated blank < 30 % LOQ  Result : 0.0 %  This % ratio (Blank vs LOQ) demontrates that there was no interference of the blank matrix at the retention time of the analyte. |
| Linearity / Calibration | ***Quantifier transition***  ***m/z 344 🡪 m/z 326 :***  The calibration curve (mg/L):   * was considered as valid over 0.02 – 1.97 mg/L.   Equation (mg/L)  y = 1000152 \* x – 67229  Correlation coefficient:  r² = 99.820  ***Quantifier 1 transition***  ***m/z 344 🡪 m/z 154 :***  The calibration curve (mg/L):   * was considered as valid over 0.02 – 1.97 mg/L.   Equation (mg/L)  y = 252293 \* x – 16876  Correlation coefficient:  r² = 99.800  ***Quantifier 2 transition***  ***m/z 344 🡪 m/z 189 :***  The calibration curve (mg/L):   * was considered as valid over 0.02 – 1.97 mg/L.   Equation (mg/L)  y = 268372 \* x - 18143  Correlation coefficient:  r² = 99.831 |
| Limit of determination (LOD) | LOD = 0.02 mg/L  Lowest calibration level |
| Limit of quantification (LOQ) | LOQ = 0.21 mg/L  Lowest fortified level |

Conclusion

The analytical method for the prothioconazole content in feeding solutions was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects and limits of determination and quantification for prothioconazole.

* + - * 1. Analytical method 6

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  In method HPLC-MS/MS detection was used. Two transitions were monitored.  The specificity of the method was assured by MS/MS detection. All validation parameters are in the currently required range.  The method is suitable for the intended purposes. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/06 |
| Report | Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Acute Toxicity to *Daphnia magna* in a 48-hour Immobilization Test under Semi-Static Exposure  Noè F.  2021a  ChemService S.r.l. Controlli e Richerche, Novate Milanese - Italy  Report No. : CH – 0229/2021  + Amendment report No.1 to final report (Amdm1\_KCP 5.1.2/06) |
| Guideline(s): | Yes : SANTE/2020/12830, rev. 1 dated 24/02/21 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The determination of Prothioconazole residues is performed by HPLC using an external standard and MS triple quadrupole detector.  Its quantification is achieved using the calibration curve obtained by plotting standard solutions concentrations and the corresponding peak area. |

Materials and methods

***1. Materials***

The determination of prothioconazole was assessed by HLPC/MS-QQQ.

|  |  |
| --- | --- |
| HPLC: | Agilent mod. 1200 |
| Column: | Zorbax Eclipse Plus C18, 3.5 µm, 100 x 4.6 mm i.d. |
| Detector | Triple quadrupole Mass Detector |
| Scan type | MRM |
| Ion mode | ESI, positive polarity |
| Electro multiplier voltage | 300 V |
| Dry gas temperature | 300 °C |
| Dry gas flow | 7 L/min |
| Nebuliser | 40 psi |
| Dwell time | 200 msec |
| Eluent flow | 0.7 mL/min |
| Volume of injection | 5 µL |
| Retention time | Approximately 3.5 minutes |
| Total analysis time | 10 minutes + 4 minutes post time |
| Mobile phase: | Water  Reconstituted water (according to OECD No. 202, 2004 guideline)  Formic acid  Ammonium formate  Acetonitrile |
| Analytical standards: | Prothioconazole, PESTANAL® analytical standard  CAS No. : 178928-70-6  Batch No. : BCBT2374  Purity : 99.6 %  Expiry date: November 1, 2021 |

***2. Methods***

The analytical method for the determination of prothioconazole in reconstituted water with *Daphnia magna* was first validated so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The method was validated in terms of linearity, precision, accuracy, specificity, limits of detection and quantification and stability.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A~~7.1~~ 34: Recovery results from method validation of prothioconazole in reconstituted water with Daphnia magna using the analytical method

| Matrix effect | Analyte | Fortification level | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| < ±20 % | Prothioconazole | Low level:  10.6 µg/L  (mean found) | 91.8 | 11 | No comments |
| < ±20 % | Prothioconazole | High level:  3.07 mg/L (mean found) | 88.5 | 12 | No comments |

Table A~~8~~ 35: Characteristics for the analytical method used for validation of prothioconazole in reconstituted water with Daphnia magna for purposes of ecotoxicological tests

|  | Prothioconazole |
| --- | --- |
| Specificity | Untreated blank < 30 % LOQ  Result : 0.0 %  This % ratio (Blank vs LOQ) demontrates that there was no interference of the blank matrix at the retention time of the analyte. |
| Linearity / Calibration | ***Quantifier transition***  ***m/z 344 🡪 m/z 326 :***  The calibration curve (µg/L):   * was considered as valid over 2.0 – 199.2 µg/L.   Correlation coefficient:  r² = 99.991  ***Quantifier 1 transition***  ***m/z 344 🡪 m/z 189.1 :***  The calibration curve (µg/L):   * was considered as valid over 2.0 – 199.2 µg/L.   Correlation coefficient:  r² = 99.962  ***Quantifier 2 transition***  ***m/z 344 🡪 m/z 125 :***  The calibration curve (µg/L):   * was considered as valid over 2.0 – 199.2 µg/L.   Correlation coefficient:  r² = 99.947  ***Time 0 hours : Prothioconazole: calibration with matrix-matched standard solutions in reconstituted water***  Equation (mg/L)  y = 63 \* x – 83  Correlation coefficient:  r² = 99.9988  ***Time 24 hours : Prothioconazole: calibration with matrix-matched standard solutions in reconstituted water***  Equation (mg/L)  y = 68 \* x – 203  Correlation coefficient:  r² = 99.965  ***Time 48 hours : Prothioconazole: calibration with matrix-matched standard solutions in reconstituted water***  Equation (mg/L)  y = 65 \* x – 243  Correlation coefficient:  r² = 99.191 |
| Limit of determination (LOD) | LOD = 2.0 µg/L  Lowest calibration level |
| Limit of quantification (LOQ) | LOQ = 11.6 µg/L  Lowest fortified level |
| Stability | Samples analyzed within 24 hours (storage at 4°C) |
| Stability of standard | Freshly prepared at each analysis day |

Conclusion

The analytical method for the prothioconazole in reconstituted water with *Daphnia magna* was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and the stability for prothioconazole.

* + - * 1. Analytical method 7

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  In method HPLC-MS/MS detection was used. Two transitions were monitored.  The specificity of the method was assured by MS/MS detection. All validation parameters are in the currently required range.  The method is suitable for the intended purposes.  Since the analysis gave both quantification and identification data, a confirmatory test is not necessary. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/07 |
| Report | Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Toxicity to Green Algae *Pseudokirchneriella subcapitata* in a Growth Inhibition Study  Noè F.  2021b  ChemService S.r.l. Controlli e Richerche, Novate Milanese - Italy  Report No. : CH – 0230/2021  + Amendment report No.1 to final report (Amdm1\_KCP 5.1.2/07) |
| Guideline(s): | Yes : SANTE/2020/12830, rev. 1 dated 24/02/21 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The determination of Prothioconazole residues is performed by HPLC using an external standard and MS triple quadrupole detector.  Its quantification is achieved using the calibration curve obtained by plotting standard solutions concentrations and the corresponding peak area. |

Materials and methods

***1. Materials***

The determination of prothioconazole was assessed by HLPC/MS-QQQ.

|  |  |
| --- | --- |
| HPLC: | Agilent mod. 1200 |
| Column: | Zorbax Eclipse Plus C18, 3.5 µm, 100 x 4.6 mm i.d. |
| Detector | Triple quadrupole Mass Detector |
| Scan type | MRM |
| Ion mode | ESI, positive polarity |
| Electro multiplier voltage | 300 V |
| Dry gas temperature | 300 °C |
| Dry gas flow | 7 L/min |
| Nebuliser | 40 psi |
| Dwell time | 200 msec |
| Eluent flow | 0.7 mL/min |
| Volume of injection | 5 µL |
| Retention time | Approximately 3.5 minutes |
| Total analysis time | 10 minutes + 4 minutes post time |
| Mobile phase: | Water  Algal growth medium (according to OECD No.201, 2011 guideline)  Formic acid  Ammonium formate  Acetonitrile |
| Analytical standards: | Prothioconazole, PESTANAL® analytical standard  CAS No. : 178928-70-6  Batch No. : BCBT2374  Purity : 99.6 %  Expiry date: November 1, 2021 |

***2. Methods***

The analytical method for the determination of prothioconazole in algal growth medium with Green Algae *Pseudokirchneriella subcapitata* was first validated so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The method was validated in terms of linearity, precision, accuracy, specificity, limits of detection and quantification and stability.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A~~7.1~~ 36:: Recovery results from method validation of prothioconazole in algal growth medium with Green Algae Pseudokirchneriella subcapitata using the analytical method

| Matrix effect | Analyte | Fortification level | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| < ±20 % | Prothioconazole | Low level:  11.4 µg/L  (mean found) | 103.7 | 6 | No comments |
| < ±20 % | Prothioconazole | High level:  15.91 mg/L (mean found) | 103.2 | 1 | No comments |

Table A~~8~~ 37: Characteristics for the analytical method used for validation of prothioconazole in algal growth medium with Green Algae Pseudokirchneriella subcapitata for purposes of ecotoxicological tests

|  | Prothioconazole |
| --- | --- |
| Specificity | Untreated blank < 30 % LOQ  Result : 0.0 %  This % ratio (Blank vs LOQ) demontrates that there was no interference of the blank matrix at the retention time of the analyte. |
| Linearity / Calibration | ***Quantifier transition***  ***m/z 344 🡪 m/z 326 :***  The calibration curve (µg/L):   * was considered as valid over 2.0 – 199.2 µg/L.   Correlation coefficient:  r² = 99.954  ***Quantifier 1 transition***  ***m/z 344 🡪 m/z 189.1 :***  The calibration curve (µg/L):   * was considered as valid over 2.0 – 199.2 µg/L.   Correlation coefficient:  r² = 99.980  ***Quantifier 2 transition***  ***m/z 344 🡪 m/z 125 :***  The calibration curve (µg/L):   * was considered as valid over 2.0 – 199.2 µg/L.   Correlation coefficient:  r² = 99.959  ***Time 0 hours : Prothioconazole: calibration with matrix-matched standard solutions in alga growth medium***  Equation (mg/L)  y = 57 \* x – 228  Correlation coefficient:  r² = 99.586  ***Time 72 hours : Prothioconazole: calibration with matrix-matched standard solutions in alga growth medium***  Equation (mg/L)  y = 73 \* x – 20  Correlation coefficient:  r² = 99.915 |
| Limit of determination (LOD) | LOD = 2.0 µg/L  Lowest calibration level |
| Limit of quantification (LOQ) | LOQ = 11.0 µg/L  Lowest fortified level |
| Stability | Samples analyzed within 24 hours (storage at 4°C) |
| Stability of standard | Freshly prepared at each analysis day |

Conclusion

The analytical method for the prothioconazole in algal growth medium with Green Algae *Pseudokirchneriella subcapitata* was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and the stability for prothioconazole.

* + - * 1. Analytical method 8

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  In method HPLC-MS/MS detection was used. Two transitions were monitored.  The specificity of the method was assured by MS/MS detection. All validation parameters are in the currently required range.  The method is suitable for the intended purposes.  Since the analysis gave both quantification and identification data, a confirmatory test is not necessary. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/08 |
| Report | Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Acute Toxicity to Zebrafish (*Brachydanio rerio*) in a 96-hour Study under Semi-Static Exposure  xxx.  2021c  xxxx  Report No. : CH – 0228/2021  + Amendment report No.1 to final report (Amdm1\_KCP 5.1.2/08) |
| Guideline(s): | Yes : SANTE/2020/12830, rev. 1 dated 24/02/21 |
| Deviations: | Yes  Deviation 1   |  |  | | --- | --- | | Change No. 1 | The study was performed with zebrafish obtained from Model Organism Facility Department of Cellular, Computational and Integrative Biology -CIBIO University of Trento instead of Research Foundation “Edmund Mach” (S. Michele all’Adige - Italy). | | Reason of change: | Availability of organisms. | | Impact on the study: | None. | |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The determination of Prothioconazole residues is performed by HPLC using an external standard and MS triple quadrupole detector.  Its quantification is achieved using the calibration curve obtained by plotting standard solutions concentrations and the corresponding peak area. |

Materials and methods

***1. Materials***

The determination of prothioconazole was assessed by HLPC/MS-QQQ.

|  |  |
| --- | --- |
| HPLC: | Agilent mod. 1200 |
| Column: | Zorbax Eclipse Plus C18, 3.5 µm, 100 x 4.6 mm i.d. |
| Detector | Triple quadrupole Mass Detector |
| Scan type | MRM |
| Ion mode | ESI, positive polarity |
| Electro multiplier voltage | 300 V |
| Dry gas temperature | 300 °C |
| Dry gas flow | 7 L/min |
| Nebuliser | 40 psi |
| Dwell time | 200 msec |
| Eluent flow | 0.7 mL/min |
| Volume of injection | 5 µL |
| Retention time | Approximately 3.5 minutes |
| Total analysis time | 10 minutes + 4 minutes post time |
| Mobile phase: | Water  Reconstituted water (according to ISO Test Water 6341 -Fish)  Formic acid  Ammonium formate  Acetonitrile |
| Analytical standards: | Prothioconazole, PESTANAL® analytical standard  CAS No. : 178928-70-6  Batch No. : BCBT2374  Purity : 99.6 %  Expiry date: November 1, 2021 |

***2. Methods***

The analytical method for the determination of prothioconazole in reconstituted water with *Brachydanio rerio* was first validated so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The method was validated in terms of linearity, precision, accuracy, specificity, limits of detection and quantification and stability.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A~~7.1~~ 38: Recovery results from method validation of prothioconazole in reconstituted water with Brachydanio rerio using the analytical method

| Matrix effect | Analyte | Fortification level | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| < ±20 % | Prothioconazole | Low level:  10.6 µg/L  (mean found) | 91.8 | 11 | No comments |
| < ±20 % | Prothioconazole | High level:  3.07 mg/L (mean found) | 88.5 | 12 | No comments |

Table A~~8~~ 39: Characteristics for the analytical method used for validation of prothioconazole in reconstituted water with Brachydanio rerio for purposes of ecotoxicological tests

|  | Prothioconazole |
| --- | --- |
| Specificity | Untreated blank < 30 % LOQ  Result : 0.0 %  This % ratio (Blank vs LOQ) demontrates that there was no interference of the blank matrix at the retention time of the analyte. |
| Linearity / Calibration | ***Quantifier transition***  ***m/z 344 🡪 m/z 326 :***  The calibration curve (µg/L):   * was considered as valid over 2.0 – 199.2 µg/L.   Correlation coefficient:  r² = 99.991  ***Quantifier 1 transition***  ***m/z 344 🡪 m/z 189.1 :***  The calibration curve (µg/L):   * was considered as valid over 2.0 – 199.2 µg/L.   Correlation coefficient:  r² = 99.962  ***Quantifier 2 transition***  ***m/z 344 🡪 m/z 125 :***  The calibration curve (µg/L):   * was considered as valid over 2.0 – 199.2 µg/L.   Correlation coefficient:  r² = 99.947  ***Time 0 hours : Prothioconazole: calibration with matrix-matched standard solutions in reconstituted water***  Equation (mg/L)  y = 76 \* x – 187  Correlation coefficient:  r² = 99.968  ***Time 24 hours : Prothioconazole: calibration with matrix-matched standard solutions in reconstituted water***  Equation (mg/L)  y = 264 \* x – 545  Correlation coefficient:  r² = 99.139  ***Time 48 hours : Prothioconazole: calibration with matrix-matched standard solutions in reconstituted water***  Equation (mg/L)  y = 456 \* x – 12  Correlation coefficient:  r² = 99.852  ***Time 72 hours : Prothioconazole: calibration with matrix-matched standard solutions in reconstituted water***  Equation (mg/L)  y = 408 \* x – 389  Correlation coefficient:  r² = 99.837  ***Time 96 hours : Prothioconazole: calibration with matrix-matched standard solutions in reconstituted water***  Equation (mg/L)  y = 958 \* x – 7023  Correlation coefficient:  r² = 99.785 |
| Limit of determination (LOD) | LOD = 2.0 µg/L  Lowest calibration level |
| Limit of quantification (LOQ) | LOQ = 11.6 µg/L  Lowest fortified level |
| Stability | Samples analyzed within 24 hours (storage at 4°C) |
| Stability of standard | Freshly prepared at each analysis day |

Conclusion

The analytical method for the prothioconazole in reconstituted water with *Brachydanio rerio* was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and the stability for prothioconazole.

* + - * 1. Analytical method 9

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  In method HPLC-MS/MS detection was used. Two transitions were monitored.  The specificity of the method was assured by MS/MS detection. All validation parameters are in the currently required range.  The method is suitable for the intended purposes.  (draft in word format submitted)  The final report of the “*Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Effects on Reproduction of Earthworm Eisenia fetida in an Artificial Soil Study*” (Dini, R.., 2021a – Report No. CH-0239/2021 - KCP 5.1.2/09) is available (in pdf format). |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/09 |
| Report | Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Effects on Reproduction of Earthworm *Eisenia fetida* in an Artificial Soil Study  Dini, R.  2021a  ChemService S.r.l. Controlli e Richerche, Novate Milanese - Italy  Report No. : CH – 0239/2021 |
| Guideline(s): | Yes : SANTE/2020/12830, rev. 1 dated 24/02/21 |
| Deviations: | Yes  Deviation 1   |  |  | | --- | --- | | Change No. 1 | Deviation in Test temperature during test period. | | Reason of change: | Due to a technical error, the temperatures were recorded from 6th August instead of 5th August.. | | Impact on the study: | None, since all validity criteria were meet. | |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The determination of Prothioconazole residues is performed by HPLC using an external standard and MS triple quadrupole detector.  Its quantification is achieved using the calibration curve obtained by plotting standard solutions concentrations and the corresponding peak area. |

Materials and methods

***1. Materials***

The determination of prothioconazole was assessed by HLPC/MS-QQQ.

|  |  |
| --- | --- |
| HPLC: | Agilent mod. 1200 |
| Column: | Zorbax Eclipse Plus C18, 3.5 µm, 100 x 4.6 mm i.d. |
| Detector | Triple quadrupole Mass Detector |
| Scan type | MRM |
| Ion mode | ESI, positive polarity |
| Interface temperature | 300 °C |
| Dry gas flow | 10 L/min |
| Nebilizing gas flow | 2.9 L/min |
| Eluent flow | 0.7 mL/min |
| Volume of injection | 10 µL |
| Retention time | Approximately 7.6 minutes |
| Total analysis time | 20 minutes |
| Mobile phase: | Water  Demineralised water  Artificial soil (according to OECD No. 222, Earthworms guideline)  Formic acid  Ammonium formate  Acetonitrile |
| Analytical standards: | Prothioconazole, PESTANAL® analytical standard  CAS No. : 178928-70-6  Batch No. : BCBT2374  Purity : 99.6 %  Expiry date: November 1, 2021 |

***2. Methods***

The analytical method for the determination of prothioconazole in Matrix-matched standard solutionswith *Eisenia fetida* was first validated so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The method was validated in terms of linearity, precision, accuracy, specificity, limits of detection and quantification and stability.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A~~7.1~~ 40: Recovery results from method validation of prothioconazole in Matrix-matched standard solutions with Eisenia fetida using the analytical method

| Matrix effect | Analyte | Fortification level | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| < ±20 % | Prothioconazole | Low level:  45.2 µg/kg  (mean found) | 89.0 | 14 | No comments |
| < ±20 % | Prothioconazole | High level:  172.13 mg/kg  (mean found) | 79.9 | 7 | No comments |

Table A~~8~~ 41: Characteristics for the analytical method used for validation of prothioconazole in Matrix-matched standard solutions with Eisenia fetida for purposes of ecotoxicological tests

|  | Prothioconazole |
| --- | --- |
| Specificity | Untreated blank < 30 % LOQ  Result : 0.0 %  This % ratio (Blank vs LOQ) demontrates that there was no interference of the blank matrix at the retention time of the analyte. |
| Linearity / Calibration | ***Quantifier transition***  ***m/z 344 🡪 m/z 326 :***  The calibration curve (µg/kg):   * was considered as valid over 14.9 – 137.3 µg/kg. * corresponding to range 7.5 – 74.7 µg/L   Correlation coefficient:  r² = 99.773  ***Quantifier 1 transition***  ***m/z 344 🡪 m/z 154 :***  The calibration curve (µg/kg):   * was considered as valid over 14.9 – 137.3 µg/kg. * corresponding to range 7.5 – 74.7 µg/L   Correlation coefficient:  r² = 99.966  ***Quantifier 2 transition***  ***m/z 344 🡪 m/z 189 :***  The calibration curve (µg/kg):   * was considered as valid over 14.9 – 137.3 µg/kg. * corresponding to range 7.5 – 74.7 µg/L   Correlation coefficient:  r² = 99.330  ***Time 0 day : Prothioconazole: calibration with matrix-matched standard solutions***  Equation (µg/kg)  y = 292 \* x – 3045  Correlation coefficient:  r² = 99.673  ***Time 28 days : Prothioconazole: calibration with matrix-matched standard solution***  Equation (µg/kg)  y = 310 \* x – 2322  Correlation coefficient:  r² = 99.781  ***Time 56 days : Prothioconazole: calibration with matrix-matched standard solutions***  Equation (µg/kg)  y = 204 \* x – 410  Correlation coefficient:  r² = 99.695 |
| Limit of determination (LOD) | LOD = 14.9 µg/kg  Lowest calibration level |
| Limit of quantification (LOQ) | LOQ = 50.8 µg/kg  Lowest fortified level |
| Stability | Recovery Mean between 70% – 120% |
| Stability (7 days) | Low level: 81.3% |
| Stability of standard | < ±10% |
| Stability of standard (3 days) | -50.6%  Not stable, the standards are prepared always freshly |

Conclusion

The analytical method for the prothioconazole in Matrix-matched standard solutions with *Eisenia fetida* was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and the stability for prothioconazole.

* + - * 1. Analytical method 10

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  The analytical method was employed to determine the difenoconazole and prothioconazole residues in soil samples coming from the biological phase of ecotoxicological test on Collembola (*Folsomia candida*).  In the method HPLC-MS/MS detection was used. Two transitions were monitored.  The specificity of the method was assured by MS/MS detection. All validation parameters are in the currently required range.  The method is suitable for the intended purposes.  (draft in word format submitted)  The final report of the “*Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Effects on Collembolan Reproduction in an Artificial Soil Study*” (Dini, R.., 2021b – Report No. CH-0240/2021 - KCP 5.1.2/10) is available (in pdf format). |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/10 |
| Report | Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Effects on Collembolan Reproduction in an Artificial Soil Study  Dini, R.  2021b  ChemService S.r.l. Controlli e Richerche, Novate Milanese - Italy  Report No. : CH – 0240/2021 |
| Guideline(s): | Yes : SANTE/2020/12830, rev. 1 dated 24/02/21 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The determination of Prothioconazole residues is performed by HPLC using an external standard and MS triple quadrupole detector.  Its quantification is achieved using the calibration curve obtained by plotting standard solutions concentrations and the corresponding peak area. |

Materials and methods

***1. Materials***

The determination of prothioconazole was assessed by HLPC/MS-QQQ.

|  |  |
| --- | --- |
| HPLC: | Agilent mod. 1200 |
| Column: | Zorbax Eclipse Plus C18, 3.5 µm, 100 x 4.6 mm i.d. |
| Detector | Triple quadrupole Mass Detector |
| Scan type | MRM |
| Ion mode | ESI, positive polarity |
| Interface temperature | 300 °C |
| Dry gas flow | 10 L/min |
| Nebilizing gas flow | 2.9 L/min |
| Eluent flow | 0.7 mL/min |
| Volume of injection | 10 µL |
| Retention time | Approximately 7.6 minutes |
| Total analysis time | 20 minutes |
| Mobile phase: | Water, HPLC grade  Demineralised water  Artificial soil (according to OECD No. 232, 2016 guideline)  Formic acid, high purity for mass spectroscopy  Ammonium formate, high purity for mass spectroscopy  Acetonitrile, HPLC grade |
| Analytical standards: | Prothioconazole, PESTANAL® analytical standard  CAS No. : 178928-70-6  Batch No. : BCBT2374  Purity : 99.6 %  Expiry date: November 1, 2021 |

***2. Methods***

The analytical method for the determination of prothioconazole in Matrix-matched standard solutionswith *Collembola* was first validated so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The method was validated in terms of linearity, precision, accuracy, specificity, limits of detection and quantification and stability.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A~~7.1~~ 42: Recovery results from method validation of prothioconazole in Matrix-matched standard solutions with Collembola using the analytical method

| Matrix effect | Analyte | Fortification level | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| < ±20 % | Prothioconazole | Low level:  45.2 µg/kg  (mean found) | 89.0 | 14 | No comments |
| < ±20 % | Prothioconazole | High level:  172.13 mg/kg  (mean found) | 79.9 | 7 | No comments |

Table A~~8~~ 43: Characteristics for the analytical method used for validation of prothioconazole in Matrix-matched standard solutions with Collembola for purposes of ecotoxicological tests

|  | Prothioconazole |
| --- | --- |
| Specificity | Untreated blank < 30 % LOQ  Result : 0.0 %  This % ratio (Blank vs LOQ) demontrates that there was no interference of the blank matrix at the retention time of the analyte. |
| Linearity / Calibration | ***Quantifier transition***  ***m/z 344 🡪 m/z 326 :***  The calibration curve (µg/kg):   * was considered as valid over 14.9 – 137.3 µg/kg. * corresponding to range 7.5 – 74.7 µg/L   Correlation coefficient:  r² = 99.773  ***Quantifier 1 transition***  ***m/z 344 🡪 m/z 154 :***  The calibration curve (µg/kg):   * was considered as valid over 14.9 – 137.3 µg/kg. * corresponding to range 7.5 – 74.7 µg/L   Correlation coefficient:  r² = 99.966  ***Quantifier 2 transition***  ***m/z 344 🡪 m/z 189 :***  The calibration curve (µg/kg):   * was considered as valid over 14.9 – 137.3 µg/kg. * corresponding to range 7.5 – 74.7 µg/L   Correlation coefficient:  r² = 99.330  ***Time 0 day : Prothioconazole: calibration with matrix-matched standard solutions***  Equation (µg/kg)  y = 256 \* x – 986  Correlation coefficient:  r² = 99.825  ***Time 13 days : Prothioconazole: calibration with matrix-matched standard solution***  Equation (µg/kg)  y = 420 \* x – 5095  Correlation coefficient:  r² = 99.000  ***Time 28 days : Prothioconazole: calibration with matrix-matched standard solutions***  Equation (µg/kg)  y = 359 \* x – 1913  Correlation coefficient:  r² = 99.748 |
| Limit of determination (LOD) | LOD = 14.9 µg/kg  Lowest calibration level |
| Limit of quantification (LOQ) | LOQ = 50.8 µg/kg  Lowest fortified level |
| Stability | Recovery Mean between 70% – 120% |
| Stability (7 days) | Low level: 81.3% |
| Stability of standard | < ±10% |
| Stability of standard (3 days) | -50.6%  Not stable, the standards are prepared always freshly |

Conclusion

The analytical method for the prothioconazole in Matrix-matched standard solutions with *Collembola* was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and the stability for prothioconazole.

* + - * 1. Analytical method 11

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  For the determination of Difenoconazole and Prothioconazole residues, all samples were analysed according to the Method No. 0668/2021 validated in the GLP Study CH - 0668/2021 for the feeding solution and according to the Method No. 0232/2021 validated in the GLP Study CH - 0232/2021 for the test chemical solution.  In the method HPLC-MS/MS (LC/MS-QQQ) detection was used. Two transitions were monitored.  The specificity of the method was assured by MS/MS detection. All validation parameters are in the currently required range.  The method is suitable for the intended purposes.  (draft in word format submitted)  The final report of the “*Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Acute Oral and Contact Toxicity to adult worker bumblebees Bombus terrestris L*.” (Ponti, B., 2021a – Report No. CH-0234/2021 - KCP 5.1.2/11) is available (in pdf format). |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/11 |
| Report | Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Acute Oral and Contact Toxicity to adult worker bumblebees *Bombus terrestris* L.  Ponti, B.  2021a  ChemService S.r.l. Controlli e Richerche, Novate Milanese - Italy  Report No. : CH – 0234/2021 |
| Guideline(s): | Yes : SANTE/2020/12830, rev. 1 dated 24/02/21 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The determination of the active ingredient Prothioconazole was performed by HPLC using an external standard and a MS-QQQ detector.  The quantification of the active ingredient is achieved by calculating its concentration in the final solutions in respect to a linear calibration obtained using the working standard solutions prepared starting from the reference material. |

Materials and methods

***1. Materials***

The determination of prothioconazole was assessed by HLPC/MS-QQQ.

|  |  |
| --- | --- |
| HPLC: | Shimadzu mod. 850 |
| Column: | Zorbax Eclipse Plus C18, 3.5 µm, 100 x 4.6 mm i.d. |
| Detector | Triple quadrupole Mass Detector |
| Scan type | MRM |
| Ion mode | ESI, positive polarity |
| Interface temperature | 300 °C |
| Dry gas flow | 10 L/min |
| Nebilizing gas flow | 2.9 L/min |
| Eluent flow | 0.7 mL/min |
| Volume of injection | 10 µL |
| Retention time | Approximately 7.4 minutes |
| Total analysis time | 20 minutes |
| Mobile phase: | Water, HPLC grade  Demineralised water  Feeding solution  Formic acid, high purity for mass spectroscopy  Ammonium formate, high purity for mass spectroscopy  Acetonitrile, HPLC grade |
| Analytical standards: | Prothioconazole, PESTANAL® analytical standard  CAS No. : 178928-70-6  Batch No. : BCBT2374  Purity : 99.6 %  Expiry date: November 1, 2021 |

***2. Methods***

The analytical method for the determination of prothioconazole in feeding solutionswith *Bombus terrestris* L. (oral test) was first validated so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The method was validated in terms of linearity, precision, accuracy, specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A~~7.1~~ 44: Recovery results from method validation of prothioconazole in feeding solutions with Bombus terrestris L. (oral test) using the analytical method

| Matrix effect | Analyte | Fortification level | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| < ±20 % | Prothioconazole | Low level:  5.63 mg/L  (mean found) | 85.7 | 2 | No comments |
| < ±20 % | Prothioconazole | High level:  5749.32 mg/L  (mean found) | 87.8 | 2 | No comments |

Table A~~8~~ 45: Characteristics for the analytical method used for validation of prothioconazole in feeding solutions with Bombus terrestris L. (oral test) for purposes of ecotoxicological tests

|  | Prothioconazole |
| --- | --- |
| Specificity | Untreated blank < 30 % LOQ  Result : 0.0 %  This % ratio (Blank vs LOQ) demontrates that there was no interference of the blank matrix at the retention time of the analyte. |
| Linearity / Calibration | ***Quantifier transition***  ***m/z 344 🡪 m/z 326 :***  The calibration curve (mg/L):   * was considered as valid over 0.02 – 1.97 mg/L.   Correlation coefficient:  r² = 99.820  ***Quantifier 1 transition***  ***m/z 344 🡪 m/z 154 :***  The calibration curve (mg/L):   * was considered as valid over 0.02 – 1.97 mg/L.   Correlation coefficient:  r² = 99.800  ***Quantifier 2 transition***  ***m/z 344 🡪 m/z 189 :***  The calibration curve (mg/L):   * was considered as valid over 0.02 – 1.97 mg/L.   Correlation coefficient:  r² = 99.831  ***Prothioconazole – oral test (feeding solution): Linear calibration :***  Equation (mg/L)  y = 435842 \* x – 33033  Correlation coefficient:  r² = 99.691 |
| Limit of determination (LOD) | LOD = 0.02 mg/L  Lowest calibration level |
| Limit of quantification (LOQ) | LOQ = 6.57 mg/L  Lowest fortified level |

Conclusion

The analytical method for the prothioconazole in feeding solutions with *Bombus terrestris* L. (oral test) was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects and limits of determination and quantification for prothioconazole.

* + - * 1. Analytical method 12

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  For the determination of Difenoconazole and Prothioconazole residues, all samples were analysed according to the Method No. 0668/2021 validated in the GLP Study CH - 0668/2021 for the feeding solution and according to the Method No. 0232/2021 validated in the GLP Study CH - 0232/2021 for the test chemical solution.  In the method HPLC-MS/MS (LC/MS-QQQ) detection was used. Two transitions were monitored.  The specificity of the method was assured by MS/MS detection. All validation parameters are in the currently required range.  The method is suitable for the intended purposes.  (draft in word format submitted)  The final report of the “*Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Acute Oral and Contact Toxicity to adult worker bumblebees Bombus terrestris L*.” (Ponti, B., 2021a – Report No. CH-0234/2021 - KCP 5.1.2/11) is available (in pdf format). |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/11 |
| Report | Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Acute Oral and Contact Toxicity to adult worker bumblebees *Bombus terrestris* L.  Ponti, B.  2021b  ChemService S.r.l. Controlli e Richerche, Novate Milanese - Italy  Report No. : CH – 0234/2021 |
| Guideline(s): | Yes : SANTE/2020/12830, rev. 1 dated 24/02/21 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The determination of Prothioconazole residues is performed by HPLC using an external standard and MS triple quadrupole detector.  Its quantification is achieved using the calibration curve obtained by plotting standard solutions concentrations and the corresponding peak area. |

Materials and methods

***1. Materials***

The determination of prothioconazole was assessed by HLPC/MS-QQQ.

|  |  |
| --- | --- |
| HPLC: | Shimadzu mod. 850 |
| Column: | Zorbax Eclipse Plus C18, 3.5 µm, 100 x 4.6 mm i.d. |
| Detector | Triple quadrupole Mass Detector |
| Scan type | MRM |
| Ion mode | ESI, positive polarity |
| Interface temperature | 300 °C |
| Dry gas flow | 10 L/min |
| Nebilizing gas flow | 2.9 L/min |
| Eluent flow | 0.7 mL/min |
| Volume of injection | 10 µL |
| Retention time | Approximately 7.4 minutes |
| Total analysis time | 20 minutes |
| Mobile phase: | Water, HPLC grade  Demineralised water  Feeding solution  Formic acid, high purity for mass spectroscopy  Ammonium formate, for HPLC  Acetonitrile, HPLC grade |
| Analytical standards: | Prothioconazole, PESTANAL® analytical standard  CAS No. : 178928-70-6  Batch No. : BCBT2374  Purity : 99.6 %  Expiry date: November 1, 2021 |

***2. Methods***

The analytical method for the determination of prothioconazole in feeding solutionswith *Bombus terrestris* L. (contact test) was first validated so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The method was validated in terms of linearity, precision, accuracy, specificity, limits of detection and quantification and stability.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A~~7.1~~ 46:: Recovery results from method validation of prothioconazole in feeding solutions with *Bombus terrestris* L. (contact test) using the analytical method

| Matrix effect | Analyte | Fortification level | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| < ±20 % | Prothioconazole | Low level:  0.16 mg/L  (mean found) | 77.4 | 2 | No comments |
| < ±20 % | Prothioconazole | High level:  10551.97 mg/L  (mean found) | 80.4 | 5 | No comments |
| < ±20 % | Prothioconazole | Ultra-High level:  89979.51 mg/L  (mean found) | 91.5 | 18 | No comments |

Table A~~8~~ 47: Characteristics for the analytical method used for validation of prothioconazole in feeding solutions with *Bombus terrestris* L. (contact test) for purposes of ecotoxicological tests

|  | Prothioconazole |
| --- | --- |
| Specificity | Untreated blank < 30 % LOQ  Result : 0.0 %  This % ratio (Blank vs LOQ) demontrates that there was no interference of the blank matrix at the retention time of the analyte. |
| Linearity / Calibration | ***Quantifier transition***  ***m/z 344 🡪 m/z 326 :***  The calibration curve (mg/L):   * was considered as valid over 0.02 – 2.03 mg/L.   Correlation coefficient:  r² = 99.852  ***Quantifier 1 transition***  ***m/z 344 🡪 m/z 154 :***  The calibration curve (mg/L):   * was considered as valid over 0.02 – 2.03 mg/L..   Correlation coefficient:  r² = 99.741  ***Quantifier 2 transition***  ***m/z 344 🡪 m/z 189 :***  The calibration curve (mg/L):   * was considered as valid over 0.02 – 2.03 mg/L..   Correlation coefficient:  r² = 99.779  ***Prothioconazole – contact test (feeding solution): Linear calibration :***  Equation (mg/L) :  y = 432216 \* x – 28748  Correlation coefficient:  r² = 99.822 |
| Limit of determination (LOD) | LOD = 0.02 mg/L  Lowest calibration level |
| Limit of quantification (LOQ) | LOQ = 0.21 mg/L  Lowest fortified level |
| Stability | Recovery Mean between 70% - 120% |
| Stability (5 days) | Low level: 71.5 % |
| Stability of standard | <± 10% |
| Stability of standard (5 days) | -153.6%  Not stable, the standards are prepared always freshly |

Conclusion

The analytical method for the prothioconazole in feeding solutions with *Bombus terrestris* L. (contact test) was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability for prothioconazole.

* + - * 1. Analytical method 13

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  The method No. 0235/2021 for Difenoconazole and Prothioconazole determination in soil samples of test item, which was validated in GLP Study CH-0235/2021 was employed. In the method HPLC-MS/MS detection was used. Two transitions were monitored. Since the analysis performed by HPLC/MS/MS gave both quantification and identification data, a confirmatory test using another instrumental technique was not necessary.  The specificity of the method was assured by MS/MS detection. All validation parameters are in the currently required range.  The method is suitable for the intended purposes.  (draft in word format submitted)  The final report of the “*Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Effects on Hypoaspis (Geolaelaps) aculeifer Reproduction in an Artificial Soil Study*” (Dini, R., 2021c – Report No. CH-0241/2021 - KCP 5.1.2/12) is available (in pdf format). |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/12 |
| Report | Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Effects on *Hypoaspis (Geolaelaps) aculeifer* Reproduction in an Artificial Soil Study  Dini, R.  2021c  ChemService S.r.l. Controlli e Richerche, Novate Milanese - Italy  Report No. : CH – 0241/2021 |
| Guideline(s): | Yes : SANTE/2020/12830, rev. 1 dated 24/02/21 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The determination of Prothioconazole residues is performed by HPLC using an external standard and MS triple quadrupole detector.  Its quantification is achieved using the calibration curve obtained by plotting standard solutions concentrations and the corresponding peak area. |

Materials and methods

***1. Materials***

The determination of prothioconazole was assessed by HLPC/MS-MS

|  |  |
| --- | --- |
| HPLC: | Shimadzu mod. 850 |
| Column: | Zorbax Eclipse Plus C18, 3.5 µm, 100 x 4.6 mm i.d. |
| Detector | Triple quadrupole Mass Detector |
| Scan type | MRM |
| Ion mode | ESI, positive polarity |
| Interface temperature | 300 °C |
| Dry gas flow | 10 L/min |
| Nebilizing gas flow | 2.9 L/min |
| Eluent flow | 0.7 mL/min |
| Volume of injection | 10 µL |
| Retention time | Approximately 7.6 minutes |
| Total analysis time | 20 minutes |
| Mobile phase: | Water, HPLC grade  Artificial soil  Formic acid, high purity for mass spectroscopy  Ammonium formate, high purity for mass spectroscopy  Acetonitrile, HPLC grade |
| Analytical standards: | Prothioconazole, PESTANAL® analytical standard  CAS No. : 178928-70-6  Batch No. : BCBT2374  Purity : 99.6 %  Expiry date: November 1, 2021 |

***2. Methods***

The analytical method for the determination of prothioconazole in artificial soilwith *Hypoaspis (Geolaelaps) aculeifer* was first validated so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The method was validated in terms of linearity, precision, accuracy, specificity, limits of detection and quantification and stability.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A~~7.1~~ 48: Recovery results from method validation of prothioconazole in artificial soil with Hypoaspis (Geolaelaps) aculeifer using the analytical method

| Matrix effect | Analyte | Fortification level | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| < ±20 % | Prothioconazole | Low level:  42.3 ug/kg  (mean found) | 83.4 | 12 | No comments |
| < ±20 % | Prothioconazole | High level:  178.34 ug/kg  (mean found) | 82.9 | 6 | No comments |

Table A~~8~~ 49: Characteristics for the analytical method used for validation of prothioconazole artificial soil with Hypoaspis (Geolaelaps) aculeifer for purposes of ecotoxicological tests

|  | Prothioconazole |
| --- | --- |
| Specificity | Untreated blank < 30 % LOQ  Result : 0.0 %  This % ratio (Blank vs LOQ) demontrates that there was no interference of the blank matrix at the retention time of the analyte. |
| Linearity / Calibration | ***Quantifier transition***  ***m/z 344 🡪 m/z 326 :***  The calibration curve (ug/kg):   * was considered as valid over 14.9 – 137.3 ug/kg. * corresponding to range 7.5 – 74.7 µg/L   Correlation coefficient:  r² = 99.979  ***Quantifier 1 transition***  ***m/z 344 🡪 m/z 154 :***  The calibration curve (ug/kg):   * was considered as valid over 14.9 – 137.3 ug/kg. * corresponding to range 7.5 – 74.7 µg/L   Correlation coefficient:  r² = 99.979  ***Quantifier 2 transition***  ***m/z 344 🡪 m/z 189 :***  The calibration curve (ug/kg):   * was considered as valid over 14.9 – 137.3 ug/kg. * corresponding to range 7.5 – 137.3 µg/L   Correlation coefficient:  r² = 99.979  ***Time 0 days : Prothioconazole –Linear calibration***  Equation (mg/kg) :  y = 301 \* x – 1322  Correlation coefficient:  r² = 99.969  ***Time 7 days : Prothioconazole –Linear calibration***  Equation (mg/kg) :  y = 480 \* x – 4837  Correlation coefficient:  r² = 99.489  ***Time 14 days : Prothioconazole –Linear calibration***  Equation (mg/kg) :  y = 376 \* x – 2664  Correlation coefficient:  r² = 99.981 |
| Limit of determination (LOD) | LOD = 14.9 ug/kg  Lowest calibration level |
| Limit of quantification (LOQ) | LOQ = 50.8 ug/kg  Lowest fortified level |
| Stability | Recovery Mean between 70% - 120% |
| Stability (5 days) | Low level: 81.3 % |
| Stability of standard | <± 10% |
| Stability of standard (5 days) | -50.6%  Not stable, the standards are prepared always freshly |

Conclusion

The analytical method for the prothioconazole in artificial soilwith *Hypoaspis (Geolaelaps) aculeifer* was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability for prothioconazole.

* + - * 1. Analytical method 14

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  The method No. 0668/2021 for Difenoconazole and Prothioconazole determination in soil samples of test item, which was validated in GLP Study CH-0668/2021 was employed. In the method HPLC/MS-QQQ detection was used. Two transitions were monitored. Since the analysis performed by HPLC/MS-QQQ gave both quantification and identification data, a confirmatory test using another instrumental technique was not necessary.  The specificity of the method was assured by MS/MS detection. All validation parameters are in the currently required range.  The method is suitable for the intended purposes.  (draft in word format submitted)  The final report of the “*Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Chronic Oral Toxicity to adult worker honeybees Apis mellifera L. (10-day feeding)”* (Ponti, B., 2021b – Report No. CH-0669/2021 - KCP 5.1.2/13) is available (in pdf format). |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/13 |
| Report | Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Chronic Oral Toxicity to adult worker honeybees Apis mellifera L. (10-day feeding)  Ponti, B.  2021b  ChemService S.r.l. Controlli e Richerche, Novate Milanese - Italy  Report No. : CH – 0669/2021 |
| Guideline(s): | Yes : SANTE/2020/12830, rev. 1 dated 24/02/21 |
| Deviations: | Yes  Deviation 1   |  |  | | --- | --- | | Change No. 1 | The expiry date of the reference item is December 01, 2025 | | Reason of change: | In the study plan was wrongly reported (typing error), as expiry date, August 01, 2025 | | Impact on the study: | None. | |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The determination of the active ingredient Prothioconazole was performed by HPLC using an external standard and a MS-QQQ detector.  The quantification of the active ingredient is achieved by calculating its concentration in the final solutions in respect to a linear calibration obtained using the working standard solutions prepared starting from the reference material. |

Materials and methods

***1. Materials***

The determination of prothioconazole was assessed by HLPC/MS-QQQ

|  |  |
| --- | --- |
| HPLC: | Shimadzu mod. 850 |
| Column: | Zorbax Eclipse Plus C18, 3.5 µm, 100 x 4.6 mm i.d. |
| Detector | Triple quadrupole Mass Detector |
| Scan type | MRM |
| Ion mode | ESI, positive polarity |
| Interface temperature | 300 °C |
| Dry gas flow | 10 L/min |
| Nebilizing gas flow | 2.9 L/min |
| Eluent flow | 0.7 mL/min |
| Volume of injection | 10 µL |
| Retention time | Approximately 5.6 minutes |
| Total analysis time | 20 minutes |
| Mobile phase: | Water, HPLC grade  Feeding solution  Formic acid, high purity for mass spectroscopy  Ammonium formate, high purity for mass spectroscopy  Acetonitrile, HPLC grade |
| Analytical standards: | Prothioconazole, PESTANAL® analytical standard  CAS No. : 178928-70-6  Batch No. : BCBT2374  Purity : 99.6 %  Expiry date: November 1, 2021 |

***2. Methods***

The analytical method for the determination of prothioconazole in feeding solutionwith *Apis mellifera* L*.* was first validated so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The method was validated in terms of linearity, precision, accuracy, specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A~~7.1~~ 50:: Recovery results from method validation of prothioconazole in feeding solution with *Apis mellifera* L. using the analytical method

| Matrix effect | Analyte | Fortification level | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| < ±20 % | Prothioconazole | Low level:  5.63 mg/L  (mean found) | 85.7 | 2 | No comments |
| < ±20 % | Prothioconazole | High level:  5749.32 mg/L  (mean found) | 87.8 | 2 | No comments |

Table A~~8~~ 51: Characteristics for the analytical method used for validation of prothioconazole in feeding solution with *Apis mellifera* L. for purposes of ecotoxicological tests

|  | Prothioconazole |
| --- | --- |
| Specificity | Untreated blank < 30 % LOQ  Result : 0.0 %  This % ratio (Blank vs LOQ) demontrates that there was no interference of the blank matrix at the retention time of the analyte. |
| Linearity / Calibration | ***Quantifier transition***  ***m/z 344 🡪 m/z 326 :***  The calibration curve (mg/L):   * was considered as valid over 0.02 – 1.97 mg/L.   Correlation coefficient:  r² = 99.820  ***Quantifier 1 transition***  ***m/z 344 🡪 m/z 154 :***  The calibration curve (mg/L):   * was considered as valid over 0.02 – 1.97 mg/L.   Correlation coefficient:  r² = 99.800  ***Quantifier 2 transition***  ***m/z 344 🡪 m/z 189 :***  The calibration curve (mg/L):   * was considered as valid over 0.02 – 1.97 mg/L.   Correlation coefficient:  r² = 99.831  ***Prothioconazole content –Linear calibration***  Equation (mg/L) :  y = 1050296 \* x – 111427  Correlation coefficient:  r² = 99.494 |
| Limit of determination (LOD) | LOD = 0.02 mg/L  Lowest calibration level |
| Limit of quantification (LOQ) | LOQ = 6.57 mg/L  Lowest fortified level |

Conclusion

The analytical method for the prothioconazole in feeding solutionwith *Apis mellifera* L. was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects and limits of determination and quantification for prothioconazole.

* + - * 1. Analytical method 15

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  The analytical method for the determination of TDMs was based on the method “Quick Method for the Analysis of Highly Polar Pesticides in Food Involving Extraction with Acidified Methanol and LC- or IC-MS/MS Measurement I. - Food of Plant Origin (QuPPe-PO-Method) - Method 8 (M8)”.  The determination was carried out using a HPLC-MS/MS system (high-performance liquid chromatography + triple quadrupole mass spectrometry) equipped with a differential mobility separation device (DMS).  The specificity of the method was assured by MS/MS detection. All validation parameters are in the currently required range.  The matrix effect was not significant according to the SANTE/2020/12830 rev.1 except for triazole-alanine in whole OSR plant.  The Study Plan was amended in the context of Extraction efficiency - On the next pages see also in A 2.1.1.1.15.3 description – the equivalent of the chapter from the study - 13.3 SAMPLE EXTRACTION.  See also into the present section B7 where this validated method was employed to generation of the data in paragraphs of Appendix 2: A 2.1.3.1.1,2,3; A 2.1.5.2.1,2,3; A 2.2.3.1.1,2,3; A 2.2.5.2.1,2,3.  Independent laboratory validation ~~is ongoing and~~ is included in study plans submitted (CH-1090/2021, CH-1085/2021, CH-1087/2021, CH-1086/2021).  The studies have been submitted – relevant paragraph for evaluation details. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/14 |
| Report | Validation of an analytical method for the quantification of Triazole Derivative Metabolites (TDMs) in wheat, barley, oilseed rape and processed commodities  Longhi, D.  2021b  ChemService S.r.l. Controlli e Richerche, Novate Milanese - Italy  Report No. : 21-108 |
| Guideline(s): | Yes : SANTE/2020/12830 rev. 1 (dated 24/02/2021) ;  SANTE2017/10632 rev. 3 (dated 22/11/2017) ;  OECD ENV/JM/MONO(2007)17 ;  “Quick Method for the Analysis of Highly Polar Pesticides in Food Involving Extraction with Acidified Methanol and LC- or IC-MS/MS Measurement - Food of Plant Origin (QuPPe-PO-Method)- Method 8 (M8)”. |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The analytical method for the determination of TDMs in the tested matrices (AM-GLP-STUDY-21-108) was based on the method “Quick Method for the Analysis of Highly Polar Pesticides in Food Involving Extraction with Acidified Methanol and LC- or IC-MS/MS Measurement I. - Food of Plant Origin (QuPPe-PO-Method) - Method 8 (M8)”.  The instrumental determination was carried out using a HPLC-MS/MS system (high-performance liquid chromatography + triple quadrupole mass spectrometry) equipped with a differential mobility separation device (DMS). |

Materials and methods

***1. Materials***

***1.A. Quantification of triazole derivative metabolites in wheat, barley, oilseed rape and processed commodities***

The quantification of triazole derivative metabolites in wheat, barley, oilseed rape and processed commodities was assessed by HLPC/MS/MS.

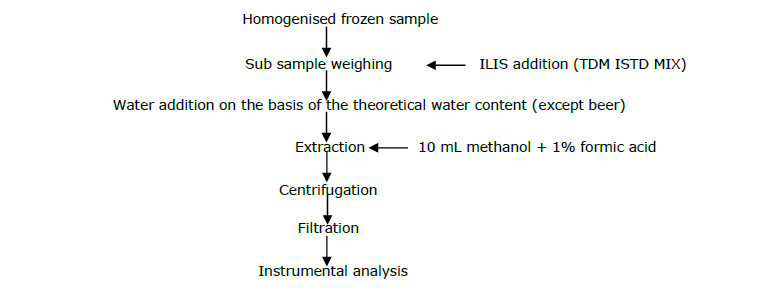
|  |  |
| --- | --- |
| HPLC: | Shimadzu LC-40 XR + spectrometer Sciex API 6500+ equipped with SelexION (Differential Mobility Separation) device |
| Column: | Thermo Hyperbare 5 μm, 2.1 x 100 mm |
| Detector: | Agilent MS spectrometer 6470A Triple Quad |
| Column temperature: | 40 °C |
| Flow rate: | 0.6 mL/min |
| Volume of injection: | 2 µL |
| Retention time: | Approximatively 2.5 minutes for difenoconazole |
| Stop time: | 10 minutes |
| Gas temperature: | 500 °C |
| Curtain Gas flow: | 30 mL/min |
| Gas flow 1: | 55 mL/min |
| Gas flow2: | 65mL/min |
| Capillary: | Positive mode 3500 V |
| Mobile phase: | A: LC-MS grade water with 1% acetic acid  B: LC-MS grade methanol with 1% acetic acid |
| Mixture-Elution: | |  |  |  | | --- | --- | --- | | % A | % B | Time (min) | | 95 | 5 | 0 | | 10 | 90 | 5 | | 10 | 90 | 6 | | 95 | 5 | 6.1 | |
| Analytical standards: | 1,2,4-triazole (1,2,4-TRZ)  CAS No. : 288-88-0  Lot: STBJ5727  Purity : 100.3% (considered 100% in the calculation)  Expiry date: December 2021  1,2,4-Triazole Alanime (TA)  CAS No. : 86362-20-1  Lot: 787796  Purity : 98.3%  Expiry date: 01/03/2024  1,2,4-Triazole lactic acid HCl (TLA)  CAS No. : 1450828-63-3  Lot: 792058  Purity : 78.5%  Expiry date: 01/11/2024  1,2,4-Triazole acetic acid (TAA)  CAS No. : 28711-29-7  Lot: BCCC0969  Purity : 95.7%  Expiry date: December 2021 |
| Isotope-labelled internal standards (ILIS): | 1,2,4-Triazole-[13C2,15N3]  CAS No. : 1261170-82-4  Lot: SL6-2012-224  Purity : 98.4%  Expiry date: 01/2023  1,2,4-Triazole Alanine [D2]  CAS No. : 2180306-38-9  Lot: 2011202L3.3  Purity : 95%  Expiry date: 20/01/2023  1,2,4-Triazole-[13C2, 15N3] Lactic Acid  CAS No. : n.d.  Lot: EFL6-2015-198A  Purity : 98.42%  Expiry date: 01/2024  1,2,4-Triazole acetic acid [13C2, 15N3]  CAS No. : n.d.  Lot: EFL6-2015-196A  Purity : 98.03%  Expiry date: 01/2024 |

***2. Methods***

The analytical method for the quantification of triazole derivatives metabolites in the tested matrices was based on the QuEChERS method (Method 8).

***2.A. Schematic diagram of the analytical method***

Plant matrices and processed commodities (rapeseed whole plant, rapeseed oil, rapeseed seeds, wheat grain, white bread (wheat), wheat straw and barley beer)



***2.B. Quantification of triazole derivative metabolites in wheat, barley, oilseed rape and processed commodities***

The analytical methods for the quantification of triazole derivatives metabolites in wheat, barley, rapeseed and processed commodities were first validated so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were validated in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |

Results and discussions

Method validation data can be summarised in Table A.1and A2.1 to A2.5. There are for each matrix a primary test. In view of the similar results between the primary and confirmatory test, a test by an independent laboratory validation (ILV) is not required.

Table A~~1~~ 52: Recovery results from method validation of triazole derivate metabolites using the analytical method

| Matrix | Analyte | Fortification level  (*n* = x) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Whole Plant (Rapeseed) | 1,2,4-TRZ | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  87.0 % | ***Primary transition***:  7.2 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  98.7 % | ***Primary transition***:  4.1 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  98.7 % | ***Primary transition***:  2.9 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  99.5 % | ***Primary transition***:  2.3 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TLA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  101.3 % | ***Primary transition***:  1.8 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  99.8 % | ***Primary transition***:  1.5 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TAA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  100.8 % | ***Primary transition***:  1.7 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  100.0 % | ***Primary transition***:  1.3 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Rapeseed seeds | 1,2,4-TRZ | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  99.0 % | ***Primary transition***:  2.3 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  93.9% | ***Primary transition***:  4.3 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  102.0 % | ***Primary transition***:  4.6 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  112.0 % | ***Primary transition***:  1.6 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TLA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  113.0 % | ***Primary transition***:  1.7 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  106.6 % | ***Primary transition***:  0.9 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TAA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  102.9 % | ***Primary transition***:  3.2 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  99.1 % | ***Primary transition***:  1.3 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Grain (wheat) | 1,2,4-TRZ | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  87.3 % | ***Primary transition***:  8.6 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  97.7 % | ***Primary transition***:  5.4 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  91.2 % | ***Primary transition***:  6.3 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  106.5 % | ***Primary transition***:  1.5 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TLA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  104.2 % | ***Primary transition***:  3.4 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  104.6 % | ***Primary transition***:  1.9 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TAA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  100.0 % | ***Primary transition***:  4.2 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  101.9 % | ***Primary transition***:  2.1 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Straw (wheat) | 1,2,4-TRZ | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  93.9 % | ***Primary transition***:  6.6 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  95.6 % | ***Primary transition***:  2.2 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  100.0 % | ***Primary transition***:  5.2 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  99.0 % | ***Primary transition***:  3.8 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TLA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  99.5 % | ***Primary transition***:  4.8 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  99.7 % | ***Primary transition***:  1.2 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TAA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  98.9 % | ***Primary transition***:  6.7 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  101.2 % | ***Primary transition***:  1.0 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Rapeseed oil | 1,2,4-TRZ | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  94.2 % | ***Primary transition***:  8.4 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  98.4 % | ***Primary transition***:  2.5 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  100.6 % | ***Primary transition***:  1.5 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  100.0 % | ***Primary transition***:  1.5 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TLA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  100.2 % | ***Primary transition***:  2.4 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  97.7 % | ***Primary transition***:  1.2 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TAA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  103.0 % | ***Primary transition***:  1.6 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  99.4 % | ***Primary transition***:  0.8 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| White bread (wheat) | 1,2,4-TRZ | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  97.7 % | ***Primary transition***:  3.4 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  98.7 % | ***Primary transition***:  4.3 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  99.9 % | ***Primary transition***:  3.8 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  100.3 % | ***Primary transition***:  1.0 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TLA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  99.9 % | ***Primary transition***:  3.8 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  100.3 % | ***Primary transition***:  1.0 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TAA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  101.1 % | ***Primary transition***:  2.5 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  98.5 % | ***Primary transition***:  0.9 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Beer (barley) | 1,2,4-TRZ | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  102.6 % | ***Primary transition***:  3.1 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  101.6 % | ***Primary transition***:  4.7 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  104.1 % | ***Primary transition***:  12.5 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  109.4 % | ***Primary transition***:  4.8 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TLA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  97.6 % | ***Primary transition***:  3.0 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  97.5 % | ***Primary transition***:  3.7% | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TAA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  100.1 % | ***Primary transition***:  17.5 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  103.1 % | ***Primary transition***:  1.6 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

Table A~~2.1~~ 53: Characteristics for the analytical method used for validation of triazole derivative metabolites residues in whole plant (rapeseed)

|  | TDMs |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  **Primarity transition:**  ***1,2,4-TRZ***  % interference mean = 6  ***TA***  % interference mean = 14.5  ***TLA***  % interference mean = 4  ***TAA***  % interference mean = 0.5 |
| Calibration (type, number of data points) | **Primarity transition:**  ***1,2,4-TRZ***  Equation : Y = 1.03477 \* x + 0.07821  Coefficient of correlation: r² = 99.984  ***TA***  Equation : Y = 0.51285 \* x + 0.02730  Coefficient of correlation: r² = 99.962  ***TLA***  Equation : Y = 1.20823 \* x + 0.02456  Coefficient of correlation: r² = 99.994  ***TAA***  Equation : Y = 0.98409 \* x + 0.01569  Coefficient of correlation: r² = 99.966 |
| Calibration range | Accepted calibration range in concentration units 0.5 – 50.0 µg/L (from 20 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes : Performed comparing the analyte response of one individual standard at L4 (or L5) prepared in solvent (water/methanol 50:50 + 1% formic acid) to one prepared in blank matrix extract at the same concentration.  ***1,2,4-TRZ***  Matrix effect = 19.5 %  Not significant  ***TA***  Matrix effect = -77.5 %  Significant in accordance to the SANTE/2020/12830 rev. 1 guideline  ***TLA***  Matrix effect = -8.1 %  Not significant  ***TAA***  Matrix effect = - 2.7 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | According to SANTE/2020/12830 rev.1, the stability of the analytes in the final extracts is not required if the extracts contain an isotope-labelled internal standard (IL-IS) for quantification, since the IL-IS compensates for losses during extract storage. Since IL-IS were used, the verification of the stability was not required. Anyway, all the extracts were analysed within 24 hours from their preparation, obtaining recoveries in the fortified samples within the acceptable range of 70-120%.  In addition, since the differences between the mean responses of the analytes in the diluted solutions prepared form stored and from freshly-prepared stock solutions was < 10%, it is possible to assess that the analytes are stable in the stock solutions prepared in water for at least 78 days, if stored in the dark at 5 ± 3°C. |

Table A~~2.2~~ 54: Characteristics for the analytical method used for validation of triazole derivatives metabolites in rapeseed oil

|  | TDMs |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  **Primarity transition:**  ***1,2,4-TRZ***  % interference mean = 5  ***TA***  % interference mean = 2.5  ***TLA***  % interference mean = 0.5  ***TAA***  % interference mean = 0 |
| Calibration (type, number of data points) | **Primarity transition:**  ***1,2,4-TRZ***  Equation : Y = 0.94482 \* x + 0.03984  Coefficient of correlation: r² = 99.912  ***TA***  Equation : Y = 2.37172 \* x + 0.01580  Coefficient of correlation: r² = 99.926  ***TLA***  Equation : Y = 1.22940 \* x – 0.00528  Coefficient of correlation: r² = 99.924  ***TAA***  Equation : Y = 0.98796 \* x + 1.07526e-4  Coefficient of correlation: r² = 99.940 |
| Calibration range | Accepted calibration range in concentration units 0.5 – 50.0 µg/L (from 20 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes : Performed comparing the analyte response of one individual standard at L4 (or L5) prepared in solvent (water/methanol 50:50 + 1% formic acid) to one prepared in blank matrix extract at the same concentration.  ***1,2,4-TRZ***  Matrix effect = -2.7 %  Not significant  ***TA***  Matrix effect = -3.2 %  Not significant  ***TLA***  Matrix effect = -5.4 %  Not significant  ***TAA***  Matrix effect = -2.5 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | According to SANTE/2020/12830 rev.1, the stability of the analytes in the final extracts is not required if the extracts contain an isotope-labelled internal standard (IL-IS) for quantification, since the IL-IS compensates for losses during extract storage. Since IL-IS were used, the verification of the stability was not required. Anyway, all the extracts were analysed within 24 hours from their preparation, obtaining recoveries in the fortified samples within the acceptable range of 70-120%.  In addition, since the differences between the mean responses of the analytes in the diluted solutions prepared form stored and from freshly-prepared stock solutions was < 10%, it is possible to assess that the analytes are stable in the stock solutions prepared in water for at least 78 days, if stored in the dark at 5 ± 3°C. |

Table A~~2.3~~ 55: Characteristics for the analytical method used for validation of triazole derivative metabolites in rapeseed seeds- grain (wheat) - white bread (wheat) (calibration in solvent 0.5-50 µg/L)

|  | TDMs |
| --- | --- |
| Specificity | **RAPESEED SEEDS**  ***For, 1,2,4-TRZ, TLA and TAA*** : No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***For TA*** : Signals were detected in amounts higher than the 30% of LOQ for this matrice and were generated by the analytes and not by other interfering substances. However, a lot of attempts were carried out by the laboratory to find samples with analytes area < 30% of LOQ, analysing a lot of commercially available commodities coming from organic farming: no samples with analytes area < 30% of LOQ were found for this matrice.  **Primarity transition:**  ***1,2,4-TRZ***  % interference mean = 8  ***TA***  % interference mean = 218.5  ***TLA***  % interference mean = 12  ***TAA***  % interference mean = 3.5  **GRAIN WHEAT**  ***For, 1,2,4-TRZ and TLA*** : No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***For TA*** ***and TAA***: Signals were detected in amounts higher than the 30% of LOQ for this matrice and were generated by the analytes and not by other interfering substanes.However, a lot of attempts were carried out by the laboratory to find samples with analytes area < 30% of LOQ, analysing a lot of commercially available commodities coming from organic farming: no samples with analytes area < 30% of LOQ were found for this matrice.  **Primarity transition:**  ***1,2,4-TRZ***  % interference mean = 28  ***TA***  % interference mean = 322.5  ***TLA***  % interference mean = 5  ***TAA***  % interference mean = 93.5  **WHITE BREAD (WHEAT)**  ***For, 1,2,4-TRZ and TLA*** : No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***For TA and TAA*** : Signals were detected in amounts higher than the 30% of LOQ for this matrice and were generated by the analytes and not by other interfering substanes.However, a lot of attempts were carried out by the laboratory to find samples with analytes area < 30% of LOQ, analysing a lot of commercially available commodities coming from organic farming: no samples with analytes area < 30% of LOQ were found for this matrice.  **Primarity transition:**  ***1,2,4-TRZ***  % interference mean = 0  ***TA***  % interference mean = 56.5  ***TLA***  % interference mean = 2  ***TAA***  % interference mean = 44.5 |
| Calibration (type, number of data points) | **Primarity transition:**  ***1,2,4-TRZ***  Equation : Y = 0.89935 \* x + 0.04423  Coefficient of correlation: r² = 99.940  ***TA***  Equation : Y = 2.76713 \* x + 0.02212  Coefficient of correlation: r² = 99.966  ***TLA***  Equation : Y = 1.02675 \* x – 9.58870e-4  Coefficient of correlation: r² = 99.898  ***TAA***  Equation : Y = 1.00664 \* x + 0.00803  Coefficient of correlation: r² = 99.996 |
| Calibration range | Accepted calibration range in concentration units 0.5 – 50.0 µg/L (from 20 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes : Performed comparing the analyte response of one individual standard at L4 (or L5) prepared in solvent (water/methanol 50:50 + 1% formic acid) to one prepared in blank matrix extract at the same concentration.  **RAPESEED SEEDS**  ***1,2,4-TRZ***  Matrix effect = -4.5 %  Not significant  ***TA***  Matrix effect = 6.0 %  Not significant  ***TLA***  Matrix effect = 6.7 %  Not significant  ***TAA***  Matrix effect = -2.1 %  Not significant  **GRAIN WHEAT**  ***1,2,4-TRZ***  Matrix effect = -7.7 %  Not significant  ***TA***  Matrix effect = -2.5 %  Not significant  ***TLA***  Matrix effect = -5.0 %  Not significant  ***TAA***  Matrix effect = -4.0 %  Not significant  **WHITE BREAD (WHEAT)**  ***1,2,4-TRZ***  Matrix effect = 3.5 %  Not significant  ***TA***  Matrix effect = 7.1 %  Not significant  ***TLA***  Matrix effect = 0.6 %  Not significant  ***TAA***  Matrix effect = -1.8 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | According to SANTE/2020/12830 rev.1, the stability of the analytes in the final extracts is not required if the extracts contain an isotope-labelled internal standard (IL-IS) for quantification, since the IL-IS compensates for losses during extract storage. Since IL-IS were used, the verification of the stability was not required. Anyway, all the extracts were analysed within 24 hours from their preparation, obtaining recoveries in the fortified samples within the acceptable range of 70-120%.  In addition, since the differences between the mean responses of the analytes in the diluted solutions prepared form stored and from freshly-prepared stock solutions was < 10%, it is possible to assess that the analytes are stable in the stock solutions prepared in water for at least 78 days, if stored in the dark at 5 ± 3°C. |

Table A~~2.4~~ 56: Characteristics for the analytical method used for validation of triazole derivative metabolites residues in straw (wheat) (calibration in solvent, 0.35 – 35 µg/L-

|  | TDMs |
| --- | --- |
| Specificity | ***For, 1,2,4-TRZ and TA*** : No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***For TLA and TAA*** : Signals were detected in amounts higher than the 30% of LOQ for this matrice and were generated by the analytes and not by other interfering substanes.However, a lot of attempts were carried out by the laboratory to find samples with analytes area < 30% of LOQ, analysing a lot of commercially available commodities coming from organic farming: no samples with analytes area < 30% of LOQ were found for this matrice. In addition no samples with analytes content < LOD, set to 20% of LOQ (28% for straw) were found. Since no matrices free from the analytes were available and the matrix effect for this matrice was negligible, it was necessary to calibrate using solvent-based standard solutions. Recoveries were necessarily evaluated subtracting the mean values measured from a duplicate analysis of the untreated samples to the values measured for the fortified ones.  **Primarity transition:**  ***1,2,4-TRZ***  % interference mean = 0  ***TA***  % interference mean = 9  ***TLA***  % interference mean = 56.5  ***TAA***  % interference mean = 126 |
| Calibration (type, number of data points) | **Primarity transition:**  ***1,2,4-TRZ***  Equation : Y = 0.97109 \* x + 0.00769  Coefficient of correlation: r² = 99.848  ***TA***  Equation : Y = 5.15827 \* x + 0.09160  Coefficient of correlation: r² = 99.988  ***TLA***  Equation : Y = 1.19902 \* x + 0.01208  Coefficient of correlation: r² = 99.956  ***TAA***  Equation : Y = 1.03861 \* x + 4.16121e-4  Coefficient of correlation: r² = 99.962 |
| Calibration range | Accepted calibration range in concentration units 0.35 – 35.0 µg/L (from 28 % of LOQ to 180 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.0028 – 0.280 mg/kg) |
| Assessment of matrix effects is presented | Yes : Performed comparing the analyte response of one individual standard at L4 (or L5) prepared in solvent (water/methanol 50:50 + 1% formic acid) to one prepared in blank matrix extract at the same concentration.  ***1,2,4-TRZ***  Matrix effect = 0.4 %  Not significant  ***TA***  Matrix effect = 1.3 %  Not significant  ***TLA***  Matrix effect = -7.9 %  Not significant  ***TAA***  Matrix effect = -3.7 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.35 µg/L  (0.0028 mg/kg) |
| Stability | According to SANTE/2020/12830 rev.1, the stability of the analytes in the final extracts is not required if the extracts contain an isotope-labelled internal standard (IL-IS) for quantification, since the IL-IS compensates for losses during extract storage. Since IL-IS were used, the verification of the stability was not required. Anyway, all the extracts were analysed within 24 hours from their preparation, obtaining recoveries in the fortified samples within the acceptable range of 70-120%.  In addition, since the differences between the mean responses of the analytes in the diluted solutions prepared form stored and from freshly-prepared stock solutions was < 10%, it is possible to assess that the analytes are stable in the stock solutions prepared in water for at least 78 days, if stored in the dark at 5 ± 3°C. |

Table A~~2.5~~ 57: Characteristics for the analytical method used for validation of triazole derivative metabolites residues in beer (barley) (calibration in solvent, 1-100 µg/L)

|  | TDMs |
| --- | --- |
| Specificity | ***For, 1,2,4-TRZ and TLA*** : No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***For TA*** ***and TAA***: Signals were detected in amounts higher than the 30% of LOQ for this matrice and were generated by the analytes and not by other interfering substanes.However, a lot of attempts were carried out by the laboratory to find samples with analytes area < 30% of LOQ, analysing a lot of commercially available commodities coming from organic farming: no samples with analytes area < 30% of LOQ were found for this matrice  **Primarity transition:**  ***1,2,4-TRZ***  % interference mean = 0  ***TA***  % interference mean = 310  ***TLA***  % interference mean = 15  ***TAA***  % interference mean = 219 |
| Calibration (type, number of data points) | **Primarity transition:**  ***1,2,4-TRZ***  Equation : Y = 0.94954 \* x + 0.00154  Coefficient of correlation: r² = 99.972  ***TA***  Equation : Y = 3.83597 \* x + 0.16176  Coefficient of correlation: r² = 99.940  ***TLA***  Equation : Y = 1.31066 \* x + 0.07797  Coefficient of correlation: r² = 99.610  ***TAA***  Equation : Y = 0.95844\* x + 0.01940  Coefficient of correlation: r² = 99.976 |
| Calibration range | Accepted calibration range in concentration units 1.00 – 100.0 µg/L (from 20 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes : Performed comparing the analyte response of one individual standard at L4 (or L5) prepared in solvent (water/methanol 50:50 + 1% formic acid) to one prepared in blank matrix extract at the same concentration.  ***1,2,4-TRZ***  Matrix effect = 5.2 %  Not significant  ***TA***  Matrix effect = 5.0 %  Not significant  ***TLA***  Matrix effect = -3.8 %  Not significant  ***TAA***  Matrix effect = 6.5 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 1 µg/L  (0.002 mg/kg) |
| Stability | According to SANTE/2020/12830 rev.1, the stability of the analytes in the final extracts is not required if the extracts contain an isotope-labelled internal standard (IL-IS) for quantification, since the IL-IS compensates for losses during extract storage. Since IL-IS were used, the verification of the stability was not required. Anyway, all the extracts were analysed within 24 hours from their preparation, obtaining recoveries in the fortified samples within the acceptable range of 70-120%.  In addition, since the differences between the mean responses of the analytes in the diluted solutions prepared form stored and from freshly-prepared stock solutions was < 10%, it is possible to assess that the analytes are stable in the stock solutions prepared in water for at least 78 days, if stored in the dark at 5 ± 3°C. |

Conclusion

The analytical method for the quantification of triazole derivative metabolites in wheat, barley, oilseed rape and processed commodities was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability for triazole derivative metabolites.

Independent laboratory validation

~~Not required.~~

Determination of Triazole Derivatives Metabolites (TMDs) in Rapeseed seeds

|  |  |
| --- | --- |
| Comments of zRMS: | The submitted ILV of the analytical method adjusted and validated in GLP study Code GLP-STUDY-21-108 by LabAnalysis s.r.l. for the determination of TDMs (1,2,4-triazole (TRZ), Triazole alanine (TA), Triazole lactic acid (TLA), Triazole acetic acid (TAA)) residues in Rapeseed seeds has been accepted.  The analytical method, using the HPLC/MS/MS in MRM mode is highly specific (2 transitions for all analytes) and gave both quantification and identification data, so the confirmatory method is not necessary.  All recovery values for each analyte at both fortification levels (LOQ and 10 x LOQ) resulted to be in the range 60-70 to 120 %, with an RSD% lower than 20-30% (SANTE/2020/12830 rev. 1) therefore the analytical method can be considered suitable to quantify TDMs residues in Rapeseed seeds samples with an established LOQ of 0.010 mg/kg. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/21 |
| Report | Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of TDM in Rapeseed seeds  Report No.: CH-1090/2021  Nichetti, S. (2022f)  ChemService S.r.l Controlli e Ricerche - Novate Milanese (MI) - Italy |
| Guideline(s): | Yes  SANTE/2020/12830 rev. 1 dated 24/02/21: Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes. |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

Materials and methods

|  |  |
| --- | --- |
| **Principle of the method** | The Test Facility conducted an Independent Laboratory Validation (ILV) of the analytical method adjusted and validated in GLP study Code GLP-STUDY-21-108 by LabAnalysis s.r.l. for the determination of triazole derivatives metabolites in rapeseed seeds: 1,2,4-triazole, triazole alanine, triazole lactic acid and triazole acetic acid. |
|  |  |

**Materials:**

|  |  |
| --- | --- |
| HPLC-MS/MS | Shimadzu mod. LC-40 XR, equipped with SelexION (Differential Mobility Separation) device and spectrometer Sciex API 6500 |
| Column: | THERMO LCN-412 Hypercarb, 5 μm, 100 x 2.1 mm i.d. |
| Detector: | MS Triple quadrupole equipped with a Differential Mobility Separation (DMS) device (MRM mode) |
| Ionisation type: | Electrospray (ESI) |
| Polarity: | Positive ion mode |
| Scan type: | MS/MS, multiple reaction monitoring |
| Drying gas temperature: | 500 °C |
| Curtain gas flow: | 30 mL/min |
| Nebulizer pressure: | 40 psi |
| Capillary voltage: | 3500 V |
|  |  |
| Analytical standards: | 1,2,4-Triazole  Batch No. : STBK4702  Purity : 100.0 %  Expiry date: October 1, 2023 |
|  | Triazole alanine dihydrochloride  Batch No. : 2017-0177604  Purity : 95 % (64.76% calculated as triazole alanine)  Expiry date: January 15, 2023 |
|  | Triazole lactic acid hydrochloride  Batch No. : 2019-0300703  Purity : 95 % (77.12% calculated as triazole lactic acid)  Expiry date: August 19, 2022  Triazole acetic acid  Batch No. : 2017-0130327  Purity : 95 %  Expiry date: April 11, 2023 |
|  |  |
| Reagents: | Water, HPLC grade, obtained by the Lab Water Purification System  Methanol, HPLC grade  Acetic acid, glacial  Formic acid, high purity for mass spectroscopy |
|  |  |
| Matrix: | Rapeseed seeds  Stored at -20°C in the dark |

**Methods:**

The analytical methods for the determination of triazole derivatives metabolites in rapeseed seeds were confirmed so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were confirmed in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A 58: Recovery results from independent laboratory validation of triazole derivative metabolites using the analytical method

| Matrix | Analyte | Fortification level (mg/kg) (n = 5) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Rapeseed seeds | 1,2,4-triazole  (product ion: 43.1 m/z) | At low level :  0.01 mg/kg (LOQ) | 102.7 | 3.88 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 102.3 | 3.16 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| 1,2,4-triazole  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 104.7 | 4.62 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 102.7 | 2.24 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole alanine  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 93.4 | 9.51 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 96.9 | 4.54 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole alanine  (product ion: 88.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 98.5 | 7.13 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 101.8 | 4.50 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole lactic acid  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 107.5 | 1.39 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 103.6 | 1.71 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole lactic acid  (product ion: 43.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 104.5 | 4.54 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 105.8 | 0.96 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole acetic acid  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 100.7 | 1.88 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 104.1 | 1.07 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole acetic acid  (product ion: 73.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 94.0 | 7.91 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 105.9 | 3.30 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

Table A 59: Characteristics for the analytical method used for independent laboratory validation of 1,2,4-triazole residues in rapeseed seeds

|  | 1,2,4-triazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Product ion (m/z = 43.1):***  % interference mean = 0.0  ***Product ion (m/z = 70.0):***  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Product ion (m/z = 43.1):***  Equation : Y = 0.0811 \* x – 0.0307  Coefficient of correlation: r² = 99.945  ***Product ion (m/z = 70.0):***  Equation : Y = 0.6901 \* x – 0.2197  Coefficient of correlation: r² = 99.972 |
| Calibration range | Accepted calibration range in concentration units 0.52 – 51.50 µg/L  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.200 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = 1 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-108 |

Table A 60: Characteristics for the analytical method used for independent laboratory validation of triazole alanine residues in rapeseed seeds

|  | Triazole alanine |
| --- | --- |
| Specificity | Since no matrix sample (Rapeseed seeds) with interference lower than 30 % of LOQ for all analytes was found, the evaluation of recovery for the Triazole-alanine analyte was carried out subtracting the contribute obtained from the Matrix Blank to Spike samples and the linear calibration was prepared in solvent.  ***Product ion (m/z = 88.0):***  % interference mean (low level) = 76.2  % interference mean (high level) = 26.2  ***Product ion (m/z = 70.0):***  % interference mean (low level) = 76.0  % interference mean (high level) = 25.6 |
| Calibration (type, number of data points) | ***Product ion (m/z = 88.0):***  Equation : Y = 0.1185 \* x – 0.0009  Coefficient of correlation: r² = 99.947  ***Product ion (m/z = 70.0):***  Equation : Y = 0.2044 \* x + 0.0261  Coefficient of correlation: r² = 99.991 |
| Calibration range | Accepted calibration range in concentration units 0.36 – 35.62 µg/L  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.200 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 19 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-108 |

Table A 61: Characteristics for the analytical method used for independent laboratory validation of triazole lactic acid in rapeseed seeds

|  | Triazole lactic acid |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Product ion (m/z = 43.0):***  % interference mean = 0.0  ***Product ion (m/z = 70.0):***  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Product ion (m/z = 43.0):***  Equation : Y = 0.0121 \* x + 0.0045  Coefficient of correlation: r² = 99.985  ***Product ion (m/z = 70.0):***  Equation : Y = 0.0965 \* x + 0.0262  Coefficient of correlation: r² = 99.988 |
| Calibration range | Accepted calibration range in concentration units 0.50 – 49.74 µg/L  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = 0 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-108 |

Table A 62: Characteristics for the analytical method used for independent laboratory validation of triazole acetic acid in rapeseed seeds

|  | Triazole acetic acid |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Product ion (m/z = 73.0):***  % interference mean = 0.0  ***Product ion (m/z = 70.0):***  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Product ion (m/z = 73.0):***  Equation : Y = 0.0035 \* x – 0.0010  Coefficient of correlation: r² = 99.860  ***Product ion (m/z = 70.0):***  Equation : Y = 0.0830 \* x + 0.0033  Coefficient of correlation: r² = 99.998 |
| Calibration range | Accepted calibration range in concentration units 0.51 – 50.83 µg/L  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = 1 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-108 |

Conclusion

The independent laboratory validation for the quantification of 1,2,4-triazole, triazole alanine, triazole lactic acid and triazole acetic acid in rapeseed seeds was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability.

Determination of Triazole Derivatives Metabolites (TMDs) in Whole plant (Rapeseed)

|  |  |
| --- | --- |
| Comments of zRMS: | The submitted ILV of the analytical method adjusted and validated in GLP study Code GLP-STUDY-21-108 by LabAnalysis s.r.l. for the determination of TDMs (1,2,4-triazole (TRZ), Triazole alanine (TA), Triazole lactic acid (TLA), Triazole acetic acid (TAA)) residues in Whole Plant (Rapeseed) has been accepted.  The analytical method, using the HPLC/MS/MS in MRM mode is highly specific (2 transitions for all analytes) and gave both quantification and identification data, so the confirmatory method is not necessary.  All recovery values for each analyte at both fortification levels (LOQ and 10 x LOQ) resulted to be in the range 60-70 to 120 %, with an RSD% lower than 20-30% (SANTE/2020/12830 rev. 1) therefore the analytical method can be considered suitable to quantify TDMs residues in Whole Plant (Rapeseed) samples with an established LOQ of 0.010 mg/kg.  Note: on page 15th of CH-1085/2021 it is stated: “the analytical method can be considered suitable to quantify Difenoconazole and Prothioconazole-desthio” It should be corrected. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/22 |
| Report | Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of TDM in Whole Plant (Rapeseed)  Report No.: CH-1085/2021  Nichetti, S. (2022g)  ChemService S.r.l Controlli e Ricerche - Novate Milanese (MI) - Italy |
| Guideline(s): | Yes  SANTE/2020/12830 rev. 1 dated 24/02/21: Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes. |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

Materials and methods

|  |  |
| --- | --- |
| **Principle of the method** | The Test Facility conducted an Independent Laboratory Validation (ILV) of the analytical method adjusted and validated in GLP study Code GLP-STUDY-21-108 by LabAnalysis s.r.l. for the determination of triazole derivatives metabolites in whole plant (rapeseed): 1,2,4-triazole, triazole alanine, triazole lactic acid and triazole acetic acid. |
|  |  |

**Materials:**

|  |  |
| --- | --- |
| HPLC-MS/MS | Shimadzu mod. LC-40 XR, equipped with SelexION (Differential Mobility Separation) device and spectrometer Sciex API 6500 |
| Column: | THERMO LCN-412 Hypercarb, 5 μm, 100 x 2.1 mm i.d. |
| Detector: | MS Triple quadrupole equipped with a Differential Mobility Separation (DMS) device (MRM mode) |
| Ionisation type: | Electrospray (ESI) |
| Polarity: | Positive ion mode |
| Scan type: | MS/MS, multiple reaction monitoring |
| Drying gas temperature: | 500 °C |
| Curtain gas flow: | 30 mL/min |
| Nebulizer pressure: | 40 psi |
| Capillary voltage: | 3500 V |
| Analytical standards: | 1,2,4-Triazole  Batch No. : STBK4702  Purity : 100.0 %  Expiry date: October 1, 2023 |
|  | Triazole alanine dihydrochloride  Batch No. : 2017-0177604  Purity : 95 % (64.76% calculated as triazole alanine)  Expiry date: January 15, 2023 |
|  | Triazole lactic acid hydrochloride  Batch No. : 2019-0300703  Purity : 95 % (77.12% calculated as triazole lactic acid)  Expiry date: August 19, 2022  Triazole acetic acid  Batch No. : 2017-0130327  Purity : 95 %  Expiry date: April 11, 2023 |
| Reagents: | Water, HPLC grade, obtained by the Lab Water Purification System  Methanol, HPLC grade  Acetic acid, glacial  Formic acid, high purity for mass spectroscopy |
| Matrix: | Whole plant (rapeseed)  Stored at -20°C in the dark |

**Methods:**

The analytical methods for the determination of triazole derivatives metabolites in whole plant (rapeseed) were confirmed so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were confirmed in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A 63: Recovery results from independent laboratory validation of triazole derivative metabolites using the analytical method

| Matrix | Analyte | Fortification level (mg/kg) (n = 5) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Whole Plant (Rapeseed) | 1,2,4-triazole  (product ion: 43.1 m/z) | At low level :  0.01 mg/kg (LOQ) | 87.5 | 5.23 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 97.1 | 3.03 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| 1,2,4-triazole  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 93.6 | 2.63 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 99.0 | 3.69 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole alanine  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 89.9 | 10.03 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 100.4 | 4.04 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole alanine  (product ion: 88.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 98.5 | 8.83 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 102.5 | 3.61 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole lactic acid  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 97.1 | 2.29 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 100.4 | 0.97 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole lactic acid  (product ion: 43.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 100.8 | 4.20 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 101.6 | 1.49 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole acetic acid  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 99.6 | 2.11 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 101.8 | 0.85 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole acetic acid  (product ion: 73.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 92.7 | 7.72 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 100.3 | 2.81 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

Table A 64: Characteristics for the analytical method used for independent laboratory validation of 1,2,4-triazole residues in whole plant (rapeseed)

|  | 1,2,4-triazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Product ion (m/z = 43.1):***  % interference mean = 0.0  ***Product ion (m/z = 70.0):***  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Product ion (m/z = 43.1):***  Equation : Y = 0.0741 \* x + 0.0238  Coefficient of correlation: r² = 99.988  ***Product ion (m/z = 70.0):***  Equation : Y = 0.6458 \* x + 0.2530  Coefficient of correlation: r² = 99.977 |
| Calibration range | Accepted calibration range in concentration units 0.53 – 52.50 µg/L  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.200 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 10 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-108 |

Table A 65: Characteristics for the analytical method used for independent laboratory validation of triazole alanine residues in whole plant (rapeseed)

|  | Triazole alanine |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Product ion (m/z = 88.0):***  % interference mean = 0.0  ***Product ion (m/z = 70.0):***  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Product ion (m/z = 88.0):***  Equation : Y = 0.0985 \* x + 0.1008  Coefficient of correlation: r² = 99.963  ***Product ion (m/z = 70.0):***  Equation : Y = 0.1743 \* x + 0.1894  Coefficient of correlation: r² = 99.982 |
| Calibration range | Accepted calibration range in concentration units 0.38 – 37.56 µg/L  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.200 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 7 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-108 |

Table A 66: Characteristics for the analytical method used for independent laboratory validation of triazole lactic acid in whole plant (rapeseed)

|  | Triazole lactic acid |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Product ion (m/z = 43.0):***  % interference mean = 0.0  ***Product ion (m/z = 70.0):***  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Product ion (m/z = 43.0):***  Equation : Y = 0.0131 \* x + 0.0028  Coefficient of correlation: r² = 99.988  ***Product ion (m/z = 70.0):***  Equation : Y = 0.1042 \* x – 0.0027  Coefficient of correlation: r² = 99.990 |
| Calibration range | Accepted calibration range in concentration units 0.48 – 48.20 µg/L  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = 3 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-108 |

Table A 67: Characteristics for the analytical method used for independent laboratory validation of triazole acetic acid in whole plant (rapeseed)

|  | Triazole acetic acid |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Product ion (m/z = 73.0):***  % interference mean = 0.0  ***Product ion (m/z = 70.0):***  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Product ion (m/z = 73.0):***  Equation : Y = 0.0037 \* x – 0.0005  Coefficient of correlation: r² = 99.960  ***Product ion (m/z = 70.0):***  Equation : Y = 0.0843 \* x + 0.0025  Coefficient of correlation: r² = 99.993 |
| Calibration range | Accepted calibration range in concentration units 0.52 – 52.25 µg/L  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = 1 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-108 |

Conclusion

The independent laboratory validation for the quantification of 1,2,4-triazole, triazole alanine, triazole lactic acid and triazole acetic acid in whole plant (rapeseed)was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability.

Determination of Triazole Derivatives Metabolites (TMDs) in Grain (wheat)

|  |  |
| --- | --- |
| Comments of zRMS: | The submitted ILV of the analytical method adjusted and validated in GLP study Code GLP-STUDY-21-108 by LabAnalysis s.r.l. for the determination of TDMs (1,2,4-triazole (TRZ), Triazole alanine (TA), Triazole lactic acid (TLA), Triazole acetic acid (TAA)) residues in Grain (wheat) has been accepted.  The analytical method, using the HPLC/MS/MS in MRM mode is highly specific (2 transitions for all analytes) and gave both quantification and identification data, so the confirmatory method is not necessary.  All recovery values for each analyte at both fortification levels (LOQ and 10 x LOQ) resulted to be in the range 60-70 to 120 %, with an RSD% lower than 20-30% (SANTE/2020/12830 rev. 1) therefore the analytical method can be considered suitable to quantify TDMs residues in Grain (wheat) samples with an established LOQ of 0.010 mg/kg. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/23 |
| Report | Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of TDM in Grain (wheat)  Report No.: CH-1087/2021  Nichetti, S. (2022h)  ChemService S.r.l Controlli e Ricerche - Novate Milanese (MI) - Italy |
| Guideline(s): | Yes  SANTE/2020/12830 rev. 1 dated 24/02/21: Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes. |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

Materials and methods

|  |  |
| --- | --- |
| **Principle of the method** | The Test Facility conducted an Independent Laboratory Validation (ILV) of the analytical method adjusted and validated in GLP study Code GLP-STUDY-21-108 by LabAnalysis s.r.l. for the determination of triazole derivatives metabolites in grain (wheat): 1,2,4-triazole, triazole alanine, triazole lactic acid and triazole acetic acid. |

**Materials:**

|  |  |
| --- | --- |
| HPLC-MS/MS | Shimadzu mod. LC-40 XR, equipped with SelexION (Differential Mobility Separation) device and spectrometer Sciex API 6500 |
| Column: | THERMO LCN-412 Hypercarb, 5 μm, 100 x 2.1 mm i.d. |
| Detector: | MS Triple quadrupole equipped with a Differential Mobility Separation (DMS) device (MRM mode) |
| Ionisation type: | Electrospray (ESI) |
| Polarity: | Positive ion mode |
| Scan type: | MS/MS, multiple reaction monitoring |
| Drying gas temperature: | 500 °C |
| Curtain gas flow: | 30 mL/min |
| Nebulizer pressure: | 40 psi |
| Capillary voltage: | 3500 V |
|  |  |
| Analytical standards: | 1,2,4-Triazole  Batch No. : STBK4702  Purity : 100.0 %  Expiry date: October 1, 2023 |
|  | Triazole alanine dihydrochloride  Batch No. : 2017-0177604  Purity : 95 % (64.76% calculated as triazole alanine)  Expiry date: January 15, 2023 |
|  | Triazole lactic acid hydrochloride  Batch No. : 2019-0300703  Purity : 95 % (77.12% calculated as triazole lactic acid)  Expiry date: August 19, 2022  Triazole acetic acid  Batch No. : 2017-0130327  Purity : 95 %  Expiry date: April 11, 2023 |
|  |  |
| Reagents: | Water, HPLC grade, obtained by the Lab Water Purification System  Methanol, HPLC grade  Acetic acid, glacial  Formic acid, high purity for mass spectroscopy |
|  |  |
| Matrix: | Grain (wheat)  Stored at -20°C in the dark |

**Methods:**

The analytical methods for the determination of triazole derivatives metabolites in grain (wheat) were confirmed so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were confirmed in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A 68: Recovery results from independent laboratory validation of triazole derivative metabolites using the analytical method

| Matrix | Analyte | Fortification level (mg/kg) (n = 5) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Grain (wheat) | 1,2,4-triazole  (product ion: 43.1 m/z) | At low level :  0.01 mg/kg (LOQ) | 97.6 | 3.49 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 99.7 | 3.06 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| 1,2,4-triazole  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 102.9 | 2.20 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 98.6 | 1.67 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole alanine  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 85.9 | 6.78 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 98.4 | 1.57 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole alanine  (product ion: 88.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 96.0 | 9.37 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 96.9 | 2.82 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole lactic acid  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 101.4 | 2.24 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 99.8 | 1.20 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole lactic acid  (product ion: 43.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 98.7 | 2.69 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 99.8 | 1.57 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole acetic acid  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 101.0 | 2.17 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 99.3 | 0.32 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole acetic acid  (product ion: 73.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 96.8 | 8.99 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 100.5 | 2.52 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

Table A 69: Characteristics for the analytical method used for independent laboratory validation of 1,2,4-triazole residues in grain (wheat)

|  | 1,2,4-triazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Product ion (m/z = 43.1):***  % interference mean = 0.0  ***Product ion (m/z = 70.0):***  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Product ion (m/z = 43.1):***  Equation : Y = 0.0730 \* x + 0.0348  Coefficient of correlation: r² = 99.972  ***Product ion (m/z = 70.0):***  Equation : Y = 0.6314 \* x + 0.0675  Coefficient of correlation: r² = 99.999 |
| Calibration range | Accepted calibration range in concentration units 0.53 – 52.50 µg/L  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.200 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 3 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-108 |

Table A 70: Characteristics for the analytical method used for independent laboratory validation of triazole alanine residues in grain (wheat)

|  | Triazole alanine |
| --- | --- |
| Specificity | Since no matrix sample (grain wheat) with interference lower than 30 % of LOQ for all analytes was found, the evaluation of recovery for the Triazole-alanine analyte was carried out subtracting the contribute obtained from the Matrix Blank to Spike samples and the linear calibration was prepared in solvent.  ***Product ion (m/z = 88.0):***  % interference mean (low level) = 53.4  % interference mean (high level) = 11.0  ***Product ion (m/z = 70.0):***  % interference mean (low level) = 55.6  % interference mean (high level) = 11.2 |
| Calibration (type, number of data points) | ***Product ion (m/z = 88.0):***  Equation : Y = 0.1175 \* x – 0.0022  Coefficient of correlation: r² = 99.942  ***Product ion (m/z = 70.0):***  Equation : Y = 0.2101 \* x – 0.0264  Coefficient of correlation: r² = 99.870 |
| Calibration range | Accepted calibration range in concentration units 0.39 – 39.18 µg/L  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.200 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = 0 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-108 |

Table A 71: Characteristics for the analytical method used for independent laboratory validation of triazole lactic acid in grain (wheat)

|  | Triazole lactic acid |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Product ion (m/z = 43.0):***  % interference mean = 0.0  ***Product ion (m/z = 70.0):***  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Product ion (m/z = 43.0):***  Equation : Y = 0.0124 \* x – 0.0010  Coefficient of correlation: r² = 99.999  ***Product ion (m/z = 70.0):***  Equation : Y = 0.0950 \* x + 0.0052  Coefficient of correlation: r² = 99.997 |
| Calibration range | Accepted calibration range in concentration units 0.48 – 48.20 µg/L  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 1 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-108 |

Table A 72: Characteristics for the analytical method used for independent laboratory validation of triazole acetic acid in grain (wheat)

|  | Triazole acetic acid |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Product ion (m/z = 73.0):***  % interference mean (low level) = 23.3  % interference mean (high level) = 3.2  ***Product ion (m/z = 70.0):***  % interference mean (low level) = 20.1  % interference mean (high level) = 2.6 |
| Calibration (type, number of data points) | ***Product ion (m/z = 73.0):***  Equation : Y = 0.0036 \* x – 0.0003  Coefficient of correlation: r² = 99.993  ***Product ion (m/z = 70.0):***  Equation : Y = 0.0851 \* x – 0.0010  Coefficient of correlation: r² = 99.992 |
| Calibration range | Accepted calibration range in concentration units 0.50 – 50.35 µg/L  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 3 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-108 |

Conclusion

The independent laboratory validation for the quantification of 1,2,4-triazole, triazole alanine, triazole lactic acid and triazole acetic acid in grain (wheat) was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability.

Determination of Triazole Derivatives Metabolites (TMDs) in ~~Grain~~ straw (wheat)

|  |  |
| --- | --- |
| Comments of zRMS: | The submitted ILV of the analytical method adjusted and validated in GLP study Code GLP-STUDY-21-108 by LabAnalysis s.r.l. for the determination of TDMs (1,2,4-triazole (TRZ), Triazole alanine (TA), Triazole lactic acid (TLA), Triazole acetic acid (TAA)) residues in Straw (wheat) has been accepted.  The analytical method, using the HPLC/MS/MS in MRM mode is highly specific (2 transitions for all analytes) and gave both quantification and identification data, so the confirmatory method is not necessary.  All recovery values for each analyte at both fortification levels (LOQ and 10 x LOQ) resulted to be in the range 60-70 to 120 %, with an RSD% lower than 20-30% (SANTE/2020/12830 rev. 1) therefore the analytical method can be considered suitable to quantify TDMs residues in Straw (wheat) samples with an established LOQ of 0.010 mg/kg |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/24 |
| Report | Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of TDM in Straw (wheat)  Report No.: CH-1086/2021  Nichetti, S. (2022i)  ChemService S.r.l Controlli e Ricerche - Novate Milanese (MI) - Italy |
| Guideline(s): | Yes  SANTE/2020/12830 rev. 1 dated 24/02/21: Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes. |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

Materials and methods

|  |  |
| --- | --- |
| **Principle of the method** | The Test Facility conducted an Independent Laboratory Validation (ILV) of the analytical method adjusted and validated in GLP study Code GLP-STUDY-21-108 by LabAnalysis s.r.l. for the determination of triazole derivatives metabolites in straw (wheat): 1,2,4-triazole, triazole alanine, triazole lactic acid and triazole acetic acid. |
|  |  |

**Materials:**

|  |  |
| --- | --- |
| HPLC-MS/MS | Shimadzu mod. LC-40 XR, equipped with SelexION (Differential Mobility Separation) device and spectrometer Sciex API 6500 |
| Column: | THERMO LCN-412 Hypercarb, 5 μm, 100 x 2.1 mm i.d. |
| Detector: | MS Triple quadrupole equipped with a Differential Mobility Separation (DMS) device (MRM mode) |
| Ionisation type: | Electrospray (ESI) |
| Polarity: | Positive ion mode |
| Scan type: | MS/MS, multiple reaction monitoring |
| Drying gas temperature: | 500 °C |
| Curtain gas flow: | 30 mL/min |
| Nebulizer pressure: | 40 psi |
| Capillary voltage: | 3500 V |
|  |  |
| Analytical standards: | 1,2,4-Triazole  Batch No. : STBK4702  Purity : 100.0 %  Expiry date: October 1, 2023 |
|  | Triazole alanine dihydrochloride  Batch No. : 2017-0177604  Purity : 95 % (64.76% calculated as triazole alanine)  Expiry date: January 15, 2023 |
|  | Triazole lactic acid hydrochloride  Batch No. : 2019-0300703  Purity : 95 % (77.12% calculated as triazole lactic acid)  Expiry date: August 19, 2022  Triazole acetic acid  Batch No. : 2017-0130327  Purity : 95 %  Expiry date: April 11, 2023 |
|  |  |
| Reagents: | Water, HPLC grade, obtained by the Lab Water Purification System  Methanol, HPLC grade  Acetic acid, glacial  Formic acid, high purity for mass spectroscopy |
|  |  |
| Matrix: | Straw (wheat)  Stored at -20°C in the dark |

**Methods:**

The analytical methods for the determination of triazole derivatives metabolites in straw (wheat) were confirmed so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were confirmed in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A 73: Recovery results from independent laboratory validation of triazole derivative metabolites using the analytical method

| Matrix | Analyte | Fortification level (mg/kg) (n = 5) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Straw (wheat) | 1,2,4-triazole  (product ion: 43.1 m/z) | At low level :  0.01 mg/kg (LOQ) | 103.2 | 5.11 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 106.8 | 4.66 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| 1,2,4-triazole  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 110.6 | 3.36 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 106.9 | 1.06 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole alanine  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 96.2 | 9.55 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 102.3 | 5.19 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole alanine  (product ion: 88.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 99.2 | 11.53 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 104.5 | 5.47 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole lactic acid  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 90.6 | 2.90 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 105.3 | 2.46 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole lactic acid  (product ion: 43.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 97.6 | 5.71 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 106.6 | 2.01 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole acetic acid  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 117.9 | 1.67 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 110.8 | 1.48 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole acetic acid  (product ion: 73.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 112.2 | 6.30 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 103.8 | 6.04 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

Table A 74: Characteristics for the analytical method used for independent laboratory validation of 1,2,4-triazole residues in straw (wheat)

|  | 1,2,4-triazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Product ion (m/z = 43.1):***  % interference mean = 0.0  ***Product ion (m/z = 70.0):***  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Product ion (m/z = 43.1):***  Equation : Y = 0.0783 \* x + 0.0279  Coefficient of correlation: r² = 99.950  ***Product ion (m/z = 70.0):***  Equation : Y = 0.6773 \* x + 0.1250  Coefficient of correlation: r² = 99.969 |
| Calibration range | Accepted calibration range in concentration units 0.35 – 34.65 µg/L  Corresponding calibration range in mass ratio units for the sample (0.0028 – 0.280 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 13 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.35 µg/L  (0.0028 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-108 |

Table A 75: Characteristics for the analytical method used for independent laboratory validation of triazole alanine residues in straw (wheat)

|  | Triazole alanine |
| --- | --- |
| Specificity | Since no matrix sample (straw wheat) with interference lower than 30 % of LOQ for all analytes was found, the evaluation of recovery for the Triazole-alanine analyte was carried out subtracting the contribute obtained from the Matrix Blank to Spike samples and the linear calibration was prepared in solvent.  ***Product ion (m/z = 88.0):***  % interference mean (low level) = 82.9  % interference mean (high level) = 36.1  ***Product ion (m/z = 70.0):***  % interference mean (low level) = 83.4  % interference mean (high level) = 35.4 |
| Calibration (type, number of data points) | ***Product ion (m/z = 88.0):***  Equation : Y = 0.1492 \* x + 0.0848  Coefficient of correlation: r² = 99.867  ***Product ion (m/z = 70.0):***  Equation : Y = 0.2649 \* x + 0.1264  Coefficient of correlation: r² = 99.837 |
| Calibration range | Accepted calibration range in concentration units 0.28 – 27.88 µg/L  Corresponding calibration range in mass ratio units for the sample (0.0028 – 0.280 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 4 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.35 µg/L  (0.0028 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-108 |

Table A 76: Characteristics for the analytical method used for independent laboratory validation of triazole lactic acid in straw (wheat)

|  | Triazole lactic acid |
| --- | --- |
| Specificity | Since no matrix sample (straw wheat) with interference lower than 30 % of LOQ for all analytes was found, the evaluation of recovery for the Triazole lactic acid analyte was carried out subtracting the contribute obtained from the Matrix Blank to Spike samples and the linear calibration was prepared in solvent.  ***Product ion (m/z = 43.0):***  % interference mean (low level) = 83.1  % interference mean (high level) = 30.4  ***Product ion (m/z = 70.0):***  % interference mean (low level) = 84.8  % interference mean (high level) = 30.6 |
| Calibration (type, number of data points) | ***Product ion (m/z = 43.0):***  Equation : Y = 0.0123 \* x + 0.0024  Coefficient of correlation: r² = 99.979  ***Product ion (m/z = 70.0):***  Equation : Y = 0.1003 \* x + 0.0139  Coefficient of correlation: r² = 99.965 |
| Calibration range | Accepted calibration range in concentration units 0.33 – 33.20 µg/L  Corresponding calibration range in mass ratio units for the sample (0.0028 – 0.280 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = 5 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.35 µg/L  (0.0028 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-108 |

Table A 77: Characteristics for the analytical method used for independent laboratory validation of triazole acetic acid in straw (wheat)

|  | Triazole acetic acid |
| --- | --- |
| Specificity | Since no matrix sample (straw wheat) with interference lower than 30 % of LOQ for all analytes was found, the evaluation of recovery for the Triazole acetic acid analyte was carried out subtracting the contribute obtained from the Matrix Blank to Spike samples and the linear calibration was prepared in solvent.  ***Product ion (m/z = 73.0):***  % interference mean (low level) = 36.6  % interference mean (high level) = 6.0  ***Product ion (m/z = 70.0):***  % interference mean (low level) = 34.8  % interference mean (high level) = 5.2 |
| Calibration (type, number of data points) | ***Product ion (m/z = 73.0):***  Equation : Y = 0.0036 \* x + 0.0004  Coefficient of correlation: r² = 99.974  ***Product ion (m/z = 70.0):***  Equation : Y = 0.0863 \* x + 0.0143  Coefficient of correlation: r² = 99.953 |
| Calibration range | Accepted calibration range in concentration units 0.34 – 34.25 µg/L  Corresponding calibration range in mass ratio units for the sample (0.0028 – 0.280 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 3 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.35 µg/L  (0.0028 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-108 |

Conclusion

The independent laboratory validation for the quantification of 1,2,4-triazole, triazole alanine, triazole lactic acid and triazole acetic acid in straw (wheat) was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability.

Confirmatory method

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  The analytical method for the determination of TDMs was based on the method “Quick Method for the Analysis of Highly Polar Pesticides in Food Involving Extraction with Acidified Methanol and LC- or IC-MS/MS Measurement I. - Food of Plant Origin (QuPPe-PO-Method) - Method 8 (M8)”.  The determination was carried out using a HPLC-MS/MS system (high-performance liquid chromatography + triple quadrupole mass spectrometry) equipped with a differential mobility separation device (DMS).  As two mass transitions were validated, the confirmatory method is not required. Duplication of the study description within the report is not necessary (this happens several times).  The specificity of the method was assured by MS/MS detection. All validation parameters are in the currently required range.  The matrix effect was not significant according to the SANTE/2020/12830 rev.1 except for triazole-alanine in whole OSR plant.  See also into the present section B7 where this validated method was employed to generation of the data in paragraphs of Appendix 2: A 2.1.3.1.1,2,3; A 2.1.5.2.1,2,3; A 2.2.3.1.1,2,3; A 2.2.5.2.1,2,3. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/14 |
| Report | Validation of an analytical method for the quantification of Triazole Derivative Metabolites (TDMs) in wheat, barley, oilseed rape and processed commodities  Longhi, D.  2021  ChemService S.r.l. Controlli e Richerche, Novate Milanese - Italy  Report No. : 21-108 |
| Guideline(s): | Yes : SANTE/2020/12830 rev. 1 (dated 24/02/2021) ;  SANTE2017/10632 rev. 3 (dated 22/11/2017) ;  OECD ENV/JM/MONO(2007)17 ;  “Quick Method for the Analysis of Highly Polar Pesticides in Food Involving Extraction with Acidified Methanol and LC- or IC-MS/MS Measurement - Food of Plant Origin (QuPPe-PO-Method)- Method 8 (M8)”. |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The confirmatory method for the determination of TDMs in the tested matrices (AM-GLP-STUDY-21-108) was based on the method “Quick Method for the Analysis of Highly Polar Pesticides in Food Involving Extraction with Acidified Methanol and LC- or IC-MS/MS Measurement I. - Food of Plant Origin (QuPPe-PO-Method) - Method 8 (M8)”.  The instrumental determination was carried out using a HPLC-MS/MS system (high-performance liquid chromatography + triple quadrupole mass spectrometry) equipped with a differential mobility separation device (DMS). |

Materials and methods

***1. Materials***

***1.A. Quantification of triazole derivative metabolites in wheat, barley, oilseed rape and processed commodities***

The quantification of triazole derivative metabolites in wheat, barley, oilseed rape and processed commodities was assessed by HLPC/MS/MS.

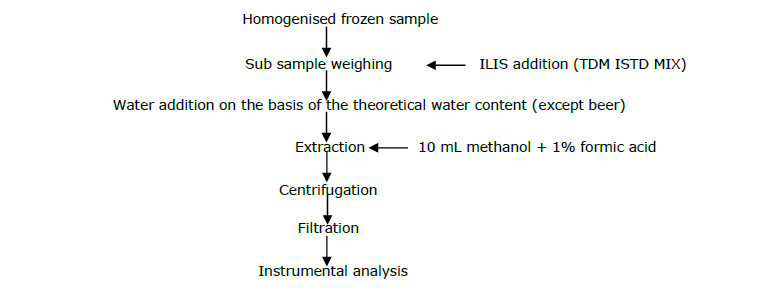
|  |  |
| --- | --- |
| HPLC: | Shimadzu LC-40 XR + spectrometer Sciex API 6500+ equipped with SelexION (Differential Mobility Separation) device |
| Column: | Thermo Hyperbare 5 μm, 2.1 x 100 mm |
| Detector: | Agilent MS spectrometer 6470A Triple Quad |
| Column temperature: | 40 °C |
| Flow rate: | 0.6 mL/min |
| Volume of injection: | 2 µL |
| Retention time: | Approximatively 2.5 minutes for difenoconazole |
| Stop time: | 10 minutes |
| Gas temperature: | 500 °C |
| Curtain Gas flow: | 30 mL/min |
| Gas flow 1: | 55 mL/min |
| Gas flow2: | 65mL/min |
| Capillary: | Positive mode 3500 V |
| Mobile phase: | A: LC-MS grade water with 1% acetic acid  B: LC-MS grade methanol with 1% acetic acid |
| Mixture-Elution: | |  |  |  | | --- | --- | --- | | % A | % B | Time (min) | | 95 | 5 | 0 | | 10 | 90 | 5 | | 10 | 90 | 6 | | 95 | 5 | 6.1 | |
| Analytical standards: | 1,2,4-triazole (1,2,4-TRZ)  CAS No. : 288-88-0  Lot: STBJ5727  Purity : 100.3% (considered 100% in the calculation)  Expiry date: December 2021  1,2,4-Triazole Alanime (TA)  CAS No. : 86362-20-1  Lot: 787796  Purity : 98.3%  Expiry date: 01/03/2024  1,2,4-Triazole lactic acid HCl (TLA)  CAS No. : 1450828-63-3  Lot: 792058  Purity : 78.5%  Expiry date: 01/11/2024  1,2,4-Triazole acetic acid (TAA)  CAS No. : 28711-29-7  Lot: BCCC0969  Purity : 95.7%  Expiry date: December 2021 |
| Isotope-labelled internal standards (ILIS): | 1,2,4-Triazole-[13C2,15N3]  CAS No. : 1261170-82-4  Lot: SL6-2012-224  Purity : 98.4%  Expiry date: 01/2023  1,2,4-Triazole Alanine [D2]  CAS No. : 2180306-38-9  Lot: 2011202L3.3  Purity : 95%  Expiry date: 20/01/2023  1,2,4-Triazole-[13C2, 15N3] Lactic Acid  CAS No. : n.d.  Lot: EFL6-2015-198A  Purity : 98.42%  Expiry date: 01/2024  1,2,4-Triazole acetic acid [13C2, 15N3]  CAS No. : n.d.  Lot: EFL6-2015-196A  Purity : 98.03%  Expiry date: 01/2024 |

***2. Methods***

The analytical method for the quantification of triazole derivatives metabolites in the tested matrices was based on the QuEChERS method (Method 8).

***2.A. Schematic diagram of the analytical method***

Plant matrices and processed commodities (rapeseed whole plant, rapeseed oil, rapeseed seeds, wheat grain, white bread (wheat), wheat straw and barley beer)



***2.B. Quantification of triazole derivative metabolites in wheat, barley, oilseed rape and processed commodities***

The analytical methods for the quantification of triazole derivatives metabolites in wheat, barley, rapeseed and processed commodities were first validated so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were validated in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |

Results and discussions

Confirmatory method validation data can be summarised in tables below ~~Table A.1and A2.1 to A2.5~~. There are for each matrix a primary test. In view of the similar results between the primary and confirmatory test, a test by an independent laboratory validation (ILV) is not required.

Table A~~1~~ 78: Recovery results from method validation of triazole derivate metabolites using the analytical method

| Matrix | Analyte | Fortification level  (*n* = x) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Whole Plant (Rapeseed) | 1,2,4-TRZ | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  92.3 % | ***Confirmatory transition***:  7.7 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  114.3 % | ***Confirmatory transition***:  3.0 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  97.8 % | ***Confirmatory transition***:  4.8 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  100.9 % | ***Confirmatory transition***:  1.3 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TLA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  99.7 % | ***Confirmatory transition***:  4.3 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  101.1 % | ***Confirmatory transition***:  1.0 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TAA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  99.9 % | ***Confirmatory transition***:  1.6 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  97.5 % | ***Confirmatory transition***:  1.6 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Rapeseed seeds | 1,2,4-TRZ | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  95.9 % | ***Confirmatory transition***:  5.0 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  92.0 % | ***Confirmatory transition***:  4.5 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  102.2 % | ***Confirmatory transition***:  6.3 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  112.1 % | ***Confirmatory transition***:  3.5 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TLA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  112.5 % | ***Confirmatory transition***:  3.3 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  107.1 % | ***Confirmatory transition***:  0.88 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TAA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  101.6 % | ***Confirmatory transition***:  5.0 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  98.6 % | ***Confirmatory transition***:  2.3 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Grain (wheat) | 1,2,4-TRZ | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  90.1 % | ***Confirmatory transition***:  3.7 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  97.7 % | ***Confirmatory transition***:  4.0 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  89.5 % | ***Confirmatory transition***:  13.1 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  107.8 % | ***Confirmatory transition***:  1.8 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TLA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  103.4 % | ***Confirmatory transition***:  6.2 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  106.5 % | ***Confirmatory transition***:  1.1 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TAA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  92.7 % | ***Confirmatory transition***:  15.6 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  102.4 % | ***Confirmatory transition***:  2.9 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Straw (wheat) | 1,2,4-TRZ | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  96.4 % | ***Confirmatory transition***:  4.0 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  99.2 % | ***Confirmatory transition***:  2.3 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  98.2 % | ***Confirmatory transition***:  6.9 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  102.1 % | ***Confirmatory transition***:  4.9 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TLA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  99.4 % | ***Confirmatory transition***:  6.6 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  96.3 % | ***Confirmatory transition***:  1.5 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TAA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  98.7 % | ***Confirmatory transition***:  5.3 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  98.3 % | ***Confirmatory transition***:  1.6 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Rapeseed oil | 1,2,4-TRZ | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  98.4 % | ***Confirmatory transition***:  5.7 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  97.9 % | ***Confirmatory transition***:  1.8 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  99.4 % | ***Confirmatory transition***:  1.7 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  100.0 % | ***Confirmatory transition***:  1.0 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TLA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  99.2 % | ***Confirmatory transition***:  1.9 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  101.0 % | ***Confirmatory transition***:  2.0 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TAA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  102.8 % | ***Confirmatory transition***:  1.4 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  98.1 % | ***Confirmatory transition***:  2.9 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| White bread (wheat) | 1,2,4-TRZ | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  94.4 % | ***Confirmatory transition***:  3.7 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  99.6 % | ***Confirmatory transition***:  1.9 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  105.7 % | ***Confirmatory transition***:  6.9 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  101.3 % | ***Confirmatory transition***:  2.7 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TLA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  99.6 % | ***Confirmatory transition***:  5.8 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  99.8 % | ***Confirmatory transition***:  2.1 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TAA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  100.9 % | ***Confirmatory transition***:  3.4 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  99.6 % | ***Confirmatory transition***:  2.3 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Beer (barley) | 1,2,4-TRZ | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  102.8 % | ***Confirmatory transition***:  2.0 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  101.9 % | ***Confirmatory transition***:  2.8 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  103.5 % | ***Confirmatory transition***:  10.3 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  111.3 % | ***Confirmatory transition***:  4.5 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TLA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  101.4 % | ***Confirmatory transition***:  5.2 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  98.0 % | ***Confirmatory transition***:  4.2 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TAA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  93.0 % | ***Confirmatory transition***:  10.3 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  102.1 % | ***Confirmatory transition***:  2.0 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

Table A~~2.1~~ 79: Characteristics for the confirmatory method used for validation of triazole derivative metabolites residues in whole plant (rapeseed)

|  | TDMs |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  **Confirmatory transition:**  ***1,2,4-TRZ***  % interference mean = 7  ***TA***  % interference mean = 16.5  ***TLA***  % interference mean = 11.5  ***TAA***  % interference mean = 2.5 |
| Calibration (type, number of data points) | **Confirmatory transition:**  ***1,2,4-TRZ***  Equation : Y = 8.97514 \* x + 0.73213  Coefficient of correlation: r² = 99.972  ***TA***  Equation : Y = 0.28175 \* x + 0.01482  Coefficient of correlation: r² = 99.928  ***TLA***  Equation : Y = 0.14667 \* x + 0.00442  Coefficient of correlation: r² = 99.956  ***TAA***  Equation : Y = 0.04289 \* x + 7.35409e-4  Coefficient of correlation: r² = 99.816 |
| Calibration range | Accepted calibration range in concentration units 0.5 – 50.0 µg/L (from 20 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes : Performed comparing the analyte response of one individual standard at L4 (or L5) prepared in solvent (water/methanol 50:50 + 1% formic acid) to one prepared in blank matrix extract at the same concentration.  ***1,2,4-TRZ***  Matrix effect = 19.5 %  Not significant  ***TA***  Matrix effect = -77.5 %  Significant in accordance to the SANTE/2020/12830 rev. 1 guideline  ***TLA***  Matrix effect = -8.1 %  Not significant  ***TAA***  Matrix effect = - 2.7 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | According to SANTE/2020/12830 rev.1, the stability of the analytes in the final extracts is not required if the extracts contain an isotope-labelled internal standard (IL-IS) for quantification, since the IL-IS compensates for losses during extract storage. Since IL-IS were used, the verification of the stability was not required. Anyway, all the extracts were analysed within 24 hours from their preparation, obtaining recoveries in the fortified samples within the acceptable range of 70-120%.  In addition, since the differences between the mean responses of the analytes in the diluted solutions prepared form stored and from freshly-prepared stock solutions was < 10%, it is possible to assess that the analytes are stable in the stock solutions prepared in water for at least 78 days, if stored in the dark at 5 ± 3°C. |

Table A~~2.2~~ 80: Characteristics for the confirmatory method used for validation of triazole derivatives metabolites in rapeseed oil

|  | TDMs |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  **Confirmatory transition:**  ***1,2,4-TRZ***  % interference mean = 5  ***TA***  % interference mean = 3  ***TLA***  % interference mean = 1  ***TAA***  % interference mean = 1 |
| Calibration (type, number of data points) | **Confirmatory transition:**  ***1,2,4-TRZ***  Equation : Y = 8.86439 \* x + 0.32346  Coefficient of correlation: r² = 99.846  ***TA***  Equation : Y = 1.29734 \* x + 0.00401  Coefficient of correlation: r² = 99.992  ***TLA***  Equation : Y = 0.14024 \* x – 8.85909e-4  Coefficient of correlation: r² = 99.868  ***TAA***  Equation : Y = 0.04240 \* x + 1.6442e-4  Coefficient of correlation: r² = 99.966 |
| Calibration range | Accepted calibration range in concentration units 0.5 – 50.0 µg/L (from 20 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes : Performed comparing the analyte response of one individual standard at L4 (or L5) prepared in solvent (water/methanol 50:50 + 1% formic acid) to one prepared in blank matrix extract at the same concentration.  ***1,2,4-TRZ***  Matrix effect = -2.7 %  Not significant  ***TA***  Matrix effect = -3.2 %  Not significant  ***TLA***  Matrix effect = -5.4 %  Not significant  ***TAA***  Matrix effect = -2.5 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | According to SANTE/2020/12830 rev.1, the stability of the analytes in the final extracts is not required if the extracts contain an isotope-labelled internal standard (IL-IS) for quantification, since the IL-IS compensates for losses during extract storage. Since IL-IS were used, the verification of the stability was not required. Anyway, all the extracts were analysed within 24 hours from their preparation, obtaining recoveries in the fortified samples within the acceptable range of 70-120%.  In addition, since the differences between the mean responses of the analytes in the diluted solutions prepared form stored and from freshly-prepared stock solutions was < 10%, it is possible to assess that the analytes are stable in the stock solutions prepared in water for at least 78 days, if stored in the dark at 5 ± 3°C. |

Table A~~2.3~~ 81: Characteristics for the confirmatory method used for validation of triazole derivative metabolites in rapeseed seeds- grain (wheat) - white bread (wheat) (calibration in solvent 0.5-50 µg/L)

|  | TDMs |
| --- | --- |
| Specificity | **RAPESEED SEEDS**  ***For, 1,2,4-TRZ, TLA and TAA*** : No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***For TA*** : Signals were detected in amounts higher than the 30% of LOQ for this matrice and were generated by the analytes and not by other interfering substanes.However, a lot of attempts were carried out by the laboratory to find samples with analytes area < 30% of LOQ, analysing a lot of commercially available commodities coming from organic farming: no samples with analytes area < 30% of LOQ were found for this matrice.  **Confirmatory transition:**  ***1,2,4-TRZ***  % interference mean = 0  ***TA***  % interference mean = 239.5  ***TLA***  % interference mean = 16  ***TAA***  % interference mean = 0.5  **GRAIN WHEAT**  ***For, 1,2,4-TRZ and TLA*** : No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***For TA*** ***and TAA***: Signals were detected in amounts higher than the 30% of LOQ for this matrice and were generated by the analytes and not by other interfering substanes.However, a lot of attempts were carried out by the laboratory to find samples with analytes area < 30% of LOQ, analysing a lot of commercially available commodities coming from organic farming: no samples with analytes area < 30% of LOQ were found for this matrice.  **Confirmatory transition:**  ***1,2,4-TRZ***  % interference mean = 25  ***TA***  % interference mean = 227  ***TLA***  % interference mean = 9  ***TAA***  % interference mean = 103.5  **WHITE BREAD (WHEAT)**  ***For, 1,2,4-TRZ and TLA*** : No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***For TA and TAA*** : Signals were detected in amounts higher than the 30% of LOQ for this matrice and were generated by the analytes and not by other interfering substanes.However, a lot of attempts were carried out by the laboratory to find samples with analytes area < 30% of LOQ, analysing a lot of commercially available commodities coming from organic farming: no samples with analytes area < 30% of LOQ were found for this matrice.  **Confirmatory transition:**  ***1,2,4-TRZ***  % interference mean = 0  ***TA***  % interference mean = 57  ***TLA***  % interference mean = 3  ***TAA***  % interference mean = 51.5 |
| Calibration (type, number of data points) | **Confirmatory transition:**  ***1,2,4-TRZ***  Equation : Y = 7.61329 \* x + 0.39044  Coefficient of correlation: r² = 99.942  ***TA***  Equation : Y = 1.53819 \* x + 0.00827  Coefficient of correlation: r² = 99.934  ***TLA***  Equation : Y = 0.12896 \* x + 2.39631e-4  Coefficient of correlation: r² = 99.930  ***TAA***  Equation : Y = 0.04310 \* x + 5.90837e-4  Coefficient of correlation: r² = 99.964 |
| Calibration range | Accepted calibration range in concentration units 0.5 – 50.0 µg/L (from 20 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes : Performed comparing the analyte response of one individual standard at L4 (or L5) prepared in solvent (water/methanol 50:50 + 1% formic acid) to one prepared in blank matrix extract at the same concentration.  **RAPESEED SEEDS**  ***1,2,4-TRZ***  Matrix effect = -4.5 %  Not significant  ***TA***  Matrix effect = 6.0 %  Not significant  ***TLA***  Matrix effect = 6.7 %  Not significant  ***TAA***  Matrix effect = -2.1 %  Not significant  **GRAIN WHEAT**  ***1,2,4-TRZ***  Matrix effect = -7.7 %  Not significant  ***TA***  Matrix effect = -2.5 %  Not significant  ***TLA***  Matrix effect = -5.0 %  Not significant  ***TAA***  Matrix effect = -4.0 %  Not significant  **WHITE BREAD (WHEAT)**  ***1,2,4-TRZ***  Matrix effect = 3.5 %  Not significant  ***TA***  Matrix effect = 7.1 %  Not significant  ***TLA***  Matrix effect = 0.6 %  Not significant  ***TAA***  Matrix effect = -1.8 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | According to SANTE/2020/12830 rev.1, the stability of the analytes in the final extracts is not required if the extracts contain an isotope-labelled internal standard (IL-IS) for quantification, since the IL-IS compensates for losses during extract storage. Since IL-IS were used, the verification of the stability was not required. Anyway, all the extracts were analysed within 24 hours from their preparation, obtaining recoveries in the fortified samples within the acceptable range of 70-120%.  In addition, since the differences between the mean responses of the analytes in the diluted solutions prepared form stored and from freshly-prepared stock solutions was < 10%, it is possible to assess that the analytes are stable in the stock solutions prepared in water for at least 78 days, if stored in the dark at 5 ± 3°C. |

Table A~~2.4~~ 82: Characteristics for the confirmatory method used for validation of triazole derivative metabolites residues in straw (wheat) (calibration in solvent, 0.35 – 35 µg/L-

|  | TDMs |
| --- | --- |
| Specificity | ***For, 1,2,4-TRZ and TA*** : No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***For TLA and TAA*** : Signals were detected in amounts higher than the 30% of LOQ for this matrice and were generated by the analytes and not by other interfering substanes.However, a lot of attempts were carried out by the laboratory to find samples with analytes area < 30% of LOQ, analysing a lot of commercially available commodities coming from organic farming: no samples with analytes area < 30% of LOQ were found for this matrice. In addition no samples with analytes content < LOD, set to 20% of LOQ (28% for straw) were found. Since no matrices free from the analytes were available and the matrix effect for this matrice was negligible, it was necessary to calibrate using solvent-based standard solutions. Recoveries were necessarily evaluated subtracting the mean values measured from a duplicate analysis of the untreated samples to the values measured for the fortified ones.  **Confirmatory transition:**  ***1,2,4-TRZ***  % interference mean = 0  ***TA***  % interference mean = 9  ***TLA***  % interference mean = 55.5  ***TAA***  % interference mean = 135.5 |
| Calibration (type, number of data points) | **Confirmatory transition:**  ***1,2,4-TRZ***  Equation : Y = 8.30803 \* x + 0.07988  Coefficient of correlation: r² = 99.928  ***TA***  Equation : Y = 2.92033 \* x + 0.05126  Coefficient of correlation: r² = 99.918  ***TLA***  Equation : Y = 0.15019 \* x + 2.79134e-4  Coefficient of correlation: r² = 99.938  ***TAA***  Equation : Y = 0.04473 \* x + 1.75957e-4  Coefficient of correlation: r² = 99.968 |
| Calibration range | Accepted calibration range in concentration units 0.35 – 35.0 µg/L (from 28 % of LOQ to 180 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.0028 – 0.280 mg/kg) |
| Assessment of matrix effects is presented | Yes : Performed comparing the analyte response of one individual standard at L4 (or L5) prepared in solvent (water/methanol 50:50 + 1% formic acid) to one prepared in blank matrix extract at the same concentration.  ***1,2,4-TRZ***  Matrix effect = 0.4 %  Not significant  ***TA***  Matrix effect = 1.3 %  Not significant  ***TLA***  Matrix effect = -7.9 %  Not significant  ***TAA***  Matrix effect = -3.7 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.35 µg/L  (0.0028 mg/kg) |
| Stability | According to SANTE/2020/12830 rev.1, the stability of the analytes in the final extracts is not required if the extracts contain an isotope-labelled internal standard (IL-IS) for quantification, since the IL-IS compensates for losses during extract storage. Since IL-IS were used, the verification of the stability was not required. Anyway, all the extracts were analysed within 24 hours from their preparation, obtaining recoveries in the fortified samples within the acceptable range of 70-120%.  In addition, since the differences between the mean responses of the analytes in the diluted solutions prepared form stored and from freshly-prepared stock solutions was < 10%, it is possible to assess that the analytes are stable in the stock solutions prepared in water for at least 78 days, if stored in the dark at 5 ± 3°C. |

Table A~~2.5~~ 83: Characteristics for the confirmatory method used for validation of triazole derivative metabolites residues in beer (barley) (calibration in solvent, 1-100 µg/L)

|  | TDMs |
| --- | --- |
| Specificity | ***For, 1,2,4-TRZ and TLA*** : No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***For TA*** ***and TAA***: Signals were detected in amounts higher than the 30% of LOQ for this matrice and were generated by the analytes and not by other interfering substanes.However, a lot of attempts were carried out by the laboratory to find samples with analytes area < 30% of LOQ, analysing a lot of commercially available commodities coming from organic farming: no samples with analytes area < 30% of LOQ were found for this matrice  **Confirmatory transition:**  ***1,2,4-TRZ***  % interference mean = 0  ***TA***  % interference mean = 314  ***TLA***  % interference mean = 16.5  ***TAA***  % interference mean = 217.5 |
| Calibration (type, number of data points) | **Confirmatory transition:**  ***1,2,4-TRZ***  Equation : Y = 8.33313 \* x + 0.11477  Coefficient of correlation: r² = 99.932  ***TA***  Equation : Y = 2.15353 \* x + 0.11609  Coefficient of correlation: r² = 99.912  ***TLA***  Equation : Y = 0.15499 \* x + 0.00652  Coefficient of correlation: r² = 99.487  ***TAA***  Equation : Y = 0.04053 \* x + 0.00126  Coefficient of correlation: r² = 99.906 |
| Calibration range | Accepted calibration range in concentration units 1.00 – 100.0 µg/L (from 20 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes : Performed comparing the analyte response of one individual standard at L4 (or L5) prepared in solvent (water/methanol 50:50 + 1% formic acid) to one prepared in blank matrix extract at the same concentration.  ***1,2,4-TRZ***  Matrix effect = 5.2 %  Not significant  ***TA***  Matrix effect = 5.0 %  Not significant  ***TLA***  Matrix effect = -3.8 %  Not significant  ***TAA***  Matrix effect = 6.5 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 1 µg/L  (0.002 mg/kg) |
| Stability | According to SANTE/2020/12830 rev.1, the stability of the analytes in the final extracts is not required if the extracts contain an isotope-labelled internal standard (IL-IS) for quantification, since the IL-IS compensates for losses during extract storage. Since IL-IS were used, the verification of the stability was not required. Anyway, all the extracts were analysed within 24 hours from their preparation, obtaining recoveries in the fortified samples within the acceptable range of 70-120%.  In addition, since the differences between the mean responses of the analytes in the diluted solutions prepared form stored and from freshly-prepared stock solutions was < 10%, it is possible to assess that the analytes are stable in the stock solutions prepared in water for at least 78 days, if stored in the dark at 5 ± 3°C. |

Conclusion

The confirmatory method for the quantification of triazole derivatives metabolites in wheat, barley, oilseed rape and processed commodities was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability for triazole derivatives metabolites.

Extraction efficiency

Extraction efficiency is guided by:

* European Commission (2017): SANTE 2017/10632 rev. 3, dated 22 November 2017: Technical Guideline on the Evaluation of Extraction Efficiency of Residue Analytical Methods.

An appropriate aliquot of each specimen was taken from the homogenised frozen samples and put in a 50 mL screw capped centrifuge PE test tube followed by the addition of 100 μL of the internal standard solution TDM ISTD MIX (2 mg/L of each internal standard) and by the following amounts of deionized water (added on the basis of QuPPe-PO-Method and considering the theoretical water content of each matrix):

|  |  |  |
| --- | --- | --- |
| **Matrix** | **Sample weight (g)** | **Water added (mL)** |
| Whole Plant (rapeseed) | 5 | 5 |
| Rapeseed seeds | 5 | 10 |
| Wheat (grain) | 5 | 10 |
| Wheat (straw) | 2.5 | 10 |
| Rapeseed oil | 5 | 10 |
| Wheat (white bread) | 5 | 10 |
| Beer (barley) | 10 | 0 |

Then, 10 mL of 1% formic acid in methanol were added and the obtained mixture was vigorously shaken for 3 minutes. The volume of the final extract is considered to be 20 mL: little variation due to the actual water content of each sample are corrected by the presence of the internal standard, that is added to produce a concentration in the final extract nominally of 10 μg/L of each compound.

The separation of the liquid phase from the solid one was achieved by centrifugation at 5000 rpm for 5 minutes. An aliquot of about 1 mL the supernatant was taken, filtered with a 0.45 μm PVDF filter and transferred in a 2 mL HPLC glass vial for the final analysis with a HPLC-DMS-MS/MS system.

* + - * 1. Analytical method 16

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  The QuEChERS method and a HPLC-MS/MS detection were used. The LOQ was set to 0.01 mg/kg. The matrix effect was considered not significant.  The specificity of the method was assured by MS/MS detection. All validation parameters are in the currently required range.  The stability of the analytes in the final extracts can be considered proven for 3 days at 5 ± 3°C in dark conditions since the recovery of the stored fortified extracts were within the range of 70-120% measured against the freshly prepared ones, as required by the SANTE/2020/12830 rev.1.  The evaluation of the extraction efficiency was done in compliance with SANTE 2017/10632 rev.3, applying a cross validation approach (comparing the amount extracted by the present and already proven method). The differences were less than 30% thus the extraction efficiency could be considered proven (see A 2.1.1.1.16.3 Extraction efficiency, on next pages).  Independent laboratory validation ~~is ongoing and~~ is included in study plan submitted (CH-1081/2021)  The studies have been submitted – relevant paragraph for evaluation details. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/15 |
| Report | Validation of an analytical method for the quantification of prothioconazole-desthio-3-hydroxy, prothioconazole-desthio-4-hydroxy, prothioconazole-desthio-5-hydroxy, prothioconazole-desthio-6-hydroxy and prothioconazole-desthio-alpha-hydroxy in cereal straw  Longhi, D.  2021c  ChemService S.r.l. Controlli e Richerche, Novate Milanese - Italy  Report No. : 21-120  + Amendment report No.1 to stduy plan (Amdm1\_KCP 5.1.2/15) |
| Guideline(s): | Yes :   * SANTE/2020/12830 rev. 1 (dated 24/02/2021) ; * SANTE2017/10632 rev. 3 (dated 22/11/2017) ; * OECD ENV/JM/MONO(2007)17 ; * EURL-SRM – Analytical Observations Report, “Analysis of Pesticides Entailing Conjugates or Esters in their Residue Definitions”, Version 2 (last update: 21.04.2021) |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The analytical method allows the determination in cereal straw of the total form (free + conjugated) of the following compounds:   * prothioconazole-desthio-3-hydroxy (PTZ-3OH) * prothioconazole-desthio-4-hydroxy (PTZ-4OH) * prothioconazole-desthio-5-hydroxy (PTZ-5OH) * prothioconazole-desthio-6-hydroxy (PTZ-6OH) * prothioconazole-desthio-alpha-hydroxy (PTZ-a-OH).   + Note: 2 diastereoisomers of this compound exist. The analytical method was developed and validated for each diastereoisomer, setting the LOQ at 0.01 mg/kg as sum of the diastereoisomers. In this study, the diastereoisomers are identified on the basis of order of elution as:   + prothioconazole-desthio-alpha-hydroxy (diastereoisomer A) (PTZ-a-OH (A))   + prothioconazole-desthio-alpha-hydroxy (diastereoisomer B) (PTZ-a-OH (B))   A hydrolytic step is carried out before the extraction in order to release the conjugated compounds. This step is based on the procedure described in the document:   * EURL-SRM - Analytical Observations Report, “Analysis of Pesticides Entailing Conjugates or Esters in their Residue Definitions”, Version 2 (last update: 21.04.2021).   The extraction of the analyte from the hydrolyzed mixture (after neutralization) is carried out using the QuEChERS method and the instrumental determination using a HPLC-MS/MS (high-performance liquid chromatography-triple-quadrupole mass spectrometry).  The limit of quantification (LOQ) is set to 0.01 mg/kg |

Materials and methods

***1. Materials***

***1.A. Quantification of prothioconazole-desthio-3-hydroxy, prothioconazole-desthio-4-hydroxy, prothioconazole-desthio-5-hydroxy, prothioconazole-desthio-6-hydroxy and prothioconazole-desthio-alpha-hydroxy in cereal straw***

The quantification of prothioconazole-desthio-3-hydroxy, prothioconazole-desthio-4-hydroxy, prothioconazole-desthio-5-hydroxy, prothioconazole-desthio-6-hydroxy and prothioconazole-desthio-alpha-hydroxy in cereal straw was assessed by HLPC/MS/MS.

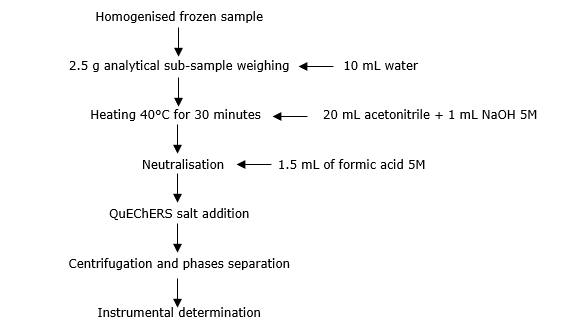
|  |  |
| --- | --- |
| HPLC: | Agilent HPLC 1290 Infinity II + Agilent MS spectrometer 6470A Triple Quad |
| Column: | Agilent Poroshell 120 EC-C18, 2.7 µm, 3 x 150 mm (x 2) |
| Column temperature: | 40 °C |
| Flow rate: | 0.6 mL/min |
| Volume of injection: | 2 µL |
| Retention time: | Approximatively 16.5 minutes for prothioconazole-desthio-3-hydroxy (PTZ-3-OH)  Approximatively 18.1 minutes for prothioconazole-desthio-4-hydroxy (PTZ-4-OH)  Approximatively 18.6 minutes for prothioconazole-desthio-5-hydroxy (PTZ-5-OH)  Approximatively 23.0 minutes for prothioconazole-desthio-6-hydroxy (PTZ-6-OH)  Approximatively 13.0 minutes for prothioconazole-desthio-alpha-hydroxy (A) (PTZ-a-OH (A))  Approximatively 13.0 minutes for prothioconazole-desthio-alpha-hydroxy (B) (PTZ-a-OH (B)) |
| Stop time: | 30 minutes |
| Post time: | 3 minutes |
| Divert valve: | 0 minute to waste  10 minutes to MS  26 minutes to waste |
| Gas temperature: | 350 °C |
| Gas flow: | 5 L//min |
| Nebulizer | 40 psi |
| Sheath gas heater: | 400 °C |
| Sheath gas flow: | 12 L/min |
| Capillary: | 3500 V |
| Mobile phase: | A: LC-MS grade water with 0.2 % formic acid and 5 mM ammonium formate  B: LC-MS grade methanol with 0.2 % formic acid and 5 mM ammonium formate |
| Mixture-Elution: | |  |  |  | | --- | --- | --- | | % A | % B | Time (min) | | 60 | 40 | 0 | | 60 | 40 | 1 | | 30 | 70 | 20 | | 0 | 100 | 25 | |
| Analytical standards: | Prothioconazole-desthio-3-hydroxy (PTZ-3-OH)  CAS No. : 856045-93-7  Lot: 3884-049A6  Purity : 99.7%  Expiry date: 03/06/2023  Prothioconazole-desthio-4-hydroxy (PTZ-4-OH)  CAS No. : 856045-88-0  Lot: 3981-001A2  Purity : 100.0%  Expiry date: 23/06/2023  Prothioconazole-desthio-5-hydroxy (PTZ-5-OH)  CAS No. : N/A  Lot: 3922-021A4  Purity : 99.3%  Expiry date: 27/05/2023  Prothioconazole-desthio-6-hydroxy (PTZ-6-OH)  CAS No. : N/A  Lot: 3967-007A3  Purity : 99.4%  Expiry date: 12/06/2023  Prothioconazole-desthio-alpha-hydroxy (PTZ-a-OH)  CAS No. : 856045-95-9  Lot: 2020-0416067  Purity : 92%  Expiry date: 20/12/2026 |

***2. Methods***

The analytical method for the quantification of prothioconazole-desthio-3-hydroxy, prothioconazole-desthio-4-hydroxy, prothioconazole-desthio-5-hydroxy, prothioconazole-desthio-6-hydroxy and prothioconazole-desthio-alpha-hydroxy in cereal straw based on the QuEChERS method.

***2.A. Schematic diagram of the analytical method***

Straw for cereals



***2.B. Quantification of prothioconazole-desthio-3-hydroxy, prothioconazole-desthio-4-hydroxy, prothioconazole-desthio-5-hydroxy, prothioconazole-desthio-6-hydroxy and prothioconazole-desthio-alpha-hydroxy in cereal straw***

The analytical methods for the quantification of prothioconazole-desthio-3-hydroxy, prothioconazole-desthio-4-hydroxy, prothioconazole-desthio-5-hydroxy, prothioconazole-desthio-6-hydroxy and prothioconazole-desthio-alpha-hydroxy in cereal straw were first validated so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were validated in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |

Results and discussions

Method validation data can be summarised in tables below ~~Table A.1 and A2. There are for each matrix a primary test. In view of the similar results between the primary and confirmatory test, a test by an independent laboratory validation (ILV) is not required.~~

Table A~~1~~ 84: Recovery results from method validation of prothioconazole-desthio-3-hydroxy, prothioconazole-desthio-4-hydroxy, prothioconazole-desthio-5-hydroxy, prothioconazole-desthio-6-hydroxy and prothioconazole-desthio-alpha-hydroxy in cereal straw using the analytical method

| Matrix | Analyte | Fortification level  (*n* = x) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Straw  (Cereals) | PTZ-3-OH | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  88.6 % | ***Primary transition***:  2.8 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  88.2 % | ***Primary transition***:  0.6 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| PTZ-4-OH | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  88.1 % | ***Primary transition***:  4.1 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  88.9 % | ***Primary transition***:  1.5 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| PTZ-5-OH | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  85.8 % | ***Primary transition***:  1.6 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  89.8 % | ***Primary transition***:  2.2 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| PTZ-6-OH | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  88.9 % | ***Primary transition***:  3.6 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  91.9 % | ***Primary transition***:  3.4 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| PTZ-a-OH (A) | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  89.8 % | ***Primary transition***:  10.3 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  90.2 % | ***Primary transition***:  4.5 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| PTZ-a-OH (B) | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  86.4 % | ***Primary transition***:  6.1 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  88.7 % | ***Primary transition***:  2.6 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

Table A~~2~~ 85: Characteristics for the analytical method used for validation of prothioconazole-desthio-3-hydroxy, prothioconazole-desthio-4-hydroxy, prothioconazole-desthio-5-hydroxy, prothioconazole-desthio-6-hydroxy and prothioconazole-desthio-alpha-hydroxy in cereal straw

|  | prothioconazole-desthio-3-hydroxy, prothioconazole-desthio-4-hydroxy, prothioconazole-desthio-5-hydroxy, prothioconazole-desthio-6-hydroxy and prothioconazole-desthio-alpha-hydroxy |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  **Primarity transition:**  ***PTZ-3-OH***  % interference mean = 1.1  ***PTZ-4-OH***  % interference mean = 1.5  ***PTZ-5-OH***  % interference mean = 4.9  ***PTZ-6-OH***  % interference mean = 0.1  ***PTZ-a-OH (A)***  % interference mean = 6.0  ***PTZ-a-OH (B)***  % interference mean = 2.8 |
| Calibration (type, number of data points) | **Primarity transition:**  ***PTZ-3-OH***  Equation : Y = 2831.351387 \* x – 179.630774  Coefficient of correlation: r² = 99.874  ***PTZ-4-OH***  Equation : Y = 4798.895229 \* x – 562.396927  Coefficient of correlation: r² = 99.685  ***PTZ-5-OH***  Equation : Y = 11404.814051 \* x – 1174.395211  Coefficient of correlation: r² = 99.671  ***PTZ-6-OH***  Equation : Y = 12783.110510 \* x – 1721.607957  Coefficient of correlation: r² = 99.789  ***PTZ-a-OH (A)***  Equation : Y = 1369.398007 \* x + 158.613802  Coefficient of correlation: r² = 99.938  ***PTZ-a-OH (B)***  Equation : Y = 1097.320208 \* x + 2.409321  Coefficient of correlation: r² = 99.896 |
| Calibration range | ***PTZ-3-OH***  Accepted calibration range in concentration units 0.303 – 25.23 µg/L (from 24 % of LOQ to 102 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.0024 – 0.202 mg/kg)  ***PTZ-4-OH***  Accepted calibration range in concentration units 0.299 – 24.95 µg/L (from 24 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.0024 – 0.200 mg/kg)  ***PTZ-5-OH***  Accepted calibration range in concentration units 0.299 – 24.88 µg/L (from 24 % of LOQ to 99 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.0024 – 0.199 mg/kg)  ***PTZ-6-OH***  Accepted calibration range in concentration units 0.299 – 24.93 µg/L (from 24 % of LOQ to 99 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.0024 – 0.199 mg/kg)  ***PTZ-a-OH (A)***  Accepted calibration range in concentration units 0.110 – 9.15 µg/L (from 24 % of LOQ to 103 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.00088 – 0.073 mg/kg)  ***PTZ-a-OH (B)***  Accepted calibration range in concentration units 0.190 – 15.83 µg/L (from 24 % of LOQ to 101 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.0015 – 0.127 mg/kg) |
| Assessment of matrix effects is presented | Yes : Performed comparing the analyte response of one individual standard at L4 prepared in solvent to one prepared in blank matrix extract at the same concentration.  ***PTZ-3-OH***  Matrix effect = 5.6 %  Not significant  ***PTZ-4-OH***  Matrix effect = -9.3 %  Not significant  ***PTZ-5-OH***  Matrix effect = -15.3 %  Not significant  ***PTZ-6-OH***  Matrix effect = - 2.3 %  Not significant  ***PTZ-a-OH (A)***  Matrix effect = - 6.1 %  Not significant  ***PTZ-a-OH (B)***  Matrix effect = 5.1 %  Not significant |
| Limit of quantification (LOQ) | ***PTZ-3-OH***  LOQ = 0.01 mg/kg  ***PTZ-4-OH***  LOQ = 0.01 mg/kg  ***PTZ-5-OH***  LOQ = 0.01 mg/kg  ***PTZ-6-OH***  LOQ = 0.01 mg/kg  ***PTZ-a-OH (sum)***  LOQ = 0.01 mg/kg  ***PTZ-a-OH (A)***  LOQ = 0.0036 mg/kg  ***PTZ-a-OH (B)***  LOQ = 0.0063 mg/kg |
| Limit of determination (LOD) | ***PTZ-3-OH***  LOD = 0.303 µg/L  (0.0024 mg/kg)  ***PTZ-4-OH***  LOD = 0.299 µg/L  (0.0024 mg/kg)  ***PTZ-5-OH***  LOD = 0.299 µg/L  (0.0024 mg/kg)  ***PTZ-6-OH***  LOD = 0.299 µg/L  (0.0024 mg/kg)  ***PTZ-a-OH (A)***  LOD = 0.110 µg/L  (0.00088 mg/kg)  ***PTZ-a-OH (B)***  LOD = 0.190 µg/L  (0.0015 mg/kg) |
| Stability in sample extracts | ***PTZ-3-OH***  % difference = 2.7  ***PTZ-4-OH***  % difference = -2.5  ***PTZ-5-OH***  % difference = - 2.3  ***PTZ-6-OH***  % difference = -8.0  ***PTZ-a-OH (A)***  % difference = 8.6  ***PTZ-a-OH (B)***  % difference = -1.0  The stability of the analytes in the final extracts can be considered proven for 3 days at 5 ± 3°C in dark conditions since the recovery of the stored spiked extracts were within the range of 70-120% measured against the freshly prepared ones, as required by the SANTE/2020/12830 rev.1 guideline |
| Stability of stock standard solutions | ***PTZ-3-OH***  % difference = 1.7  ***PTZ-4-OH***  % difference = 3.5  ***PTZ-5-OH***  % difference = -0.9  ***PTZ-6-OH***  % difference = 0.7  ***PTZ-a-OH (A)***  % difference = -7.5  ***PTZ-a-OH (B)***  % difference = 9.3  In addition, since the differences between the mean responses of the analytes in the diluted solutions prepared form stored and from freshly-prepared stock solutions was < 10%, it is possible to assess that the analytes are stable in the stock solutions prepared in water for at least 78 days, if stored in the dark at 5 ± 3°C. |

Conclusion

The analytical method for the quantification of prothioconazole-desthio-3-hydroxy, prothioconazole-desthio-4-hydroxy, prothioconazole-desthio-5-hydroxy, prothioconazole-desthio-6-hydroxy and prothioconazole-desthio-alpha-hydroxy in cereal straw was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability for prothioconazole-desthio-3-hydroxy, prothioconazole-desthio-4-hydroxy, prothioconazole-desthio-5-hydroxy, prothioconazole-desthio-6-hydroxy and prothioconazole-desthio-alpha-hydroxy.

Independent laboratory validation

~~Not required.~~

Determination of Prothioconazole-desthio metabolites in cereals straw

|  |  |
| --- | --- |
| Comments of zRMS: | The submitted ILV of the analytical method adjusted and validated in GLP study Code GLP-STUDY-21-120 by LabAnalysis s.r.l. for the determination of Prothioconazole-desthio metabolites (Prothioconazole-desthio-3-hydroxy, Prothioconazole-desthio-4-hydroxy, Prothioconazole-desthio-5-hydroxy, Prothioconazole-desthio-6-hydroxy, Prothioconazole-desthio-alpha-hydroxy) residues in Cereal straw has been accepted.  The analytical method, using the HPLC/MS/MS in MRM mode is highly specific and gave both quantification and identification data, so the confirmatory method is not necessary.  All recovery values for each analyte at both fortification levels (L.O.Q and 10 x LOQ) resulted to be in the range 70 to 120 %, with an RSD% lower than 20% therefore the analytical method can be considered suitable to quantify Prothioconazole-desthio metabolites residues in Cereal straw samples with an established LOQ of 0.010 mg/kg. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/20 |
| Report | Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of Prothioconazole-desthio metabolites in Cereal straw  Report No.: CH-1091/2021  Nichetti, S. (2022e)  ChemService S.r.l Controlli e Ricerche - Novate Milanese (MI) - Italy |
| Guideline(s): | Yes  SANTE/2020/12830 rev. 1 dated 24/02/21: Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes. |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

Materials and methods

|  |  |
| --- | --- |
| **Principle of the method** | The Test Facility conducted an Independent Laboratory Validation (ILV) of the analytical method adjusted and validated in GLP study Code GLP-STUDY-21-120 by LabAnalysis s.r.l. for the determination of prothioconazole-desthio metabolites in cereal straw: prothioconazole-desthio-3-hydroxy, prothioconazole-desthio-4-hydroxy, prothioconazole-desthio-5-hydroxy, prothioconazole-desthio-6-hydroxy and prothioconazole-desthio-α-hydroxy |
|  |  |

**Materials:**

|  |  |
| --- | --- |
| UHPLC/MS-QQQ: | Agilent mod. 1290, equipped with binary pump, autosampler coupled with an Agilent Jet Stream (AJS) ESI |
| Column: | Kinetex C18 100 Å, 1.7 μm, 50 x 2.1 mm i.d. |
| Detector: | MS Triple quadrupole |
| Ionisation type: | Electrospray (ESI) |
| Polarity: | Positive ion mode |
| Scan type: | MS/MS, multiple reaction monitoring |
| Drying gas temperature: | 350 °C |
| Drying gas flow: | 5 L/min |
| Nebulizer pressure: | 40 psi |
| Capillary voltage: | 3500 V |
|  |  |
| Analytical standards: | Prothioconazole-desthio-3-hydroxy  Batch No. : 3884-049A6  Purity : 99.4 %  Expiry date: June 3, 2023 |
|  | Prothioconazole-desthio-4-hydroxy  Batch No. : 3981-001A2  Purity : 99.7 %  Expiry date: June 23, 2023 |
|  | Prothioconazole-desthio-5-hydroxy  Batch No. : 3922-021A4  Purity : 96.5 %  Expiry date: May 27, 2023  Prothioconazole-desthio-6-hydroxy  Batch No. : 3967-007A3  Purity : 98.2 %  Expiry date: June 12, 2023 |
|  | Prothioconazole-desthio-α-hydroxy  Batch No. : 2020-0416067  Purity : 92 %  Expiry date: December 20, 2026 |
|  |  |
| Reagents: | Water, HPLC grade, obtained by the Lab Water Purification System  Methanol, HPLC grade  Acetonitrile, HPLC grade  Acetone, pesticide residue analysis  Sodium hydroxide pellets GR, analytical grade  Ammonium formate, high purity (>99%) for mass spectroscopy  Formic acid, high purity for mass spectroscopy  Formic acid, ACS reagent |
|  |  |
| Matrix: | Cereal straw  Stored at -20°C in the dark |

**Methods:**

The analytical methods for the determination of prothioconazole-desthio metabolites in cereal straw were confirmed so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were confirmed in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A 86: Recovery results from independent laboratory validation of prothioconazole-desthio metabolites using the analytical method

| Matrix | Analyte | Fortification level (mg/kg) (n = 5) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Cereal straw | Prothioconazole-desthio-3-hydroxy  (product ion: 141.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 100.5 | 2.85 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 96.3 | 1.20 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Prothioconazole-desthio-3-hydroxy  (product ion: 70.0 m/z)\* | At low level :  0.01 mg/kg (LOQ) | 111.6 | 1.88 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 94.4 | 0.38 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Prothioconazole-desthio-4-hydroxy  (product ion: 141.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 117.7 | 1.46 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 93.6 | 1.06 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Prothioconazole-desthio-4-hydroxy  (product ion: 70.0 m/z)\* | At low level :  0.01 mg/kg (LOQ) | 117.0 | 1.87 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 94.3 | 1.16 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Prothioconazole-desthio-5-hydroxy  (product ion: 70.0 m/z)\* | At low level :  0.01 mg/kg (LOQ) | 115.0 | 2.04 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 94.5 | 0.53 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Prothioconazole-desthio-5-hydroxy  (product ion: 141.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 112.5 | 5.94 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 94.2 | 2.45 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Prothioconazole-desthio-6-hydroxy  (product ion: 70.0 m/z)\* | At low level :  0.01 mg/kg (LOQ) | 115.1 | 1.32 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 95.4 | 0.88 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Prothioconazole-desthio-6-hydroxy  (product ion: 141.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 117.2 | 2.61 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 94.0 | 1.09 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Prothioconazole-desthio-α-hydroxy (A)  (product ion: 75.0 m/z)\* | At low level :  0.01 mg/kg (LOQ) | 107.9 | 8.62 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 97.4 | 2.58 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Prothioconazole-desthio- α -hydroxy (A)  (product ion: 70.0 m/z)\* | At low level :  0.01 mg/kg (LOQ) | 104.2 | 2.61 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 94.7 | 2.58 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Prothioconazole-desthio-α-hydroxy (B)  (product ion: 75.0 m/z)\* | At low level :  0.01 mg/kg (LOQ) | 105.2 | 4.04 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 94.8 | 2.61 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Prothioconazole-desthio- α -hydroxy (B)  (product ion: 70.0 m/z)\* | At low level :  0.01 mg/kg (LOQ) | 116.6 | 2.07 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 94.2 | 1.32 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

\* For the prothioconazole-desthio metabolites, it was not possible to find both products ions with m/z > 100, as required by SANTE/2020/12830 rev.1. This analyte fragmented only in two products ions, one of them with m/z < 100.

Table A 87: Characteristics for the analytical method used for independent laboratory validation of prothioconazole-desthio-3-hydroxy residues in cereal straw

|  | Prothioconazole-desthio-3-hydroxy |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Product ion (m/z = 141.0):***  % interference mean = 0.0  ***Product ion (m/z = 70.0):***  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Product ion (m/z = 141.0):***  Equation : Y = 2536 \* x - 870  Coefficient of correlation: r² = 99.899  ***Product ion (m/z = 70.0):***  Equation : Y = 8858 \* x - 3777  Coefficient of correlation: r² = 99.890 |
| Calibration range | Accepted calibration range in concentration units 0.30 – 24.85 µg/L  Corresponding calibration range in mass ratio units for the sample (0.0024 – 0.199 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 10 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.30 µg/L  (0.0024 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-120 |

Table A 88: Characteristics for the analytical method used for independent laboratory validation of prothioconazole-desthio-4-hydroxy residues in cereal straw

|  | Prothioconazole-desthio-4-hydroxy |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Product ion (m/z = 141.0):***  % interference mean = 0.0  ***Product ion (m/z = 70.0):***  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Product ion (m/z = 141.0):***  Equation : Y = 5979 \* x - 3671  Coefficient of correlation: r² = 99.835  ***Product ion (m/z = 70.0):***  Equation : Y = 11931 \* x - 6650  Coefficient of correlation: r² = 99.884 |
| Calibration range | Accepted calibration range in concentration units 0.34 – 28.41 µg/L  Corresponding calibration range in mass ratio units for the sample (0.0027 – 0.227 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 11 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.30 µg/L  (0.0024 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-120 |

Table A 89: Characteristics for the analytical method used for independent laboratory validation of prothioconazole-desthio-5-hydroxy residues in cereal straw

|  | Prothioconazole-desthio-5-hydroxy |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Product ion (m/z = 141.0):***  % interference mean = 0.0  ***Product ion (m/z = 70.0):***  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Product ion (m/z = 141.0):***  Equation : Y = 2950 \* x - 1578  Coefficient of correlation: r² = 99.853  ***Product ion (m/z = 70.0):***  Equation : Y = 10287 \* x - 5453  Coefficient of correlation: r² = 99.886 |
| Calibration range | Accepted calibration range in concentration units 0.32 – 27.02 µg/L  Corresponding calibration range in mass ratio units for the sample (0.0026 – 0.216 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 11 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.30 µg/L  (0.0024 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-120 |

Table A 90: Characteristics for the analytical method used for independent laboratory validation of prothioconazole-desthio-6-hydroxy residues in cereal straw

|  | Prothioconazole-desthio-6-hydroxy |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Product ion (m/z = 141.0):***  % interference mean = 0.0  ***Product ion (m/z = 70.0):***  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Product ion (m/z = 141.0):***  Equation : Y = 6296 \* x - 3645  Coefficient of correlation: r² = 99.860  ***Product ion (m/z = 70.0):***  Equation : Y = 17222 \* x -9647  Coefficient of correlation: r² = 99.904 |
| Calibration range | Accepted calibration range in concentration units 0.34 – 28.48 µg/L  Corresponding calibration range in mass ratio units for the sample (0.0027 – 0.228 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 11 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.30 µg/L  (0.0024 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-120 |

Table A 91: Characteristics for the analytical method used for independent laboratory validation of prothioconazole-desthio-α-hydroxy residues in cereal straw

|  | Prothioconazole-desthio-α-hydroxy |
| --- | --- |
| Specificity | **Prothioconazole-desthio-α-hydroxy (A)**  No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Product ion (m/z = 75.0):***  % interference mean = 0.0  ***Product ion (m/z = 70.0):***  % interference mean = 0.0  **Prothioconazole-desthio-α-hydroxy (B)**  No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Product ion (m/z = 75.0):***  % interference mean = 0.0  ***Product ion (m/z = 70.0):***  % interference mean = 0.0 |
| Calibration (type, number of data points) | **Prothioconazole-desthio-α-hydroxy (A)**  ***Product ion (m/z = 75.0):***  Equation : Y = 1725 \* x - 173  Coefficient of correlation: r² = 99.954  ***Product ion (m/z = 70.0):***  Equation : Y = 12614 \* x - 1952  Coefficient of correlation: r² = 99.920  **Prothioconazole-desthio-α-hydroxy (B)**  ***Product ion (m/z = 75.0):***  Equation : Y = 1094 \* x - 147  Coefficient of correlation: r² = 99.946  ***Product ion (m/z = 70.0):***  Equation : Y = 8641 \* x - 2930  Coefficient of correlation: r² = 99.847 |
| Calibration range | **Prothioconazole-desthio-α-hydroxy (A)**  Accepted calibration range in concentration units 0.111 – 9.28 µg/L  Corresponding calibration range in mass ratio units for the sample (0.0009 – 0.074 mg/kg)  **Prothioconazole-desthio-α-hydroxy (B)**  Accepted calibration range in concentration units 0.192 – 16.04 µg/L  Corresponding calibration range in mass ratio units for the sample (0.0015 – 0.128 mg/kg) |
| Assessment of matrix effects is presented | Yes  **Prothioconazole-desthio-α-hydroxy (A)**  Matrix effect = - 10 %  Not significant  **Prothioconazole-desthio-α-hydroxy (B)**  Matrix effect = - 3 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.30 µg/L  (0.0024 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-120 |

Conclusion

The independent laboratory validation for the quantification of prothioconazole-desthio metabolites (prothioconazole-desthio-3-hydroxy, prothioconazole-desthio-4-hydroxy, prothioconazole-desthio-5-hydroxy, prothioconazole-desthio-6-hydroxy and prothioconazole-desthio-α-hydroxy) in cereal straw was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability.

Confirmatory method

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  The QuEChERS method and a HPLC-MS/MS detection were used. The LOQ was set to 0.01 mg/kg. The matrix effect was considered not significant.  The specificity of the method was assured by MS/MS detection. All validation parameters are in the currently required range. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/15 |
| Report | Validation of an analytical method for the quantification of prothioconazole-desthio-3-hydroxy, prothioconazole-desthio-4-hydroxy, prothioconazole-desthio-5-hydroxy, prothioconazole-desthio-6-hydroxy and prothioconazole-desthio-alpha-hydroxy in cereal straw  Longhi, D.  2021c  ChemService S.r.l. Controlli e Richerche, Novate Milanese - Italy  Report No. : 21-120 |
| Guideline(s): | Yes :   * SANTE/2020/12830 rev. 1 (dated 24/02/2021) ; * SANTE2017/10632 rev. 3 (dated 22/11/2017) ; * OECD ENV/JM/MONO(2007)17 ; * EURL-SRM – Analytical Observations Report, “Analysis of Pesticides Entailing Conjugates or Esters in their Residue Definitions”, Version 2 (last update: 21.04.2021) |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The analytical method allows the determination in cereal straw of the total form (free + conjugated) of the following compounds:   * prothioconazole-desthio-3-hydroxy (PTZ-3OH) * prothioconazole-desthio-4-hydroxy (PTZ-4OH) * prothioconazole-desthio-5-hydroxy (PTZ-5OH) * prothioconazole-desthio-6-hydroxy (PTZ-6OH) * prothioconazole-desthio-alpha-hydroxy (PTZ-a-OH). Note: 2 diastereoisomers of this compound exist. The analytical method was developed and validated for each diastereoisomer, setting the LOQ at 0.01 mg/kg as sum of the diastereoisomers. In this study, the diastereoisomers are identified on the basis of order of elution as:   + prothioconazole-desthio-alpha-hydroxy (diastereoisomer A) (PTZ-a-OH (A))   + prothioconazole-desthio-alpha-hydroxy (diastereoisomer B) (PTZ-a-OH (B))   A hydrolytic step is carried out before the extraction in order to release the conjugated compounds. This step is based on the procedure described in the document:   * EURL-SRM - Analytical Observations Report, “Analysis of Pesticides Entailing Conjugates or Esters in their Residue Definitions”, Version 2 (last update: 21.04.2021).   The extraction of the analyte from the hydrolyzed mixture (after neutralization) is carried out using the QuEChERS method and the instrumental determination using a HPLC-MS/MS (high-performance liquid chromatography-triple-quadrupole mass spectrometry).  The limit of quantification (LOQ) is set to 0.01 mg/kg |

Materials and methods

***1. Materials***

***1.A. Quantification of prothioconazole-desthio-3-hydroxy, prothioconazole-desthio-4-hydroxy, prothioconazole-desthio-5-hydroxy, prothioconazole-desthio-6-hydroxy and prothioconazole-desthio-alpha-hydroxy in cereal straw***

The quantification of prothioconazole-desthio-3-hydroxy, prothioconazole-desthio-4-hydroxy, prothioconazole-desthio-5-hydroxy, prothioconazole-desthio-6-hydroxy and prothioconazole-desthio-alpha-hydroxy in cereal straw was assessed by HLPC/MS/MS.

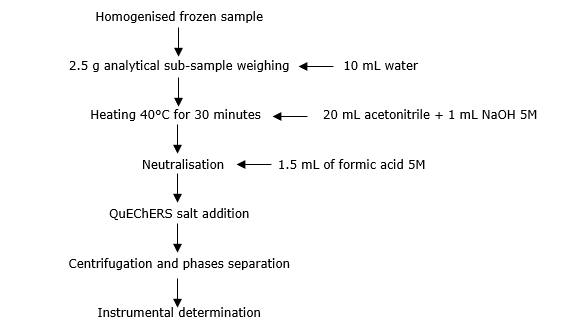
|  |  |
| --- | --- |
| HPLC: | Agilent HPLC 1290 Infinity II + Agilent MS spectrometer 6470A Triple Quad |
| Column: | Agilent Poroshell 120 EC-C18, 2.7 µm, 3 x 150 mm (x 2) |
| Column temperature: | 40 °C |
| Flow rate: | 0.6 mL/min |
| Volume of injection: | 2 µL |
| Retention time: | Approximatively 16.5 minutes for prothioconazole-desthio-3-hydroxy (PTZ-3-OH)  Approximatively 18.1 minutes for prothioconazole-desthio-4-hydroxy (PTZ-4-OH)  Approximatively 18.6 minutes for prothioconazole-desthio-5-hydroxy (PTZ-5-OH)  Approximatively 23.0 minutes for prothioconazole-desthio-6-hydroxy (PTZ-6-OH)  Approximatively 13.0 minutes for prothioconazole-desthio-alpha-hydroxy (A) (PTZ-a-OH (A))  Approximatively 13.0 minutes for prothioconazole-desthio-alpha-hydroxy (B) (PTZ-a-OH (B)) |
| Stop time: | 30 minutes |
| Post time: | 3 minutes |
| Divert valve: | 0 minute to waste  10 minutes to MS  26 minutes to waste |
| Gas temperature: | 350 °C |
| Gas flow: | 5 L//min |
| Nebulizer | 40 psi |
| Sheath gas heater: | 400 °C |
| Sheath gas flow: | 12 L/min |
| Capillary: | 3500 V |
| Mobile phase: | A: LC-MS grade water with 0.2 % formic acid and 5 mM ammonium formate  B: LC-MS grade methanol with 0.2 % formic acid and 5 mM ammonium formate |
| Mixture-Elution: | |  |  |  | | --- | --- | --- | | % A | % B | Time (min) | | 60 | 40 | 0 | | 60 | 40 | 1 | | 30 | 70 | 20 | | 0 | 100 | 25 | |
| Analytical standards: | Prothioconazole-desthio-3-hydroxy (PTZ-3-OH)  CAS No. : 856045-93-7  Lot: 3884-049A6  Purity : 99.7%  Expiry date: 03/06/2023  Prothioconazole-desthio-4-hydroxy (PTZ-4-OH)  CAS No. : 856045-88-0  Lot: 3981-001A2  Purity : 100.0%  Expiry date: 23/06/2023  Prothioconazole-desthio-5-hydroxy (PTZ-5-OH)  CAS No. : N/A  Lot: 3922-021A4  Purity : 99.3%  Expiry date: 27/05/2023  Prothioconazole-desthio-6-hydroxy (PTZ-6-OH)  CAS No. : N/A  Lot: 3967-007A3  Purity : 99.4%  Expiry date: 12/06/2023  Prothioconazole-desthio-alpha-hydroxy (PTZ-a-OH)  CAS No. : 856045-95-9  Lot: 2020-0416067  Purity : 92%  Expiry date: 20/12/2026 |

***2. Methods***

The analytical method for the quantification of prothioconazole-desthio-3-hydroxy, prothioconazole-desthio-4-hydroxy, prothioconazole-desthio-5-hydroxy, prothioconazole-desthio-6-hydroxy and prothioconazole-desthio-alpha-hydroxy in cereal straw based on the QuEChERS method.

***2.A. Schematic diagram of the analytical method***

Straw for cereals



***2.B. Quantification of prothioconazole-desthio-3-hydroxy, prothioconazole-desthio-4-hydroxy, prothioconazole-desthio-5-hydroxy, prothioconazole-desthio-6-hydroxy and prothioconazole-desthio-alpha-hydroxy in cereal straw***

The analytical methods for the quantification of prothioconazole-desthio-3-hydroxy, prothioconazole-desthio-4-hydroxy, prothioconazole-desthio-5-hydroxy, prothioconazole-desthio-6-hydroxy and prothioconazole-desthio-alpha-hydroxy in cereal straw were first validated so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were validated in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |

Results and discussions

Method validation data can be summarised in tables below ~~Table A.1 and~~ A2. There are for each matrix a primary test. In view of the similar results between the primary and confirmatory test, a test by an independent laboratory validation (ILV) is not required.

Table A~~1~~ 92: Recovery results from method validation of prothioconazole-desthio-3-hydroxy, prothioconazole-desthio-4-hydroxy, prothioconazole-desthio-5-hydroxy, prothioconazole-desthio-6-hydroxy and prothioconazole-desthio-alpha-hydroxy in cereal straw using the confirmatory method

| Matrix | Analyte | Fortification level  (*n* = x) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Straw  (Cereals) | PTZ-3-OH | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  83.3 % | ***Confirmatory transition***:  1.1 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  88.1 % | ***Confirmatory transition***:  2.4 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| PTZ-4-OH | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  90.3 % | ***Confirmatory transition***:  1.4 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  91.2 % | ***Confirmatory transition***:  1.9 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| PTZ-5-OH | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  88.4 % | ***Confirmatory transition***:  5.3 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  88.8 % | ***Confirmatory transition***:  3.1 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| PTZ-6-OH | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  84.6 % | ***Confirmatory transition***:  3.4 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  90.8 % | ***Confirmatory transition***:  3.1 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| PTZ-a-OH (A) | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  85.9 % | ***Confirmatory transition***:  3.0 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  85.9 % | ***Confirmatory transition***:  3.3 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| PTZ-a-OH (B) | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  85.3 % | ***Confirmatory transition***:  2.1 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  87.4 % | ***Confirmatory transition***:  1.3 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

Table A~~2~~ 93: Characteristics for the confirmatory method used for validation of prothioconazole-desthio-3-hydroxy, prothioconazole-desthio-4-hydroxy, prothioconazole-desthio-5-hydroxy, prothioconazole-desthio-6-hydroxy and prothioconazole-desthio-alpha-hydroxy in cereal straw

|  | prothioconazole-desthio-3-hydroxy, prothioconazole-desthio-4-hydroxy, prothioconazole-desthio-5-hydroxy, prothioconazole-desthio-6-hydroxy and prothioconazole-desthio-alpha-hydroxy |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  **Confirmatory transition:**  ***PTZ-3-OH***  % interference mean = 0.9  ***PTZ-4-OH***  % interference mean = 1.2  ***PTZ-5-OH***  % interference mean = 17.6  ***PTZ-6-OH***  % interference mean = 29.9  ***PTZ-a-OH (A)***  % interference mean = 6.7  ***PTZ-a-OH (B)***  % interference mean = 25.8 |
| Calibration (type, number of data points) | **Confirmatory transition:**  ***PTZ-3-OH***  Equation : Y = 9667.495664 \* x + 133.797658  Coefficient of correlation: r² = 99.770  ***PTZ-4-OH***  Equation : Y = 9612.080447 \* x – 1010.508794  Coefficient of correlation: r² = 99.882  ***PTZ-5-OH***  Equation : Y = 3240.631036 \* x – 88.763505  Coefficient of correlation: r² = 99.819  ***PTZ-6-OH***  Equation : Y = 4572.564454 \* x + 550.925657  Coefficient of correlation: r² = 99.550  ***PTZ-a-OH (A)***  Equation : Y = 10147.236480 \* x + 918.117746  Coefficient of correlation: r² = 99.876  ***PTZ-a-OH (B)***  Equation : Y = 8344.465 \* x + 175.691  Coefficient of correlation: r² = 99.685 |
| Calibration range | ***PTZ-3-OH***  Accepted calibration range in concentration units 0.303 – 25.23 µg/L (from 24 % of LOQ to 102 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.0024 – 0.202 mg/kg)  ***PTZ-4-OH***  Accepted calibration range in concentration units 0.299 – 24.95 µg/L (from 24 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.0024 – 0.200 mg/kg)  ***PTZ-5-OH***  Accepted calibration range in concentration units 0.299 – 24.88 µg/L (from 24 % of LOQ to 99 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.0024 – 0.199 mg/kg)  ***PTZ-6-OH***  Accepted calibration range in concentration units 0.299 – 24.93 µg/L (from 24 % of LOQ to 99 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.0024 – 0.199 mg/kg)  ***PTZ-a-OH (A)***  Accepted calibration range in concentration units 0.110 – 9.15 µg/L (from 24 % of LOQ to 103 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.00088 – 0.073 mg/kg)  ***PTZ-a-OH (B)***  Accepted calibration range in concentration units 0.190 – 15.83 µg/L (from 24 % of LOQ to 101 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.0015 – 0.127 mg/kg) |
| Assessment of matrix effects is presented | Yes : Performed comparing the analyte response of one individual standard at L4 prepared in solvent to one prepared in blank matrix extract at the same concentration.  ***PTZ-3-OH***  Matrix effect = 5.6 %  Not significant  ***PTZ-4-OH***  Matrix effect = -9.3 %  Not significant  ***PTZ-5-OH***  Matrix effect = -15.3 %  Not significant  ***PTZ-6-OH***  Matrix effect = - 2.3 %  Not significant  ***PTZ-a-OH (A)***  Matrix effect = - 6.1 %  Not significant  ***PTZ-a-OH (B)***  Matrix effect = 5.1 %  Not significant |
| Limit of quantification (LOQ) | ***PTZ-3-OH***  LOQ = 0.01 mg/kg  ***PTZ-4-OH***  LOQ = 0.01 mg/kg  ***PTZ-5-OH***  LOQ = 0.01 mg/kg  ***PTZ-6-OH***  LOQ = 0.01 mg/kg  ***PTZ-a-OH (sum)***  LOQ = 0.01 mg/kg  ***PTZ-a-OH (A)***  LOQ = 0.0036 mg/kg  ***PTZ-a-OH (B)***  LOQ = 0.0063 mg/kg |
| Limit of determination (LOD) | ***PTZ-3-OH***  LOD = 0.303 µg/L  (0.0024 mg/kg)  ***PTZ-4-OH***  LOD = 0.299 µg/L  (0.0024 mg/kg)  ***PTZ-5-OH***  LOD = 0.299 µg/L  (0.0024 mg/kg)  ***PTZ-6-OH***  LOD = 0.299 µg/L  (0.0024 mg/kg)  ***PTZ-a-OH (A)***  LOD = 0.110 µg/L  (0.00088 mg/kg)  ***PTZ-a-OH (B)***  LOD = 0.190 µg/L  (0.0015 mg/kg) |
| Stability in sample extracts | ***PTZ-3-OH***  % difference = 2.7  ***PTZ-4-OH***  % difference = -2.5  ***PTZ-5-OH***  % difference = - 2.3  ***PTZ-6-OH***  % difference = -8.0  ***PTZ-a-OH (A)***  % difference = 8.6  ***PTZ-a-OH (B)***  % difference = -1.0  The stability of the analytes in the final extracts can be considered proven for 3 days at 5 ± 3°C in dark conditions since the recovery of the stored spiked extracts were within the range of 70-120% measured against the freshly prepared ones, as required by the SANTE/2020/12830 rev.1 guideline |
| Stability of stock standard solutions | ***PTZ-3-OH***  % difference = 1.7  ***PTZ-4-OH***  % difference = 3.5  ***PTZ-5-OH***  % difference = -0.9  ***PTZ-6-OH***  % difference = 0.7  ***PTZ-a-OH (A)***  % difference = -7.5  ***PTZ-a-OH (B)***  % difference = 9.3  In addition, since the differences between the mean responses of the analytes in the diluted solutions prepared form stored and from freshly-prepared stock solutions was < 10%, it is possible to assess that the analytes are stable in the stock solutions prepared in water for at least 78 days, if stored in the dark at 5 ± 3°C. |

Conclusion

The confirmatory method for the quantification of prothioconazole-desthio-3-hydroxy, prothioconazole-desthio-4-hydroxy, prothioconazole-desthio-5-hydroxy, prothioconazole-desthio-6-hydroxy and prothioconazole-desthio-alpha-hydroxy in cereal straw was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability for prothioconazole-desthio-3-hydroxy, prothioconazole-desthio-4-hydroxy, prothioconazole-desthio-5-hydroxy, prothioconazole-desthio-6-hydroxy and prothioconazole-desthio-alpha-hydroxy.

Extraction efficiency

Extraction efficiency is guided by:

* European Commission (2017): SANTE 2017/10632 rev. 3, dated 22 November 2017: Technical Guideline on the Evaluation of Extraction Efficiency of Residue Analytical Methods.

Aliquots of the same homogenised sample were extracted and analysed according to the extraction procedure of the method under validation and the extraction method reported in the cited RAR, here below described.

Aliquots of 2.5 g of homogenised straw sample were weighed in a 50 mL screw capped centrifuge PE test tube, and then submitted to the following extraction procedure:

* Extraction 1: 20 mL of water/methanol 50:50 were added to the pellet and the extraction was performed by manually shaking for 1 minute. After centrifugation, the obtained liquid phase was separated and the solid one was submitted to extraction 2.
* Extraction 2: the solid phase was extracted in the same way using 20 mL of methanol/dichloromethane 50:50. After centrifugation:
  + The liquid phase of Extraction 2 was pooled to the liquid phase of Extraction 1. Then, 40 mL of dichloromethane were added to facilitate the separation of the phases. After shaking, the aqueous layer (phase A) was separated from the organic phase (phase B1).
    - The aqueous layer (phase A) was submitted to a hydrolysis step adding 1 mL of hydrochloric acid 6M and heating at about 40°C for 1 hour. The resulting mixture was extracted with 20 mL of dichloromethane: after separation, the organic phase (phase B2) was separated from the aqueous one, that was discarded.
  + The solid was submitted to the Extraction 3:
* Extraction 3: the exhausted solid obtained from the Extraction 2 was submitted to an extraction with 20 mL of methanol under reflux for 1 h. After centrifugation, the obtained liquid phase (methanol, phase B3) was separated and the solid one was submitted to Extraction 4.
* Extraction 4: the solid was submitted to a hydrolysis step adding 20 mL of hydrochloric acid 6M and heating at about 40°C for 1 hour. The resulting mixture was extracted with 20 mL of dichloromethane: after separation, the organic phase (phase B4) was separated from the aqueous one, that was discarded.

The organic phases B1, B2, B3, B4 were then joint and evaporated to dryness using a rotary evaporator and a nitrogen stream. The residue was dissolved in acetonitrile and analysed by a HPLC-MS/MS according to the instrumental method (the change of solvent was necessary since the stationary phase of the HPLC column was not compatible with dichloromethane).

Results and discussions

Extraction efficiency evaluation can be summarised in Table A.1. There are for each matrix a primary test. In view of the similar results between the primary and confirmatory test, a test by an independent laboratory validation (ILV) is not required.

Table A 94: Recovery results from method validation of prothioconazole-desthio-3-hydroxy, prothioconazole-desthio-4-hydroxy, prothioconazole-desthio-5-hydroxy, prothioconazole-desthio-6-hydroxy and prothioconazole-desthio-alpha-hydroxy in cereal straw using the confirmatory method

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Straw (barley)** | | | | | |  |
| **Analyte** | **METHOD UNDER VALIDATION**  **(AM-GLP-STUDY-21-120)** | | | **METABOLISM METHOD** | | |  |
| **GLP-SMPL-21-**  **2890 MB-M1** | **GLP-SMPL-21-588  SMPL-M1** (from the  study KCP 6.3/03) | | **GLP-SMPL-21-**  **2890 MB-M2** | **GLP-SMPL-21-588 SMPL-M2** (from the  study KCP 6.3/03) | |  |
| **Result**  **(mg/kg)** | **Result**  **(mg/kg)** | **Result corrected  for the recovery**  **(mg/kg)** | **Result**  **(mg/kg)** | **Result**  **(mg/kg)** | **Result corrected  for the recovery**  **(mg/kg)** | **DIFFERENCE (%) (limit: +/- 30%)** |
| PTZ-3-OH | < LOD | 0.19991 | 0.2140 | < LOD | 0.09599 | 0.2401 | **-10.9** |
| PTZ-4-OH | < LOD | 0.22317 | 0.2329 | < LOD | 0.09225 | 0.2454 | **-5.1** |
| PTZ-5-OH | < LOD | 0.08572 | 0.0858 | < LOD | 0.03705 | 0.1012 | **-15.2** |
| PTZ-6-OH | < LOD | 0.01945 | 0.0201 | < LOD | 0.01000 | 0.0256 | **-21.5** |
| PTZ-a-OH (A) | < LOD | 0.03769 | 0.0405 | < LOD | 0.02004 | 0.0494 | **-18.1** |
| PTZ-a-OH (B) | < LOD | 0.07779 | 0.0827 | < LOD | 0.03516 | 0.0914 | **-9.5** |

Conclusion

The difference of the amounts of analytes found in the sample with the analytical method under validation and those found applying the extraction procedure of the metabolism study is less than 30%: the extraction efficiency can be considered proven.

* + - * 1. Analytical method 17

|  |  |
| --- | --- |
| Comments of zRMS: | The LC/MS/MS analytical method has been accepted.  2 transitions were monitored. Validation results are presented below:      The validation parameters are in the required range. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/25 |
| Report | Validation of the Analytical Method for the Determination of Prothioconazole-desthio Residues in Aqueous Samples coming from the Ecotoxicological tests  Garagna, D.  2022a  ChemService S.r.l. Controlli e Ricerche, Novate Milanese – Italy  Report No. : CH – 0949/2021 |
| Guideline(s): | Yes :   * SANTE/2020/12830, rev. 1 dated 24/02/21 * OECD No. 201 “*Freshwater algae and cyanobacteria growth inhibition test*” (2011) |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The determination of Prothioconazole-desthio residues is performed by HPLC using an external standard and MS triple quadrupole detector.  Its quantification is achieved using the calibration curve obtained by plotting standard solutions concentrations and the corresponding peak area. |

Materials and methods

***1. Materials***

***1.A. Determination of prothioconazole-desthio residues in aqueous samples***

The determination of prothioconazole-desthio residues in aqueous samples was assessed by HLPC/MS-QQQ.

|  |  |
| --- | --- |
| HPLC: | Agilent Technologies 1200 System |
| Column: | Zorbax Eclipse Plus C18, 3.5 µm, 100 x 4.6 mm i.d. |
| Detector: | MS Triple quadrupole (Scan in MRM mode) |
| Mobile phase: | Water, HPLC grade  Formic acid, high purity for mass spectroscopy  Ammonium formate, for HPLC  Acetonitrile, HPLC grade |
| Eluent flow: | 0.4 mL/min |
| Elution mode: | Gradient condition |
| Mixture: | |  |  |  | | --- | --- | --- | | A% | B% | Time (min) | | 30 | 70 | 0 | | 5 | 95 | 10 | | 30 | 70 | 15 | |
|  |  |
| Volume of injection: | 1 µL |
| Retention time: | About 5.2 minutes |
| Total analysis time: | 15 minutes + 2 minutes post run |
| Ion mode: | ESI, positive polarity |
| Scan type: | MRM |
| Electro multiplier voltage: | 400 V |
| Dry gas temperature: | 350 °C |
| Dry gas flow: | 10 L/min |
| Nebuliser: | 45 psi |
| Capillary current: | 4000 V |
| Dwell time: | 200 msec |
|  |  |
| Analytical standards: | Prothioconazole-desthio  CAS No. : 120983-64-4  Batch No. : BCCC7798  Purity : 98.4 %  Expiry date: February 1, 2023 |

***2. Methods***

***2.A. Preparation of aqueous samples***

Aqueous samples prepared in algal growth medium as suggest by the guideline OECD No. 201 (2011).

No particular treatment is necessary to prepare the samples for the analysis, only a dilution in matrix is performed before the injection.

***2.B. Determination of prothioconazole-desthio residues in aqueous samples***

The analytical methods for the determination of prothioconazole-desthio residues in aqueous samples were first validated so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were validated in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A 95: Matric effects results from method validation of prothioconazole-desthio using the analytical method

| Matrix effect | Analyte | Slope of Algal growth medium | Slope of solvent (Acetonitrile) | Comments |
| --- | --- | --- | --- | --- |
| Result  -19.1 % | Prothioconazole-desthio | 289 | 357 | No comments |

Table A 96: Recovery results from method validation of prothioconazole-desthio using the analytical method

| Matrix | Analyte | Fortification level  (n = x) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Alga growth medium | Prothioconazole-desthio | Low level:  10.6 µg/L  (mean found)  N = 5 | 72.1 | 4 | No comments |
| High level:  24.15 mg/L (mean found)  N = 5 | 97.4 | 3 | No comments |

Table A 97: Characteristics for the analytical method used for validation of prothioconazole-desthio residues for purposes of aqueous ecotoxicological tests

|  | Prothioconazole-desthio |
| --- | --- |
| Specificity | Untreated blank < 30 % LOQ  Result : 0.0 % for Alga growth medium  These % ratio (Blank vs LOQ) demontrate that there was no interference of the blank matrix at the retention time of the analyte. |
| Linearity / Calibration | **Alga growth medium**  ***Quantifier1 transition***  ***m/z 312 🡪 m/z 70 :***  The calibration curve (µg/L):   * was considered as valid over 4.8 – 482.2 µg/L.   Equation (µg/L)  y = 286 \* x + 1112  Correlation coefficient:  r² = 99.768  ***Quantifier2 transition***  ***m/z 312 🡪 m/z 125 :***  The calibration curve (µg/L):   * was considered as valid over 4.8 – 482.2 µg/L.   Equation (µg/L)  y = 35 \* x +185  Correlation coefficient:  r² = 99.564 |
| Limit of determination (LOD) | **Alga growth medium**  LOD = 4.8 µg Prothioconazole-desthio/L  Lowest calibration level |
| Limit of quantification (LOQ) | **Alga growth medium**  LOD = 14.8 µg Prothioconazole-desthio/L in Alga growth medium (corresponding to 15.0 µg test item/L)  Lowest fortified level |
| Stability  (after 3 days) | Recovery Mean between 70 % - 120 %  High level: 94.5 % |
| Stability of standard  (after 3 days) | 8.5 % |

Conclusion

The analytical method for the quantification of prothioconazole-desthio residues in aqueous samples was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability for prothioconazole-desthio.

* + - * 1. Analytical method 18

|  |  |
| --- | --- |
| Comments of zRMS: | The analytical method validation has been accepted.  The LC-MS/MS method was applied in tunnel test. 2 transitions were monitored. The confirmatory method is not necessary. The validation parameters are in the required range. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/26 |
| Report | Difenoconazole 130 g/L + Prothioconazole 250 g/L EC- IN233C1560 : Validation of the Analytical Method for the Determination of Difenoconazole and Prothioconazole Residues in Pollen and Nectar from Ecotoxicological Study  Report No.: CH-0223/2022  Garagna, D. (2022b)  ChemService S.r.l Controlli e Ricerche - Novate Milanese (MI) - Italy |
| Guideline(s): | Yes  SANTE/2020/12830 rev. 1 dated 24/02/21: Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes. |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

Materials and methods

|  |  |
| --- | --- |
| **Principle of the method** | The analytical phase was conducted to determine Prothioconazole and Difenoconazole concentrations in samples coming from the biological phase of the ecotoxicological test on Honeybee *Apis Mellifera L*. |

**Materials:**

|  |  |
| --- | --- |
| UHPLC/MS-QQQ: | Shimadzu Technologies, mod. 8050, equipped with binary pump, autosampler, coupled with an ESI |
| Column: | Zorbax Eclipse Plus C18, 3.5 µm, 100 x 4.6 mm i.d. |
| Detector: | Triple Quadrupole Mass Detector |
| Ionisation type: | Electrospray (ESI) |
| Polarity: | Positive ion mode |
| Eluent: | A = Water / Formic acid 0.1% / Ammonium formate 10 mM  B = Acetonitrile |
| Eluent flow: | 0.7 mL/min |
| Elution mode: | Gradient condition   |  |  |  | | --- | --- | --- | | % A | % B | Time (min) | | 40 | 60 | 0 | | Waste on *during the samples analysis the matrix is send to waste, during wash analysis not.* | | 4.5 | | 40 | 60 | 8 | | 10 | 90 | 8.1 | | Waste off *during the samples analysis the matrix is send to waste, during wash analysis not* | | 9 | | 10 | 90 | 12 | | 40 | 60 | 16 | | 40 | 60 | 18 | |
| Volume of injection: | 10 µL |
| Retention time: | Approximately 5.3 minutes for a total analysis time of 18 minutes |
| Scan type: | Multiple reaction monitoring |
| Interface temperature: | 300 °C |
| DL temperature: | 250 °C |
| Heat Block: | 30 °C |
| Drying gas flow: | 10 L/min |
| Nebulizer Gas Flow: | 2.9 L/min |
| Heating Gas Flow: | 5 L/min |
|  |  |
| Analytical standards: | Prothioconazole  Batch No. : BCCB2271  Purity : 99.9 %  Expiry date: February 1, 2024 |
|  |  |
| Reagents: | Water, HPLC grade  Acetonitrile, HPLC grade  Ammonium formate  Formic acid  QuEChERS Extraction Salt Packet |
|  |  |

**Methods:**

The analytical methods for the determination of prothioconazole in pollen and in nectar were confirmed so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were confirmed in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A 98: Recovery results from validation method of prothioconazole using the analytical method

| Matrix | Analyte | Fortification level (mg/kg) (n = 5) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Pollen | Prothioconazole | At low level :  55.5 µg/kg (LOQ) | 101.1 | 5.0 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  555 µg/kg (10\*LOQ) | 81.5 | 5.0 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Nectar | Prothioconazole | At low level :  55.5 µg/kg (LOQ) | 76.0 | 7.0 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  555 µg/kg (10\*LOQ) | 82.3 | 4.0 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

Table A 99: Characteristics for the analytical method used for validation method of prothioconazole residues in pollen

|  | Prothioconazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Quantifier transition m/z 344 🡪 m/z 326***  Equation (µg/L) : Y = 4814 \* x – 6300  Equation (µg/kg) : Y = 241 \* x – 6300  Coefficient of correlation: r² = 99.798  ***Quantifier 1 transition m/z 344 🡪 m/z 154***  Equation (µg/L) : Y = 1129 \* x – 1469  Equation (µg/kg) : Y = 56 \* x – 1469  Coefficient of correlation: r² = 99.475  ***Quantifier 2 transition m/z 344 🡪 m/z 189***  Equation (µg/L) : Y = 1178 \* x – 1836  Equation (µg/kg) : Y = 59 \* x – 1836  Coefficient of correlation: r² = 99.759 |
| Calibration range | Accepted calibration range in concentration units 2.0 – 99.9 µg/L  Corresponding calibration range in mass ratio units for the sample (40.0 – 1998.0 µg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = 40.2 %  Significant matrix effects for Prothioconazole residues in pollen matrix were found (> ± 20%).  Therefore, the quantification should be performed using matrix-matched standard solutions prepared in pollen (matrix matched calibration). |
| Limit of quantification (LOQ) | LOQ = 55.5 µg/kg |
| Limit of determination (LOD) | LOD = 2.0 µg/L |
| Stability | Analysis performed within 24 hours from preparation; stability check not performed.  Standard prepared freshly; stability check not performed. |
| Residue amount | Residue results calculated as values lower than the LOD are classified as not detected (n.d.).  Residue results calculated as higher than the limit of detection but less than limit of quantification, are designated as < LOQ. |

Table A 100: Characteristics for the analytical method used for validation method of prothioconazole residues in nectar

|  | Prothioconazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Quantifier transition m/z 344 🡪 m/z 326***  Equation (µg/L) : Y = 3591 \* x – 2597  Equation (µg/kg) : Y = 180 \* x – 2597  Coefficient of correlation: r² = 99.666  ***Quantifier 1 transition m/z 344 🡪 m/z 154***  Equation (µg/L) : Y = 843 \* x – 200  Equation (µg/kg) : Y = 42 \* x – 200  Coefficient of correlation: r² = 99.767  ***Quantifier 2 transition m/z 344 🡪 m/z 189***  Equation (µg/L) : Y = 897 \* x – 597  Equation (µg/kg) : Y = 45 \* x – 597  Coefficient of correlation: r² = 99.830 |
| Calibration range | Accepted calibration range in concentration units 2.0 – 99.9 µg/L  Corresponding calibration range in mass ratio units for the sample (40.0 – 1998.0 µg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = 26.9 %  Significant matrix effects for Prothioconazole residues in pollen matrix were found (> ± 20%).  Therefore, the quantification should be performed using matrix-matched standard solutions prepared in pollen (matrix matched calibration). |
| Limit of quantification (LOQ) | LOQ = 55.5 µg/kg |
| Limit of determination (LOD) | LOD = 2.0 µg/L |
| Stability | Analysis performed within 24 hours from preparation; stability check not performed.  Standard prepared freshly; stability check not performed. |
| Residue amount | Residue results calculated as values lower than the LOD are classified as not detected (n.d.).  Residue results calculated as higher than the limit of detection but less than limit of quantification, are designated as < LOQ. |

Conclusion

The validation method for the quantification of prothioconazole in pollen and nectar was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability.

* + - * 1. Analytical method 19

|  |  |
| --- | --- |
| Comments of zRMS: | The analytical method validation has been accepted.  The LC-MS/MS method 0223/2022 (see previous study) was applied in tunnel test. 2 transitions were monitored. The confirmatory method is not necessary. The validation parameters are in the required range. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/27 |
| Report | Difenoconazole 130 g/L + Prothioconazole 250 g/L EC- IN233C1560: Effects on Honey Bee Brood (Apis Mellifera L.) under Semi-Field Conditions – Tunnel Test (Analytical Phase)  Report No.: 168191033  Test site study Report No.: CH-0695/2022  Garagna, D. (2022c)  Ibacom GmBH, Rossdorf - Germany |
| Guideline(s): | Yes  SANTE/2020/12830 rev. 1 dated 24/02/21: Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes. |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

Materials and methods

|  |  |
| --- | --- |
| **Principle of the method** | The analytical phase was conducted to determine Prothioconazole concentrations in samples coming from the biological phase of the ecotoxicological test on Honeybee *Apis Mellifera L*. |

**Materials:**

|  |  |
| --- | --- |
| UHPLC/MS-QQQ: | Shimadzu Technologies, mod. 8050, equipped with binary pump, autosampler, coupled with an ESI |
| Column: | Zorbax Eclipse Plus C18, 3.5 µm, 100 x 4.6 mm i.d. |
| Detector: | Triple Quadrupole Mass Detector |
| Ionisation type: | Electrospray (ESI) |
| Polarity: | Positive ion mode |
| Eluent: | A = Water / Formic acid 0.1% / Ammonium formate 10 mM  B = Acetonitrile |
| Eluent flow: | 0.7 mL/min |
| Elution mode: | Gradient condition   |  |  |  | | --- | --- | --- | | % A | % B | Time (min) | | 40 | 60 | 0 | | Waste on *during the samples analysis the matrix is send to waste, during wash analysis not.* | | 4.5 | | 40 | 60 | 8 | | 10 | 90 | 8.1 | | Waste off *during the samples analysis the matrix is send to waste, during wash analysis not* | | 9 | | 10 | 90 | 12 | | 40 | 60 | 16 | | 40 | 60 | 18 | |
| Volume of injection: | 10 µL |
| Retention time: | Approximately 5.3 minutes for a total analysis time of 18 minutes |
| Scan type: | Multiple reaction monitoring |
| Interface temperature: | 300 °C |
| DL temperature: | 250 °C |
| Heat Block: | 30 °C |
| Drying gas flow: | 10 L/min |
| Nebulizer Gas Flow: | 2.9 L/min |
| Heating Gas Flow: | 5 L/min |
|  |  |
| Analytical standards: | Prothioconazole  Batch No. : BCCB2271  Purity : 99.9 %  Expiry date: February 1, 2024 |
|  |  |
| Reagents: | Water, HPLC grade  Acetonitrile, HPLC grade  Ammonium formate  Formic acid  QuEChERS Extraction Salt Packet |
|  |  |

**Methods:**

The analytical methods for the determination of prothioconazole in pollen and in nectar were confirmed so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were confirmed in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A 101: Recovery results from validation method of prothioconazole using the analytical method

| Matrix | Analyte | Fortification level (mg/kg) (n = 5) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Pollen | Prothioconazole | At low level :  55.5 µg/kg (LOQ) | 101.1 | 5.0 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  555 µg/kg (10\*LOQ) | 81.5 | 5.0 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Nectar | Prothioconazole | At low level :  55.5 µg/kg (LOQ) | 76.0 | 7.0 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  555 µg/kg (10\*LOQ) | 82.3 | 4.0 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

Table A 102: Characteristics for the analytical method used for validation method of prothioconazole residues in pollen

|  | Prothioconazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Pollen***  Equation: Y = 8933 \* x – 3225  Coefficient of correlation: r² = 99.953  ***Pollen (diluted samples)***  Equation: Y = 7750 \* x – 3031  Coefficient of correlation: r² = 99.883 |
| Calibration range | Accepted calibration range in concentration units 2.0 – 99.9 µg/L  Corresponding calibration range in mass ratio units for the sample (0.02 – 1 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = 40.2 %  Significant matrix effects for Prothioconazole residues in pollen matrix were found (> ± 20%).  Therefore, the quantification should be performed using matrix-matched standard solutions prepared in pollen (matrix matched calibration). |
| Limit of quantification (LOQ) | LOQ = 55.5 µg/kg |
| Limit of determination (LOD) | LOD = 2.0 µg/L |
| Stability | Analysis performed within 24 hours from preparation; stability check not performed.  Standard prepared freshly; stability check not performed. |
| Residue amount | Residue results calculated as values lower than the LOD are classified as not detected (n.d.).  Residue results calculated as higher than the limit of detection but less than limit of quantification, are designated as < LOQ. |

Table A 103: Characteristics for the analytical method used for validation method of prothioconazole residues in nectar

|  | Prothioconazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  % interference mean = 0.0 |
| Calibration (type, number of data points) | Equation: Y = 4797 \* x – 3184  Coefficient of correlation: r² = 99.865 |
| Calibration range | Accepted calibration range in concentration units 2.0 – 99.9 µg/L  Corresponding calibration range in mass ratio units for the sample (0.02 – 1 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = 26.9 %  Significant matrix effects for Prothioconazole residues in pollen matrix were found (> ± 20%).  Therefore, the quantification should be performed using matrix-matched standard solutions prepared in pollen (matrix matched calibration). |
| Limit of quantification (LOQ) | LOQ = 55.5 µg/kg |
| Limit of determination (LOD) | LOD = 2.0 µg/L |
| Stability | Analysis performed within 24 hours from preparation; stability check not performed.  Standard prepared freshly; stability check not performed. |
| Residue amount | Residue results calculated as values lower than the LOD are classified as not detected (n.d.).  Residue results calculated as higher than the limit of detection but less than limit of quantification, are designated as < LOQ. |

Conclusion

The validation method for the quantification of prothioconazole in pollen and nectar was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability.

* + - * 1. Analytical method 20

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  The LC-MS/MS detection were used. 2 transitions were monitored. The specificity of the method was assured by MS/MS detection. The limit of quantification (LOQ) was set to 0.01 mg/kg. All validation parameters are in the currently required range. |

|  |  |
| --- | --- |
| Reference: | KCP 5.3.2.8/01 |
| Report | Validation of an analytical method for the quantification of Difenoconazole, Prothioconazole and Prothioconazole-desthio in honey  Report No.: LBN-0092-2023  Longhi, D. 2023a  LabAnalysis s.r.l., Casanova Lonati (PV) - Italy |
| Guideline(s): | Yes  SANTE/2020/12830 rev. 2: Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes. |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

Materials and methods

|  |  |
| --- | --- |
| **Principle of the method** | The analytical method was based on the method “European Committee for Standardisation (CEN) EN 15662:2018. Foods of plant origin - Multimethod for the determination of pesticide residues using GC- and LC-based analysis following acetonitrile extraction/partitioning and clean-up by dispersive SPE - Modular QuEChERS-method”.  The analytical method was based on an instrumental determination using a HPLC-MS/MS (high-performance liquid chromatography + triple-quadrupole mass spectrometry).  The limit of quantification (LOQ) was set to 0.01 mg/kg.  The analytical method was validated under GLP compliance according to SANTE/2020/12830 Rev.2. |

**Materials:**

|  |  |
| --- | --- |
| HPLC-MS/MS | Agilent HPLC 1290 Infinity II + Agilent MS spectrometer 6470A Triple Quad |
| Column: | Phenomenex Kinetex C18, 1.7 μm, 2.1 x 50 mm |
| Column temperature: | 40°C |
| Flow: | 0.6 mL/min |
| Injection volume: | 2.5 µL |
| Mobile phase:  Elution: | A = LC-MS grade water with 0.2% formic acid and 5 mM ammonium formate  B = LC-MS grade methanol with 0.2% formic acid and 5 mM ammonium formate   |  |  |  | | --- | --- | --- | | Time (min) | % A | % B | | 0 | 70 | 30 | | 0.5 | 70 | 30 | | 3.0 | 0 | 100 | |
| Stop time: | 5 min |
| Post time: | 1 min |
| Divert value: | 0 min. to waste, 2 min to MS, 3.5 min. to waste |
| Source type: | ESI |
| Gas temperature: | 350°C |
| Gas flow: | 8 L/min |
| Nebulizer: | 40 psi |
| Sheath gas heater: | 400°C |
| Sheath gas flow: | 12 L/min |
| Capillary: | Positive mode 3500V  Negative mode 3000V |
| Vcharging: | 0 |
| Acquiring mode: | ESI positive and ESI negative, MRM (multi-reaction monitoring) |

|  |  |
| --- | --- |
| Analytical standards: | Prothioconazole  CAS No.: 179828-70-6  Batch No. : BCCB2271  Purity : 99.9% with 2.2% water (purity corrected for the water content: 97.7 %)  Expiry date: February, 2024 |
|  |  |
|  | Prothioconazole-desthio  CAS No.: 120983-64-4  Batch No. : BCCJ7867  Purity : 98.4% with 0.1% water (purity corrected for the water content: 98.3 %)  Expiry date: November, 2025 |
|  |  |
| Reagents: | Water, LC-MS grade  Cysteine HCl  Formic acid  Acetonitrile  Unbuffered QuEChERS Extraction Salt Packet  Ammonium formate  Methanol |
| Buffer,  reference standard: | Buffer, pH 4.00 ± 0.01 (25°C)  Batch No.: MKCR2269  Product No.: B5020  Certified value (pH); 4.01  Expiry date: January, 2024  Buffer, pH 7.00 ± 0.01 (25°C)  Batch No.: MKCR0856  Product No.: B4770  Certified value (pH); 7.00  Expiry date: December, 2023 |
| Matrix: | Honey (multiflower origin) purchased in a local market Esselunga, Broni (PV), Italy  Storage: frozen  Measured pH-value: 4.2 according to method CIPC MT 75.3 |

**Methods:**

The analytical method for the quantification of prothioconazole and prothioconazole-desthio in the honey are presented below.

***2.A. Schematic diagram of the analytical method***

***Une image contenant texte, capture d’écran, Police, ligne

Description générée automatiquement***

***2.B. Quantification of prothioconazole and prothioconazole-desthio in honey***

The analytical methods for the determination of prothioconazole and prothioconazole-desthio were confirmed so as to comply with the Guidance Document SANTE/2020/12830 rev.2. The methods were confirmed in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A 104: Recovery results from validation method of prothioconazole and prothioconazole-desthio using the analytical method

| Matrix | Analyte | Fortification level (mg/kg) (n = 5) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Honey | Prothioconazole  (product ion: 100 m/z) | At low level :  0.01 mg/kg (LOQ) | 112 | 3.0 | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 104 | 1.8 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Prothioconazole-desthio  (product ion: 125 m/z) | At low level :  0.01 mg/kg (LOQ) | 107 | 3.1 | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 107 | 3.0 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

Table A 105: Characteristics for the analytical method used for validation method of prothioconazole residues in honey

|  | prothioconazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 2.  ***Product ion (m/z = 100):***  % interference mean = 2.0 |
| Calibration (type, number of data points) | ***Product ion (m/z = 100):***  Equation : Y = 256.537655 \* x + 74.712665  Coefficient of correlation: r² = 99.63 |
| Calibration range | Accepted calibration range in concentration units 0.50 – 50. µg/L  Corresponding calibration range in mass ratio units for the sample (0.0020 – 0.200 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 0.06 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.5 µg/L  (0.002 mg/kg)  497.1 S/N at LOD level |
| Stability | Λ% = 1.3 |
| Stability of standard | % difference = - 3.5%  Since the differences between the mean responses of the analytes in the solutions prepared form stored and from freshly-prepared stock solutions was < 10%, it is possible to assess that the analyte Prothioconazole is stable in the stock solutions prepared in acetonitrile for 55 days, if stored in the dark at 5 ± 3°C. |

Table A 106: Characteristics for the analytical method used for validation method of prothioconazole-desthio residues in honey

|  | Prothioconazole-desthio |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 2.  ***Product ion (m/z = 125):***  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Product ion (m/z = 125):***  Equation : Y = 864.321197 \* x – 24.703398  Coefficient of correlation: r² = 99.85 |
| Calibration range | Accepted calibration range in concentration units 0.50 – 50. µg/L  Corresponding calibration range in mass ratio units for the sample (0.0020 – 0.200 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 10.5 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.5 µg/L  (0.002 mg/kg)  59.1 S/N at LOD level |
| Stability | Λ% = -1.8 |
| Stability of standard | The stability of the Prothioconazole-desthio in the stock standard solutions was verified in the concurrent study GLP-STUDY-21-31 |

Conclusion

The analytical method for the quantification of prothioconazole and prothioconazole-desthio residues in honey was successfully validated according to Guidance Document SANTE/2020/12830 rev. 2 by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability for prothioconazole and prothioconazole-desthio.

Confirmatory method

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  The LC-MS/MS detection were used. 2 transitions were monitored. The specificity of the method was assured by MS/MS detection. The limit of quantification (LOQ) was set to 0.01 mg/kg. All validation parameters are in the currently required range |

|  |  |
| --- | --- |
| Reference: | KCP 5.3.2.8/01 |
| Report | Validation of an analytical method for the quantification of Difenoconazole, Prothioconazole and Prothioconazole-desthio in honey  Report No.: LBN-0092-2023  Longhi, D. 2023a  LabAnalysis s.r.l., Casanova Lonati (PV) - Italy |
| Guideline(s): | Yes  SANTE/2020/12830 rev. 2: Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes. |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

Materials and methods

|  |  |
| --- | --- |
| **Principle of the method** | The analytical method was based on the method “European Committee for Standardisation (CEN) EN 15662:2018. Foods of plant origin - Multimethod for the determination of pesticide residues using GC- and LC-based analysis following acetonitrile extraction/partitioning and clean-up by dispersive SPE - Modular QuEChERS-method”.  The analytical method was based on an instrumental determination using a HPLC-MS/MS (high-performance liquid chromatography + triple-quadrupole mass spectrometry).  The limit of quantification (LOQ) was set to 0.01 mg/kg.  The analytical method was validated under GLP compliance according to SANTE/2020/12830 Rev.2. |

**Materials:**

|  |  |
| --- | --- |
| HPLC-MS/MS | Agilent HPLC 1290 Infinity II + Agilent MS spectrometer 6470A Triple Quad |
| Column: | Phenomenex Kinetex C18, 1.7 μm, 2.1 x 50 mm |
| Column temperature: | 40°C |
| Flow: | 0.6 mL/min |
| Injection volume: | 2.5 µL |
| Mobile phase:  Elution: | A = LC-MS grade water with 0.2% formic acid and 5 mM ammonium formate  B = LC-MS grade methanol with 0.2% formic acid and 5 mM ammonium formate   |  |  |  | | --- | --- | --- | | Time (min) | % A | % B | | 0 | 70 | 30 | | 0.5 | 70 | 30 | | 3.0 | 0 | 100 | |
| Stop time: | 5 min |
| Post time: | 1 min |
| Divert value: | 0 min. to waste, 2 min to MS, 3.5 min. to waste |
| Source type: | ESI |
| Gas temperature: | 350°C |
| Gas flow: | 8 L/min |
| Nebulizer: | 40 psi |
| Sheath gas heater: | 400°C |
| Sheath gas flow: | 12 L/min |
| Capillary: | Positive mode 3500V  Negative mode 3000V |
| Vcharging: | 0 |
| Acquiring mode: | ESI positive and ESI negative, MRM (multi-reaction monitoring) |
| Analytical standards: | Prothioconazole  CAS No.: 179828-70-6  Batch No. : BCCB2271  Purity : 99.9% with 2.2% water (purity corrected for the water content: 97.7 %)  Expiry date: February, 2024 |
|  | Prothioconazole-desthio  CAS No.: 120983-64-4  Batch No. : BCCJ7867  Purity : 98.4% with 0.1% water (purity corrected for the water content: 98.3 %)  Expiry date: November, 2025 |
| Reagents: | Water, LC-MS grade  Cysteine HCl  Formic acid  Acetonitrile  Unbuffered QuEChERS Extraction Salt Packet  Ammonium formate  Methanol |
| Buffer,  reference standard: | Buffer, pH 4.00 ± 0.01 (25°C)  Batch No.: MKCR2269  Product No.: B5020  Certified value (pH); 4.01  Expiry date: January, 2024  Buffer, pH 7.00 ± 0.01 (25°C)  Batch No.: MKCR0856  Product No.: B4770  Certified value (pH); 7.00  Expiry date: December, 2023 |
| Matrix: | Honey (multiflower origin) purchased in a local market Esselunga, Broni (PV), Italy  Storage: frozen  Measured pH-value: 4.2 according to method CIPC MT 75.3 |

**Methods:**

The analytical method for the quantification of prothioconazole and prothioconazole-desthio in the honey are presented below.

***2.A. Schematic diagram of the analytical method***

***Une image contenant texte, capture d’écran, Police, ligne

Description générée automatiquement***

***2.B. Quantification of prothioconazole and prothioconazole-desthio in honey***

The analytical methods for the determination of prothioconazole and prothioconazole-desthio were confirmed so as to comply with the Guidance Document SANTE/2020/12830 rev.2. The methods were confirmed in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A 104: Recovery results from confirmatory method of prothioconazole and prothioconazole-desthio using the analytical method

| Matrix | Analyte | Fortification level (mg/kg) (n = 5) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Honey | Prothioconazole  (product ion: 264 m/z) | At low level :  0.01 mg/kg (LOQ) | 115 | 3.3 | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 104 | 2.5 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Prothioconazole-desthio  (product ion: 69.8 m/z)\* | At low level :  0.01 mg/kg (LOQ) | 108 | 4.9 | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 107 | 2.3 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

\*for the analyte Prothioconazole-desthio it was not possible to find both the product ions with m/z > 100, as required by SANTE/2020/12830, rev.2; this analyte fragmented only in two product ions, one of them with m/z < 100.

Table A 105: Characteristics for the analytical method used for confirmatory method of prothioconazole residues in honey

|  | prothioconazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 2.  ***Product ion (m/z = 264):***  % interference mean = 2.0 |
| Calibration (type, number of data points) | ***Product ion (m/z = 265):***  Equation : Y = 187.811210 \* x + 37.233145  Coefficient of correlation: r² = 99.559 |
| Calibration range | Accepted calibration range in concentration units 0.50 – 50. µg/L  Corresponding calibration range in mass ratio units for the sample (0.0020 – 0.200 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 0.06 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.5 µg/L  (0.002 mg/kg)  149.3 S/N at LOD level |

Table A 106: Characteristics for the analytical method used for confirmatory method of prothioconazole-desthio residues in honey

|  | Prothioconazole-desthio |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 2.  ***Product ion (m/z = 2):***  % interference mean = 1.5 |
| Calibration (type, number of data points) | ***Product ion (m/z = 69.8):***  Equation : Y = 1357.479071\* x – 86.286598  Coefficient of correlation: r² = 99.86 |
| Calibration range | Accepted calibration range in concentration units 0.50 – 50. µg/L  Corresponding calibration range in mass ratio units for the sample (0.0020 – 0.200 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 10.5 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.5 µg/L  (0.002 mg/kg)  19.4 S/N at LOD level |

Conclusion

The confirmatory method for the quantification of prothioconazole and prothioconazole-desthio residues in honey was successfully validated according to Guidance Document SANTE/2020/12830 rev. 2 by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification for prothioconazole and prothioconazole-desthio.

Independent laboratory validation

|  |  |
| --- | --- |
| Comments of zRMS: | The ILV of the analytical method developed and validated in GLP studies Code LBN-0092-2023 has been accepted.  The Prothioconazole, Prothioconazole-desthio and difenoconazole determination was conducted by LC-MS/MS in MRM mode, monitoring two MS/MS ion mass transitions. The validation parameters were in the required range. |

|  |  |
| --- | --- |
| Reference: | KCP 5.3.2.8/03 |
| Report | Independent Laboratory Validation (ILV) of the analytical Method for the Determination of Difenoconazole, Prothioconazole, Prothioconazole-desthio and Triazole Derivatives Metabolites (TDMs) in Honey  Report No.: CH-0859-2023  Mattioli, B. 2023  ChemService S.r.l. Controlli e Ricerche, Novate Milanese (MI) - Italy |
| Guideline(s): | Yes  SANTE/2020/12830 rev. 2: Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes. |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

Materials and methods

|  |  |
| --- | --- |
| **Aim of the method** | The aim of this study was to perform an Independent Laboratory Validation (ILV) of the analytical method developed and validated in GLP studies Code LBN-0092-2023 (Determination of Difenoconazole, Prothioconazole, Prothioconazole-desthio and difenoconazole in Honey) performed by LabAnalysis s.r.l.  The Test Facility ChemService Srl Controlli e Ricerche had re-validate the section of “Recovery and Repeatability” and of “Selectivity and Specificity” of the analytical method already adjusted and validated by LabAnalysis in GLP studies Code LBN-0092-2023.  The analytical method was based on an instrumental determination using a HPLC-MS/MS (high-performance liquid chromatography + triple-quadrupole mass spectrometry).  The limit of quantification (LOQ) was set to 0.01 mg/kg.  The analytical method was validated under GLP compliance according to SANTE/2020/12830 Rev.2. |

**Materials:**

|  |  |
| --- | --- |
| HPLC-MS/MS | Agilent HPLC 1290 Infinity II + Agilent MS spectrometer 6470A Triple Quad |
| Column: | Phenomenex Kinetex C18, 1.7 μm, 2.1 x 50 mm |
| Column temperature: | 40°C |
| Eluent Flow: | 0.6 mL/min |
| Injection volume: | 2.5 µL |
| Eluent:  Solvent composition: | C = Methanol with 0.2% formic acid and 5 mM ammonium formate  D = Water with 0.2% formic acid and 5 mM ammonium formate   |  |  |  | | --- | --- | --- | | Time (min) | % C | % D | | 0 | 30 | 70 | | 0.5 | 30 | 70 | | 3 | 10 | 90 | | 5 | 30 | 70 | |
| Retention time: | Prothioconazole – about 3.2 minutes  Prothioconazole-desthio – About 3.0 minutes |
| Total analysis time: | 5 minutes + 1.0 minutes as post time |
| Source type: | ESI |
| Dry Gas temperature: | 300°C |
| Dry Gas flow: | 8 L/min |
| Nebulizer: | 40 psi |
| Sheath gas temp: | 400°C |
| Sheath gas flow: | 12 L/min |
| Capillary current: | 3000V |
| Vcharging: | 1500V |
| Dwell time: | 50 msec |

|  |  |
| --- | --- |
| Analytical standards: | Prothioconazole  CAS No.: 179828-70-6  Batch No. : BCCB2271  Purity : 99.9%  Expiry date: February 01, 2024 |
|  |  |
|  | Prothioconazole-desthio  CAS No.: 120983-64-4  Batch No. : G1043839  Purity : 99.5%  Expiry date: November 21, 2025 |
| Reagents: | Water, LC-MS grade  Cysteine HCl  Formic acid  Acetonitrile, HPLC grade  Unbuffered QuEChERS Extraction Salt Packet  Ammonium formate  Methanol, LC-MS grade |
| Matrix: | Honey  Expiry date: September 13, 2024  Storage: frozen |

**Methods:**

The analytical method for the quantification of prothioconazole and prothioconazole-desthio in the honey are presented below.

***2.A. Quantification of prothioconazole and prothioconazole-desthio in honey***

The analytical methods for the determination of prothioconazole and prothioconazole-desthio were confirmed so as to comply with the Guidance Document SANTE/2020/12830 rev.2. The methods were confirmed in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A 110: Recovery results from independent laboratory validation of prothioconazole and prothioconazole-desthio using the analytical method

| Matrix | Analyte | Fortification level (mg/kg) (n = 5) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Honey | Prothioconazole  (product ion: 100 m/z)  Primary dectection | At low level :  0.01 mg/kg (LOQ) | 76.4 | 3.2 | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 98.1 | 9.3 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Prothioconazole-desthio  (product ion: 125 m/z)  Primary dectection | At low level :  0.01 mg/kg (LOQ) | 107.4 | 10.0 | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 89.1 | 14.7 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Prothioconazole  (product ion: 264 m/z)  Confirmatory detection | At low level :  0.01 mg/kg (LOQ) | 86.3 | 2.7 | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 96.5 | 9.7 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Prothioconazole-desthio  (product ion: 69.8 m/z)\*  Confirmatory detection | At low level :  0.01 mg/kg (LOQ) | 105.1 | 16.5 | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 87.2 | 15.0 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

\* For the analyte Prothioconazole-desthio it was not possible to find both the product ions with m/z > 100, as required by SANTE/2020/12830, rev.2; this analyte fragmented only in two product ions, one of them with m/z < 100

Table A 111: Characteristics for the analytical method used for independent validation method of prothioconazole residues in honey

|  | prothioconazole |
| --- | --- |
| Specificity | n.d. |
| Calibration (type, number of data points) | ***Product ion (m/z = 100) – primary dectection:***  Equation : Y = 91.5 \* x + 123.3  Coefficient of correlation: r² = 99.708  ***Product ion (m/z = 264) – confirmatory dectection:***  Equation : Y = 47.2 \* x + 48.4  Coefficient of correlation: r² = 99.879 |
| Calibration range | Accepted calibration range in concentration units 0.50 – 50. µg/L  Corresponding calibration range in mass ratio units for the sample (0.0020 – 0.200 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 0.06 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.5 µg/L  (0.002 mg/kg) |
| Stability of samples extracts | % residual analyte after storage = 101% |
| Stability of standard solutions | % difference = - 3.5%  Since the differences between the mean responses of the analytes in the solutions prepared form stored and from freshly-prepared stock solutions was < 10%, it is possible to assess that the analyte Prothioconazole is stable in the stock solutions prepared in acetonitrile for 55 days, if stored in the dark at 5 ± 3°C. |

Table A 112: Characteristics for the analytical method used for independent validation method of prothioconazole-desthio residues in honey

|  | Prothioconazole-desthio |
| --- | --- |
| Specificity | n.d. |
| Calibration (type, number of data points) | ***Product ion (m/z = 125) – primary dectection:***  Equation : Y = 189.3 \* x + 190.6  Coefficient of correlation: r² = 99.991  ***Product ion (m/z = 69.4) – confirmatory dectection:***  Equation : Y = 625.8 \* x + 753.8  Coefficient of correlation: r² = 99.997 |
| Calibration range | Accepted calibration range in concentration units 0.50 – 50. µg/L  Corresponding calibration range in mass ratio units for the sample (0.0020 – 0.200 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 10.5 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.5 µg/L  (0.002 mg/kg) |
| Stability of samples extracts | % residual analyte after storage = 98.2% |
| Stability of standard solutions | No degradation higher than 10% was observed during this storage period. |

Conclusion

The independent laboratory validation for the quantification of prothioconazole and prothioconazole-desthio in honey was successfully validated according to Guidance Document SANTE/2020/12830 rev. 2 by definition of the accuracy, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability.

* + - * 1. Analytical method 21

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| --- | --- |
| Comments of zRMS: | The validation of the analytical method has been accepted.  TDMs determination was conducted by LC-MS/MS (MRM mode), monitoring two mass transitions. The limit of quantification (LOQ) was set to 0.01 mg/kg. The validation parameters were in the required range. |

|  |  |
| --- | --- |
| Reference: | KCP 5.3.2.8/02 |
| Report | Validation of an analytical method for the quantification of Triazole Derivatives Metabolites (TDMs) in honey  Report No.: LBN-0093-2023  Longhi, D. 2023b  LabAnalysis s.r.l., Casanova Lonati (PV) - Italy |
| Guideline(s): | Yes  SANTE/2020/12830 rev. 2: Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes. |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

Materials and methods

|  |  |
| --- | --- |
| **Principle of the method** | The analytical method was based on the method “Quick Method for the Analysis of Highly Polar Pesticides in Food Involving Extraction with Acidified Methanol and LC- or IC-MS/MS Measurement - Food of Plant Origin (QuPPe-PO-Method) - Method 8 (M8)”.  The analytical method was based on an extraction with a mixture of water/methanol with 1% of formic acid and on an instrumental determination using a HPLC-DMS-MS/MS (high-performance liquid chromatography + Differential ion mobility-triple-quadrupole mass spectrometry).  The limit of quantification (LOQ) was set to 0.01 mg/kg.  The analytical method was validated under GLP compliance according to SANTE/2020/12830 Rev.2. |

**Materials:**

|  |  |
| --- | --- |
| HPLC-MS/MS | Shimadzu LC-40 XR + spectrometer Sciex API 6500+ equipped with SelexION (Differential Mobility Separation) device |
| Column: | Thermo Hypercarb 5 μm, 2.1 x 100 mm |
| Column temperature: | 40°C |
| Flow: | 0.6 mL/min |
| Injection volume: | 2 µL |
| Mobile phase:  Elution: | A = LC-MS grade water with 1% acetic acid  B = LC-MS grade methanol with 1% acetic acid   |  |  |  | | --- | --- | --- | | Time (min) | % A | % B | | 0 | 100 | 0 | | 6.00 | 10 | 90 | | 7.00 | 10 | 90 | | 7.10 | 100 | 0 | |
| Stop time: | 10 min |
| Source type: | ESI |
| Curtain gas flow: | 30 mL/min |
| Gas temperature: | 500°C |
| Gas 1: | 55 mL/min |
| Gas 2: | 65 mM/min |
| Capillary: | Positive mode 3500V |
| Acquiring mode: | ESI positive and ESI negative, MRM (multi-reaction monitoring) |

|  |  |
| --- | --- |
| Analytical standards: | 1,2,4-Triazole (1,2,4-T or TRZ)  CAS No.: 288-88-0  Batch No. : STBK4702  Purity : 100.0 %  Expiry date: October 1, 2023 |
|  | Triazole alanine (TA)  CAS No.: 86362-20-1  Batch No. : 787796  Purity : 98.3%  Expiry date: March 1, 2024 |
|  | 1,2,4-Triazole lactic acid (TLA) HCl  CAS No.: 1450828-63-3  Batch No. : 792058  Purity : 78.5%  Expiry date: November 1, 2024 |
|  | Triazole acetic acid (TAA)  CAS No.: 28711-29-7  Batch No. : 711657210-1-1  Purity : 97%  Expiry date: November 9, 2026 |
| Isotope-labelled  internal standard (ILIS) | 1,2,4-Triazole-[13C2, 15N3]  CAS No.: 1261170-82-4  Batch No. : SL6-2012-224  Purity : 98.4%  Expiry date: July 2024 |
|  | Triazole alanine [1,2,4-D2]  CAS No.: 2180306-38-9  Batch No. : 239DCDA135  Actual concentration: 999.68 ± 11.23 uncertainty  Expiry date: August 14, 2024 |
|  | Triazole-[13C2, 15N3] lactic acid  CAS No.: n.d.  Batch No. : EFL6-2015-198A  Purity : 98.42%  Expiry date: July 2024 |
|  | Triazole acetic acid [13C2, 15N3]  CAS No.: n.d.  Batch No. : EFL6-2015-196A  Purity : 98.03%  Expiry date: July 2024 |
| Reagents: | Water, LC-MS grade  Cysteine HCl  Formic acid  Acetonitrile  Unbuffered QuEChERS Extraction Salt Packet  Ammonium formate  Methanol |
| Buffer,  reference standard: | Buffer, pH 4.00 ± 0.01 (25°C)  Batch No.: MKCR2269  Product No.: B5020  Certified value (pH); 4.01  Expiry date: January, 2024  Buffer, pH 7.00 ± 0.01 (25°C)  Batch No.: MKCR0856  Product No.: B4770  Certified value (pH); 7.00  Expiry date: December, 2023 |
| Matrix: | Honey (multiflower origin) purchased in a local market Esselunga, Broni (PV), Italy  Storage: frozen  Measured pH-value: 4.2 according to method CIPC MT 75.3 |

**Methods:**

The analytical method for the quantification of triazole derivatives metabolites in the honey are presented below.

***2.A. Schematic diagram of the analytical method***

***Une image contenant texte, capture d’écran, Police, ligne

Description générée automatiquement***

***2.B. Quantification of triazole derivatives metabolites in honey***

The analytical methods for the determination of triazole derivatives metabolites were confirmed so as to comply with the Guidance Document SANTE/2020/12830 rev.2. The methods were confirmed in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A 110: Recovery results from validation method of triazole derivative metabolite using the analytical method

| Matrix | Analyte | Fortification level (mg/kg) (n = 5) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Honey | 1,2,4-triazole  (product ion: 43.1 m/z) | At low level :  0.01 mg/kg (LOQ) | 99.6 | 3.6 | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 103 | 1.5 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole alanine  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 103 | 7.7 | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 96.9 | 3.4 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole lactic acid  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 106 | 2.9 | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 105 | 2.8 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole acetic acid  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 104 | 3.5 | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 98.0 | 1.6 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

Table A 111: Characteristics for the analytical method used for validation method of 1,2,4-triazole residues in honey

|  | 1,2,4-triazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 2.  ***Product ion (m/z = 264):***  % interference mean = 2.2 |
| Calibration (type, number of data points) | ***Product ion (m/z = 43.1):***  Equation : Y = 0.73354 \* x + 0.00864  Coefficient of correlation: r² = 99.994 |
| Calibration range | Accepted calibration range in concentration units 0.50 – 50. µg/L  Corresponding calibration range in mass ratio units for the sample (0.0020 – 0.200 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = + 2.4 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.5 µg/L  (0.002 mg/kg)  3.9 S/N at LOD level |
| Stability | According to SANTE/2020/12830 rev.2, the stability of the analytes in the final extracts is not required if the extracts contain an isotope-labelled internal standard (ILIS) for quantification, since the ILIS compensates for losses during extract storage. Since ILIS were used, the verification of the stability was not required. Anyway, all the extracts were analysed within 24 hours from their preparation, obtaining recoveries in the fortified samples within the acceptable range of 70-120%. |
| Stability of standard | The stability of the analytes in the stock standard solutions was verified in the study GLP-STUDY-21-108 “Validation of an analytical method for the quantification of Triazole Derivative Metabolites (TDMs) in wheat, barley, oilseed rape and processed commodities”. |

Table A 112: Characteristics for the analytical method used for validation method of triazole alanine residues in honey

|  | triazole alanine |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 2.  ***Product ion (m/z = 264):***  % interference mean = 27.6 |
| Calibration (type, number of data points) | ***Product ion (m/z = 70.0):***  Equation : Y = 2.08566 \* x – 0.00732  Coefficient of correlation: r² = 99.990 |
| Calibration range | Accepted calibration range in concentration units 0.50 – 50. µg/L  Corresponding calibration range in mass ratio units for the sample (0.0020 – 0.200 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 7.2 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.5 µg/L  (0.002 mg/kg)  41.7 S/N at LOD level |
| Stability | According to SANTE/2020/12830 rev.2, the stability of the analytes in the final extracts is not required if the extracts contain an isotope-labelled internal standard (ILIS) for quantification, since the ILIS compensates for losses during extract storage. Since ILIS were used, the verification of the stability was not required. Anyway, all the extracts were analysed within 24 hours from their preparation, obtaining recoveries in the fortified samples within the acceptable range of 70-120%. |
| Stability of standard | The stability of the analytes in the stock standard solutions was verified in the study GLP-STUDY-21-108 “Validation of an analytical method for the quantification of Triazole Derivative Metabolites (TDMs) in wheat, barley, oilseed rape and processed commodities”. |

Table A 113: Characteristics for the analytical method used for validation method of triazole lactic acid residues in honey

|  | triazole lactic acid |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 2.  ***Product ion (m/z = 264):***  % interference mean = 5.3 |
| Calibration (type, number of data points) | ***Product ion (m/z = 70.0):***  Equation : Y = 0.99184 \* x + 0.00209  Coefficient of correlation: r² = 99.998 |
| Calibration range | Accepted calibration range in concentration units 0.50 – 50. µg/L  Corresponding calibration range in mass ratio units for the sample (0.0020 – 0.200 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = + 2.1 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.5 µg/L  (0.002 mg/kg)  26.3 S/N at LOD level |
| Stability | According to SANTE/2020/12830 rev.2, the stability of the analytes in the final extracts is not required if the extracts contain an isotope-labelled internal standard (ILIS) for quantification, since the ILIS compensates for losses during extract storage. Since ILIS were used, the verification of the stability was not required. Anyway, all the extracts were analysed within 24 hours from their preparation, obtaining recoveries in the fortified samples within the acceptable range of 70-120%. |
| Stability of standard | The stability of the analytes in the stock standard solutions was verified in the study GLP-STUDY-21-108 “Validation of an analytical method for the quantification of Triazole Derivative Metabolites (TDMs) in wheat, barley, oilseed rape and processed commodities”. |

Table A 114: Characteristics for the analytical method used for validation method of triazole acetic acid residues in honey

|  | triazole acetic acid |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 2.  ***Product ion (m/z = 264):***  % interference mean = 27.2 |
| Calibration (type, number of data points) | ***Product ion (m/z = 70.0):***  Equation : Y = 0.95773 \* x + 0.00453  Coefficient of correlation: r² = 99.996 |
| Calibration range | Accepted calibration range in concentration units 0.50 – 50. µg/L  Corresponding calibration range in mass ratio units for the sample (0.0020 – 0.200 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 2.5 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.5 µg/L  (0.002 mg/kg)  35.6 S/N at LOD level |
| Stability | According to SANTE/2020/12830 rev.2, the stability of the analytes in the final extracts is not required if the extracts contain an isotope-labelled internal standard (ILIS) for quantification, since the ILIS compensates for losses during extract storage. Since ILIS were used, the verification of the stability was not required. Anyway, all the extracts were analysed within 24 hours from their preparation, obtaining recoveries in the fortified samples within the acceptable range of 70-120%. |
| Stability of standard | The stability of the analytes in the stock standard solutions was verified in the study GLP-STUDY-21-108 “Validation of an analytical method for the quantification of Triazole Derivative Metabolites (TDMs) in wheat, barley, oilseed rape and processed commodities”. |

Conclusion

The analytical method for the quantification of triazole derivatives metabolites residues in honey was successfully validated according to Guidance Document SANTE/2020/12830 rev. 2 by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability for triazole derivatives metabolites.

Confirmatory method

|  |  |
| --- | --- |
| Comments of zRMS: | The validation of the analytical method has been accepted above.  TDMs determination was conducted by LC-MS/MS (MRM mode), monitoring two mass transitions. The limit of quantification (LOQ) was set to 0.01 mg/kg. The validation parameters were in the required range. |

|  |  |
| --- | --- |
| Reference: | KCP 5.3.2.8/02 |
| Report | Validation of an analytical method for the quantification of Triazole Derivatives Metabolites (TDMs) in honey  Report No.: LBN-0093-2023  Longhi, D. 2023b  LabAnalysis s.r.l., Casanova Lonati (PV) - Italy |
| Guideline(s): | Yes  SANTE/2020/12830 rev. 2: Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes. |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

Materials and methods

|  |  |
| --- | --- |
| **Principle of the method** | The analytical method was based on the method “Quick Method for the Analysis of Highly Polar Pesticides in Food Involving Extraction with Acidified Methanol and LC- or IC-MS/MS Measurement - Food of Plant Origin (QuPPe-PO-Method) - Method 8 (M8)”.  The analytical method was based on an extraction with a mixture of water/methanol with 1% of formic acid and on an instrumental determination using a HPLC-DMS-MS/MS (high-performance liquid chromatography + Differential ion mobility-triple-quadrupole mass spectrometry).  The limit of quantification (LOQ) was set to 0.01 mg/kg.  The analytical method was validated under GLP compliance according to SANTE/2020/12830 Rev.2. |

**Materials:**

|  |  |
| --- | --- |
| HPLC-MS/MS | Shimadzu LC-40 XR + spectrometer Sciex API 6500+ equipped with SelexION (Differential Mobility Separation) device |
| Column: | Thermo Hypercarb 5 μm, 2.1 x 100 mm |
| Column temperature: | 40°C |
| Flow: | 0.6 mL/min |
| Injection volume: | 2 µL |
| Mobile phase:  Elution: | A = LC-MS grade water with 1% acetic acid  B = LC-MS grade methanol with 1% acetic acid   |  |  |  | | --- | --- | --- | | Time (min) | % A | % B | | 0 | 100 | 0 | | 6.00 | 10 | 90 | | 7.00 | 10 | 90 | | 7.10 | 100 | 0 | |
| Stop time: | 10 min |
| Source type: | ESI |
| Curtain gas flow: | 30 mL/min |
| Gas temperature: | 500°C |
| Gas 1: | 55 mL/min |
| Gas 2: | 65 mM/min |
| Capillary: | Positive mode 3500V |
| Acquiring mode: | ESI positive and ESI negative, MRM (multi-reaction monitoring) |

|  |  |
| --- | --- |
| Analytical standards: | 1,2,4-Triazole (1,2,4-T or TRZ)  CAS No.: 288-88-0  Batch No. : STBK4702  Purity : 100.0 %  Expiry date: October 1, 2023 |
|  | Triazole alanine (TA)  CAS No.: 86362-20-1  Batch No. : 787796  Purity : 98.3%  Expiry date: March 1, 2024 |
|  | 1,2,4-Triazole lactic acid (TLA) HCl  CAS No.: 1450828-63-3  Batch No. : 792058  Purity : 78.5%  Expiry date: November 1, 2024 |
|  | Triazole acetic acid (TAA)  CAS No.: 28711-29-7  Batch No. : 711657210-1-1  Purity : 97%  Expiry date: November 9, 2026 |
| Isotope-labelled  internal standard (ILIS) | 1,2,4-Triazole-[13C2, 15N3]  CAS No.: 1261170-82-4  Batch No. : SL6-2012-224  Purity : 98.4%  Expiry date: July 2024 |
|  | Triazole alanine [1,2,4-D2]  CAS No.: 2180306-38-9  Batch No. : 239DCDA135  Actual concentration: 999.68 ± 11.23 uncertainty  Expiry date: August 14, 2024 |
|  | Triazole-[13C2, 15N3] lactic acid  CAS No.: n.d.  Batch No. : EFL6-2015-198A  Purity : 98.42%  Expiry date: July 2024 |
|  | Triazole acetic acid [13C2, 15N3]  CAS No.: n.d.  Batch No. : EFL6-2015-196A  Purity : 98.03%  Expiry date: July 2024 |
| Reagents: | Water, LC-MS grade  Cysteine HCl  Formic acid  Acetonitrile  Unbuffered QuEChERS Extraction Salt Packet  Ammonium formate  Methanol |
| Buffer,  reference standard: | Buffer, pH 4.00 ± 0.01 (25°C)  Batch No.: MKCR2269  Product No.: B5020  Certified value (pH); 4.01  Expiry date: January, 2024  Buffer, pH 7.00 ± 0.01 (25°C)  Batch No.: MKCR0856  Product No.: B4770  Certified value (pH); 7.00  Expiry date: December, 2023 |
| Matrix: | Honey (multiflower origin) purchased in a local market Esselunga, Broni (PV), Italy  Storage: frozen  Measured pH-value: 4.2 according to method CIPC MT 75.3 |

**Methods:**

The confirmatory method for the quantification of triazole derivatives metabolites in the honey are presented below.

***2.A. Schematic diagram of the analytical method***

***Une image contenant texte, capture d’écran, Police, ligne

Description générée automatiquement***

***2.B. Quantification of triazole derivatives metabolites in honey***

The confirmatory methods for the determination of triazole derivatives metabolites were confirmed so as to comply with the Guidance Document SANTE/2020/12830 rev.2. The methods were confirmed in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A 115: Recovery results from confirmatory method of triazole derivative metabolite using the analytical method

| Matrix | Analyte | Fortification level (mg/kg) (n = 5) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Honey | 1,2,4-triazole  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 101 | 3.1 | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 102 | 3.3 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole alanine  (product ion: 88.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 103 | 4.7 | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 98.1 | 3.1 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole lactic acid  (product ion: 43.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 107 | 4.8 | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 103 | 2.3 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole acetic acid  (product ion: 43.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 116 | 4.4 | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 98.7 | 2.4 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

Table A 116: Characteristics for the analytical method used for confirmatory method of 1,2,4-triazole residues in honey

|  | 1,2,4-triazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 2.  ***Product ion (m/z = 70.0):***  % interference mean = 3.2 |
| Calibration (type, number of data points) | ***Product ion (m/z = 70.0):***  Equation : Y = 6.82752 \* x + 0.06506  Coefficient of correlation: r² = 99.967 |
| Calibration range | Accepted calibration range in concentration units 0.50 – 50. µg/L  Corresponding calibration range in mass ratio units for the sample (0.0020 – 0.200 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = + 2.4 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.5 µg/L  (0.002 mg/kg)  11.9 S/N at LOD level |

Table A 117: Characteristics for the analytical method used for confirmatory method of triazole alanine residues in honey

|  | triazole alanine |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 2.  ***Product ion (m/z = 88.0):***  % interference mean = 27.2 |
| Calibration (type, number of data points) | ***Product ion (m/z = 88.0):***  Equation : Y = 1.13230 \* x – 0.00656  Coefficient of correlation: r² = 99.954 |
| Calibration range | Accepted calibration range in concentration units 0.50 – 50. µg/L  Corresponding calibration range in mass ratio units for the sample (0.0020 – 0.200 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 7.2 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.5 µg/L  (0.002 mg/kg)  33.6 S/N at LOD level |

Table A 118: Characteristics for the analytical method used for confirmatory method of triazole lactic acid residues in honey

|  | triazole lactic acid |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 2.  ***Product ion (m/z = 43.0):***  % interference mean = 4.4 |
| Calibration (type, number of data points) | ***Product ion (m/z = 43.0):***  Equation : Y = 0.12362 \* x + 3.1519e-4  Coefficient of correlation: r² = 99.991 |
| Calibration range | Accepted calibration range in concentration units 0.50 – 50. µg/L  Corresponding calibration range in mass ratio units for the sample (0.0020 – 0.200 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = + 2.1 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.5 µg/L  (0.002 mg/kg)  8.0 S/N at LOD level |

Table A 119: Characteristics for the analytical method used for confirmatory method of triazole acetic acid residues in honey

|  | triazole acetic acid |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 2.  ***Product ion (m/z = 43.0):***  % interference mean = 22.7 |
| Calibration (type, number of data points) | ***Product ion (m/z = 43.0):***  Equation : Y = 0.04068 \* x – 3.75358e-4  Coefficient of correlation: r² = 99.976 |
| Calibration range | Accepted calibration range in concentration units 0.50 – 50. µg/L  Corresponding calibration range in mass ratio units for the sample (0.0020 – 0.200 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 2.5 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.5 µg/L  (0.002 mg/kg)  7.3 S/N at LOD level |

Conclusion

The confirmatory method for the quantification of triazole derivatives metabolites residues in honey was successfully validated according to Guidance Document SANTE/2020/12830 rev. 2 by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification for triazole derivatives metabolites.

Independent laboratory validation

|  |  |
| --- | --- |
| Comments of zRMS: | The ILV of the analytical method developed and validated in GLP studies Code LBN-0093-2023 has been accepted.  TDMs determination was conducted by LC-MS/MS in MRM mode, monitoring two MS/MS ion mass transitions. The validation parameters were in the required range. |

|  |  |
| --- | --- |
| Reference: | KCP 5.3.2.8/03 |
| Report | Independent Laboratory Validation (ILV) of the analytical Method for the Determination of Difenoconazole, Prothioconazole, Prothioconazole-desthio and Triazole Derivatives Metabolites (TDMs) in Honey  Report No.: CH-0859-2023  Mattioli, B. 2023  ChemService S.r.l. Controlli e Ricerche, Novate Milanese (MI) - Italy |
| Guideline(s): | Yes  SANTE/2020/12830 rev. 2: Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes. |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

Materials and methods

|  |  |
| --- | --- |
| **Aim of the method** | The aim of this study was to perform an Independent Laboratory Validation (ILV) of the analytical method developed and validated in GLP studies Code LBN-0093-2023 (Determination of Triazole Derivative Metabolites in Honey) performed by LabAnalysis s.r.l.  The Test Facility ChemService Srl Controlli e Ricerche had re-validate the section of “Recovery and Repeatability” and of “Selectivity and Specificity” of the analytical method already adjusted and validated by LabAnalysis in GLP studies Code LBN-0093-2023.  The analytical method was based on an instrumental determination using a HPLC-MS/MS (high-performance liquid chromatography + triple-quadrupole mass spectrometry).  The limit of quantification (LOQ) was set to 0.01 mg/kg.  The analytical method was validated under GLP compliance according to SANTE/2020/12830 Rev.2. |

**Materials:**

|  |  |
| --- | --- |
| HPLC-MS/MS | Shimadzu LC-40 XR + spectrometer Sciex API 6500+ equipped with SelexION (Differential Mobility Separation) device |
| Column: | Thermo Hypercarb 5 μm, 2.1 x 100 mm |
| Column temperature: | 40°C |
| Eluent Flow: | 0.6 mL/min |
| Injection volume: | 2 µL |
| Solvent composition:  Elution: | A = Water with 1% acetic acid  B = Methanol with 1% acetic acid   |  |  |  | | --- | --- | --- | | Time (min) | % A | % B | | 0 | 100 | 0 | | 1 | 100 | 0 | | 6 | 10 | 90 | | 7. | 10 | 90 | | 7.1 | 100 | 0 | |
| Retention time: | 1,2,4-Triazole (TRZ) – About 0.90 minutes  Triazole alanine (TA) – About 1.05 minutes  Triazole lactic acid (TLA) – About 3.50 minutes  Triazole acetic acid (TAA) – About 3.80 minutes |
| Total analysis time: | 10 min |
| Source type: | ESI |
| Scant type: | MRM |
| Curtain gas flow: | 30 mL/min |
| Gas temperature: | 500°C |
| Gas 1 flow: | 55 mL/min |
| Gas 2 flow: | 65 mM/min |
| Capillary: | 3500V |

|  |  |
| --- | --- |
| Analytical standards: | 1,2,4-Triazole (1,2,4-T or TRZ)  CAS No.: 288-88-0  Batch No. : STBK9756  Purity : 99.7%  Expiry date: November 01, 2024 |
|  | Triazole alanine (TA)  CAS No.: 4819-36-7  Batch No. : 212NVN  Purity : 99.6%  Expiry date: November 19, 2027 |
|  | Triazole lactic acid (TLA) HCl  CAS No.: 2126162-19-2  Batch No. : 814842  Purity : 95.88% (77.82% calculated as triazole lactic acid)  Expiry date: December 01, 2027 |
|  | Triazole acetic acid (TAA)  CAS No.: 28711-29-7  Batch No. : BCCG3086  Purity : 95.8%  Expiry date: July 01, 2024 |
| Isotope-labelled  internal standard (ILIS) | 1,2,4-Triazole-[13C2, 15N3]  CAS No.: 1261170-82-4  Batch No. : SL6-2012-224  Purity : 98.4%  Expiry date: March 28, 2025 |
|  | Triazole alanine [1,2,4-D2]  CAS No.: 2180306-38-9  Batch No. : 239DCDA135  Purity: 99.0%  Expiry date: August 14, 2024 |
|  | Triazole-[13C2, 15N3] lactic acid  CAS No.: n.d.  Batch No. : EFL6-2015-198A  Purity : 98.42%  Expiry date: March 28, 2025 |
|  | Triazole acetic acid [13C2, 15N3]  CAS No.: n.d.  Batch No. : EFL6-2015-196A  Purity : 98.03%  Expiry date: March 28, 2025 |
| Reagents: | Water, LC-MS grade  Cysteine HCl  Formic acid  Acetonitrile, HPLC grade  Unbuffered QuEChERS Extraction Salt Packet  Ammonium formate  Methanol, LC-MS grade |
| Matrix: | Honey  Expiry date: September 13, 2024  Storage: frozen |

**Methods:**

The analytical method for the quantification of triazole derivatives metabolites in the honey are presented below.

***2.A. Quantification of triazole derivatives metabolites in honey***

The analytical methods for the determination of triazole derivatives metabolites were confirmed so as to comply with the Guidance Document SANTE/2020/12830 rev.2. The methods were confirmed in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A 123: Recovery results from independent validation method of triazole derivative metabolite using the analytical method

| Matrix | Analyte | Fortification level (mg/kg) (n = 5) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Honey | 1,2,4-triazole  (product ion: 43.1 m/z)  Primary detection | At low level :  0.01 mg/kg (LOQ) | 109.9 | 6.1 | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 101.4 | 2.8 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole alanine  (product ion: 70.0 m/z)  Primary detection | At low level :  0.01 mg/kg (LOQ) | 80.5 | 9.1 | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 85.8 | 4.7 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole lactic acid  (product ion: 70.0 m/z)  Primary detection | At low level :  0.01 mg/kg (LOQ) | 103.3 | 5.8 | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 102.9 | 2.5 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole acetic acid  (product ion: 70.0 m/z)  Primary detection | At low level :  0.01 mg/kg (LOQ) | 104.2 | 4.4 | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 97.6 | 1.4 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| 1,2,4-triazole  (product ion: 70 m/z)  Confirmatory detection | At low level :  0.01 mg/kg (LOQ) | 101.8 | 3.3 | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 101.5 | 2.0 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole alanine  (product ion: 70.0 m/z)  Confirmatory detection | At low level :  0.01 mg/kg (LOQ) | 83.8 | 9.0 | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 87.1 | 3.9 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole lactic acid  (product ion: 70.0 m/z)  Confirmatory detection | At low level :  0.01 mg/kg (LOQ) | 111.9 | 5.8 | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 112.1 | 2.6 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole acetic acid  (product ion: 73.0 m/z)  Confirmatory detection | At low level :  0.01 mg/kg (LOQ) | 98.1 | 7.1 | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 94.6 | 4.8 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

Table A 124: Characteristics for the analytical method used for independent validation method of 1,2,4-triazole residues in honey

|  | 1,2,4-triazole |
| --- | --- |
| Specificity | n.d. |
| Calibration (type, number of data points) | ***Product ion (m/z = 43.1) – primary detection:***  Equation : Y = 0.0622 \* x – 0.0077  Coefficient of correlation: r² = 99.994  ***Product ion (m/z = 70.0) – confirmatory detection:***  Equation : Y = 0.5586 \* x + 0.0687  Coefficient of correlation: r² = 99.999 |
| Calibration range | Accepted calibration range in concentration units 0.50 – 50. µg/L  Corresponding calibration range in mass ratio units for the sample (0.0020 – 0.200 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = + 2.4 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.5 µg/L  (0.002 mg/kg) |
| Stability of sample extract | Not required since Isotope-Labelled Internal Standards (ILIS) were used. |
| Stability of standard solution | No degradation higher than 10% was observed during this storage period |

Table A 125: Characteristics for the analytical method used for independent validation method of triazole alanine residues in honey

|  | triazole alanine |
| --- | --- |
| Specificity | n.d. |
| Calibration (type, number of data points) | ***Product ion (m/z = 70.0) – primary detection:***  Equation : Y = 1.3679 \* x + 0.7700  Coefficient of correlation: r² = 99.816  ***Product ion (m/z = 88.0) – confirmatory detection:***  Equation : Y = 0.7341 \* x + 0.3037  Coefficient of correlation: r² = 99.926 |
| Calibration range | Accepted calibration range in concentration units 0.50 – 50. µg/L  Corresponding calibration range in mass ratio units for the sample (0.0020 – 0.200 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 7.2 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.5 µg/L  (0.002 mg/kg) |
| Stability of sample extract | Not required since Isotope-Labelled Internal Standards (ILIS) were used. |
| Stability of standard solution | No degradation higher than 10% was observed during this storage period |

Table A 126: Characteristics for the analytical method used for independent validation method of triazole lactic acid residues in honey

|  | triazole lactic acid |
| --- | --- |
| Specificity | n.d. |
| Calibration (type, number of data points) | ***Product ion (m/z = 70.0) – primary detection:***  Equation : Y = 0.0171 \* x + 0.0009  Coefficient of correlation: r² = 99.991  ***Product ion (m/z = 43.0) – confirmatory detection:***  Equation : Y = 0.0021 \* x + 0.0011  Coefficient of correlation: r² = 99.869 |
| Calibration range | Accepted calibration range in concentration units 0.50 – 50. µg/L  Corresponding calibration range in mass ratio units for the sample (0.0020 – 0.200 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = + 2.1 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.5 µg/L  (0.002 mg/kg) |
| Stability of sample extract | Not required since Isotope-Labelled Internal Standards (ILIS) were used. |
| Stability of standard solution | No degradation higher than 10% was observed during this storage period |

Table A 127: Characteristics for the analytical method used for independent validation method of triazole acetic acid residues in honey

|  | triazole acetic acid |
| --- | --- |
| Specificity | n.d. |
| Calibration (type, number of data points) | ***Product ion (m/z = 70.0) – primary detection:***  Equation : Y = 0.1130 \* x – 0.0223  Coefficient of correlation: r² = 99.989  ***Product ion (m/z = 73.0) – confirmatory detection:***  Equation : Y = 0.0049 \* x – 0.0021  Coefficient of correlation: r² = 99.968 |
| Calibration range | Accepted calibration range in concentration units 0.50 – 50. µg/L  Corresponding calibration range in mass ratio units for the sample (0.0020 – 0.200 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 2.5 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.5 µg/L  (0.002 mg/kg) |
| Stability of sample extract | Not required since Isotope-Labelled Internal Standards (ILIS) were used. |
| Stability of standard solution | No degradation higher than 10% was observed during this storage period |

Conclusion

The independent analytical method for the quantification of triazole derivatives metabolites residues in honey was successfully validated according to Guidance Document SANTE/2020/12830 rev. 2 by definition of the accuracy, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability for triazoles derivatives metabolites.

* + - * 1. Analytical method 22

|  |  |
| --- | --- |
| Comments of zRMS: | The validation of the analytical method has been accepted.  Difenoconazole, prothioconazole, prothioconazole-desthio and the triazole-derivative metabolites (TDMs): triazole-alanine (TA), 1,2,4-triazole (1,2,4-T), triazole lactic acid (TLA), triazole acetic acid (TAA) in honey were determined by LC-MS/MS (MRM mode), monitoring two mass transitions.  Samples were analysed according to the analytical method AM1-LBN-0092-2023 and AM-LBN-0093-2023 (see relevant validations).  The limits of quantification (LOQs) were set to 0.01 mg/kg. The validation parameters were in the required range. |

|  |  |
| --- | --- |
| Reference: | KCP 5.3.2.8/04 |
| Report | Analytical phase report – Magnitude of the residue of difenoconazole, prothioconazole, prothioconazole-desthio and triazole-derivative metabolites (TDMs) in honey after one application of IN233C1560 380 EC on Phacelia crop under semi-field conditions in four trials in Northern Europe and Southern Europe – 2023.  Multisite study 1111.4F.SAG23  Analytical Phase Report No.: LBN-0108-2023  Rovetto, I. 2023  LabAnalysis s.r.l., Casanova Lonati (PV) - Italy |
| Guideline(s): | Yes  SANTE/2020/12830 rev. 2: Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes. |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

Materials and methods

|  |  |
| --- | --- |
| **Principle of the method** | The analytical method to quantify Prothioconazole and Prothioconazole-desthio in honey was based on the QuEChERS method (EN 15662\_2018). The instrumental determination was carried out using a HPLC-MS/MS system (high-performance liquid chromatography + triple quadrupole mass spectrometry).  The analyses were carried out according to the following analytical methods validated under GLP compliance  according to SANTE/2020/12830 rev.2:  - Analytical method AM1-LBN-0092-2023 “Determination of Difenoconazole, Prothioconazole and  Prothioconazole-desthio in honey” validated under GLP compliance in the GLP study LBN-0092-2023  “Validation of an analytical method for the quantification of Difenoconazole, Prothioconazole and  Prothioconazole-desthio in honey”, Test Facility: LabAnalysis s.r.l., Study Director: Diego Longhi. |

**Materials:**

|  |  |
| --- | --- |
| HPLC-MS/MS | Agilent HPLC 1290 Infinity II + Agilent MS spectrometer 6470A Triple Quad |
| Column: | Phenomenex Kinetex C18, 1.7 μm, 2.1 x 50 mm |
| Column temperature: | 40°C |
| Flow: | 0.6 mL/min |
| Injection volume: | 2.5 µL |
| Mobile phase:  Elution: | A = LC-MS grade water with 0.2% formic acid and 5 mM ammonium formate  B = LC-MS grade methanol with 0.2% formic acid and 5 mM ammonium formate   |  |  |  | | --- | --- | --- | | Time (min) | % A | % B | | 0 | 70 | 30 | | 0.5 | 70 | 30 | | 3.0 | 0 | 100 | |
| Stop time: | 5 min |
| Post time: | 1 min |
| Divert value: | 0 min. to waste, 2 min to MS, 3.5 min. to waste |
| Source type: | ESI |
| Gas temperature: | 350°C |
| Gas flow: | 8 L/min |
| Nebulizer: | 40 psi |
| Sheath gas heater: | 400°C |
| Sheath gas flow: | 12 L/min |
| Capillary: | Positive mode 3500V  Negative mode 3000V |
| Vcharging: | 0 |
| Acquiring mode: | ESI positive and ESI negative, MRM (multi-reaction monitoring) |

|  |  |
| --- | --- |
| Analytical standards: | Prothioconazole  CAS No.: 179828-70-6  Batch No. : BCCB2271  Purity : 99.9% with 2.2% water (purity corrected for the water content: 97.7 %)  Expiry date: February, 2024 |
|  | Prothioconazole-desthio  CAS No.: 120983-64-4  Batch No. : BCCJ7867  Purity : 98.4% with 0.1% water (purity corrected for the water content: 98.3 %)  Expiry date: November, 2025 |
|  |  |
| Reagents: | Water, LC-MS grade  Cysteine HCl  Formic acid  Acetonitrile  Unbuffered QuEChERS Extraction Salt Packet  Ammonium formate  Methanol |
| Buffer,  reference standard: | Buffer, pH 4.00 ± 0.01 (25°C)  Batch No.: MKCR2269  Product No.: B5020  Certified value (pH); 4.01  Expiry date: January, 2024  Buffer, pH 7.00 ± 0.01 (25°C)  Batch No.: MKCR0856  Product No.: B4770  Certified value (pH); 7.00  Expiry date: December, 2023 |
| Matrix: | Honey (multiflower origin) purchased in a local market Esselunga, Broni (PV), Italy  Storage: frozen  Measured pH-value: 4.2 according to method CIPC MT 75.3 |

**Methods:**

The analytical method for the quantification of prothioconazole and prothioconazole-desthio in the honey are presented below.

***2.A. Schematic diagram of the analytical method***

***Une image contenant texte, capture d’écran, Police, ligne

Description générée automatiquement***

***2.B. Quantification of prothioconazole and prothioconazole-desthio in honey***

The analytical methods for the determination of prothioconazole and prothioconazole-desthio were confirmed so as to comply with the Guidance Document SANTE/2020/12830 rev.2. The methods were confirmed in terms of linearity, accuracy and limits of quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |

Results and discussions

Table A 120: Recovery results from analytical phase of prothioconazole and prothioconazole-desthio using the analytical method for honey residue trials

| Matrix | Analyte | Fortification level (mg/kg) (n = 5) | Mean  recovery (%) | Comments |
| --- | --- | --- | --- | --- |
| Honey  CDS-23-1469 | Prothioconazole  (product ion: 100 m/z) | At low level :  0.01 mg/kg (LOQ) | 79.3 | Recoveries range of 60 – 120 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 70.2 | Recoveries range of 70 – 120 % |
| Prothioconazole-desthio  (product ion: 125 m/z) | At low level :  0.01 mg/kg (LOQ) | 96.7 | Recoveries range of 60 – 120 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 109 | Recoveries range of 70 – 120 % |
| Honey  CDS-23-1565 | Prothioconazole  (product ion: 100 m/z) | At low level :  0.01 mg/kg (LOQ) | 97.8 | Recoveries range of 60 – 120 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 108 | Recoveries range of 70 – 120 % |
| Prothioconazole-desthio  (product ion: 125 m/z) | At low level :  0.01 mg/kg (LOQ) | 95.9 | Recoveries range of 60 – 120 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 101 | Recoveries range of 70 – 120 % |

Table A 121: Characteristics for the analytical method used for analytical phase of prothioconazole in honey residue trials

|  | prothioconazole |
| --- | --- |
| Calibration (type, number of data points) | ***Product ion (m/z = 100):***  Equation : Y = 18.433477 \* x – 1.901495  Coefficient of correlation: r² = 99.826763 |
| Calibration range | Accepted calibration range in concentration units 0.50 – 50. µg/L  Corresponding calibration range in mass ratio units for the sample (0.0020 – 0.200 mg/kg) |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Extraction | Samples were extracted and analysed within 30 days from the sampling.  All the extracts were analysed within 24 hours from their preparation, keeping them at a temperature of 5 ± 3°C until the analysis. |

Table A 121: Characteristics for the analytical method used for analytical phase of prothioconazole-desthio in honey residue trials

|  | prothioconazole |
| --- | --- |
| Calibration (type, number of data points) | ***Product ion (m/z = 125):***  Equation : Y = 517.190437 \* x + 274.256963  Coefficient of correlation: r² = 99.971858 |
| Calibration range | Accepted calibration range in concentration units 0.50 – 50. µg/L  Corresponding calibration range in mass ratio units for the sample (0.0020 – 0.200 mg/kg) |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Extraction | Samples were extracted and analysed within 30 days from the sampling.  All the extracts were analysed within 24 hours from their preparation, keeping them at a temperature of 5 ± 3°C until the analysis. |

Conclusion

The analytical phase according to the analytical validation method for the quantification of prothioconazole and prothioconazole-desthio in honey residue study was successfully validated according to Guidance Document SANTE/2020/12830 rev. 2 by definition of the calibration (linearity), limits of quantification and extraction for prothioconazole and prothioconazole-desthio.

* + - * 1. Analytical method 23

|  |  |
| --- | --- |
| Comments of zRMS: | The validation of the analytical methods has been accepted.  Difenoconazole, prothioconazole, prothioconazole-desthio and the triazole-derivative metabolites (TDMs): triazole-alanine (TA), 1,2,4-triazole (1,2,4-T), triazole lactic acid (TLA), triazole acetic acid (TAA) in honey were determined by LC-MS/MS (MRM mode), monitoring two mass transitions.  Samples were analysed according to the analytical method AM1-LBN-0092-2023 and AM-LBN-0093-2023 (see relevant validations).  The limits of quantification (LOQs) were set to 0.01 mg/kg. The validation parameters were in the required range. |

|  |  |
| --- | --- |
| Reference: | KCP 5.3.2.8/04 |
| Report | Analytical phase report – Magnitude of the residue of difenoconazole, prothioconazole, prothioconazole-desthio and triazole-derivative metabolites (TDMs) in honey after one application of IN233C1560 380 EC on Phacelia crop under semi-field conditions in four trials in Northern Europe and Southern Europe – 2023.  Multisite study 1111.4F.SAG23  Report No.: LBN-0108-2023  Rovetto, I. 2023  LabAnalysis s.r.l., Casanova Lonati (PV) - Italy |
| Guideline(s): | Yes  SANTE/2020/12830 rev. 2: Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes. |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

Materials and methods

|  |  |
| --- | --- |
| **Principle of the method** | The analytical method was based on the method “Quick Method for the Analysis of Highly Polar Pesticides in Food Involving Extraction with Acidified Methanol and LC- or IC-MS/MS Measurement - Food of Plant Origin (QuPPe-PO-Method) - Method 8 (M8)”. The instrumental determination was carried out using a HPLC-MS/MS system (high-performance liquid chromatography + triple quadrupole mass spectrometry) equipped with a differential mobility separation device (DMS).  The analyses were carried out according to the following analytical methods validated under GLP compliance  according to SANTE/2020/12830 rev.2:  Analytical method AM-LBN-0093-2023 “Determination of Triazole Derivative Metabolites (TDMs) in  honey”, validated under GLP compliance in the study LBN-0093-2023 “Validation of an analytical  method for the quantification of Triazole Derivative Metabolites (TDMs) in honey” (Test Facility:  LabAnalysis s.r.l., Study Director: Diego Longhi). |

**~~Materials:~~**

**Materials:**

|  |  |
| --- | --- |
| HPLC-MS/MS | Shimadzu LC-40 XR + spectrometer Sciex API 6500+ equipped with SelexION (Differential Mobility Separation) device |
| Column: | Thermo Hypercarb 5 μm, 2.1 x 100 mm |
| Column temperature: | 40°C |
| Flow: | 0.6 mL/min |
| Injection volume: | 2 µL |
| Mobile phase:  Elution: | A = LC-MS grade water with 1% acetic acid  B = LC-MS grade methanol with 1% acetic acid   |  |  |  | | --- | --- | --- | | Time (min) | % A | % B | | 0 | 100 | 0 | | 6.00 | 10 | 90 | | 7.00 | 10 | 90 | | 7.10 | 100 | 0 | |
| Stop time: | 10 min |
| Source type: | ESI |
| Curtain gas flow: | 30 mL/min |
| Gas temperature: | 500°C |
| Gas 1: | 55 mL/min |
| Gas 2: | 65 mM/min |
| Capillary: | Positive mode 3500V |
| Acquiring mode: | ESI positive and ESI negative, MRM (multi-reaction monitoring) |
| Analytical standards: | 1,2,4-Triazole (1,2,4-T or TRZ)  CAS No.: 288-88-0  Batch No. : STBK4702  Purity : 100.0 %  Expiry date: October 1, 2023 |
|  | Triazole alanine (TA)  CAS No.: 86362-20-1  Batch No. : 787796  Purity : 98.3%  Expiry date: March 1, 2024 |
|  | 1,2,4-Triazole lactic acid (TLA) HCl  CAS No.: 1450828-63-3  Batch No. : 792058  Purity : 78.5%  Expiry date: November 1, 2024 |
|  | Triazole acetic acid (TAA)  CAS No.: 28711-29-7  Batch No. : 711657210-1-1  Purity : 97%  Expiry date: November 9, 2026 |
| Isotope-labelled  internal standard (ILIS) | 1,2,4-Triazole-[13C2, 15N3]  CAS No.: 1261170-82-4  Batch No. : SL6-2012-224  Purity : 98.4%  Expiry date: July 2024 |
|  | Triazole alanine [1,2,4-D2]  CAS No.: 2180306-38-9  Batch No. : 239DCDA135  Actual concentration: 999.68 ± 11.23 uncertainty  Expiry date: August 14, 2024 |
|  | Triazole-[13C2, 15N3] lactic acid  CAS No.: n.d.  Batch No. : EFL6-2015-198A  Purity : 98.42%  Expiry date: July 2024 |
|  | Triazole acetic acid [13C2, 15N3]  CAS No.: n.d.  Batch No. : EFL6-2015-196A  Purity : 98.03%  Expiry date: July 2024 |
| Reagents: | Water, LC-MS grade  Cysteine HCl  Formic acid  Acetonitrile  Unbuffered QuEChERS Extraction Salt Packet  Ammonium formate  Methanol |
| Buffer,  reference standard: | Buffer, pH 4.00 ± 0.01 (25°C)  Batch No.: MKCR2269  Product No.: B5020  Certified value (pH); 4.01  Expiry date: January, 2024  Buffer, pH 7.00 ± 0.01 (25°C)  Batch No.: MKCR0856  Product No.: B4770  Certified value (pH); 7.00  Expiry date: December, 2023 |
| Matrix: | Honey (multiflower origin) purchased in a local market Esselunga, Broni (PV), Italy  Storage: frozen  Measured pH-value: 4.2 according to method CIPC MT 75.3 |

**Methods:**

The analytical method for the quantification of triazole derivatives metabolites in the honey are presented below.

***2.A. Schematic diagram of the analytical method***

***Une image contenant texte, capture d’écran, Police, ligne

Description générée automatiquement***

***2.B. Quantification of triazole derivatives metabolites in honey***

The analytical methods for the determination of triazole derivatives metabolites were confirmed so as to comply with the Guidance Document SANTE/2020/12830 rev.2. The methods were confirmed in terms of linearity, accuracy and limits of quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |

Results and discussions

Table A 123: Recovery results from analytical phase of triazole derivative metabolites using the analytical method for honey residue trials

| Matrix | Analyte | Fortification level (mg/kg) (n = 5) | Mean  recovery (%) | Comments |
| --- | --- | --- | --- | --- |
| Honey  CDS-23-1469 | 1,2,4-triazole  (product ion: 43.1 m/z) | At low level :  0.01 mg/kg (LOQ) | 93.7 | Recoveries range of 60 – 120 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 95.7 | Recoveries range of 70 – 120 % |
| Triazole alanine  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 98.8 | Recoveries range of 60 – 120 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 87.4 | Recoveries range of 70 – 120 % |
| Triazole lactic acid  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 94.4 | Recoveries range of 60 – 120 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 102 | Recoveries range of 70 – 120 % |
| Triazole acetic acid  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 96.1 | Recoveries range of 60 – 120 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 101 | Recoveries range of 70 – 120 % |
| Honey  CDS-23-1565 | 1,2,4-triazole  (product ion: 43.1 m/z) | At low level :  0.01 mg/kg (LOQ) | 80.9 | Recoveries range of 60 – 120 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 98.8 | Recoveries range of 70 – 120 % |
| Triazole alanine  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 77.2 | Recoveries range of 60 – 120 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 87.5 | Recoveries range of 70 – 120 % |
| Triazole lactic acid  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 78.8 | Recoveries range of 60 – 120 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 101 | Recoveries range of 70 – 120 % |
| Triazole acetic acid  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 103 | Recoveries range of 60 – 120 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 101 | Recoveries range of 70 – 120 % |

Table A 124: Characteristics for the analytical method used for analytical phase of 1,2,4-triazole in honey residue trials

|  | 1,2,4-triazole |
| --- | --- |
| Calibration (type, number of data points) | ***Product ion (m/z = 43.1):***  Equation : Y = 0.64316 \* x + 0.00558  Coefficient of correlation: r² = 99.924 |
| Calibration range | Accepted calibration range in concentration units 0.50 – 50. µg/L  Corresponding calibration range in mass ratio units for the sample (0.0020 – 0.200 mg/kg) |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Extraction | Samples were extracted and analysed within 30 days from the sampling.  All the extracts were analysed within 24 hours from their preparation, keeping them at a temperature of 5 ± 3°C until the analysis. |

Table A 125: Characteristics for the Characteristics for the analytical method used for analytical phase of triazole alanine in honey residue trials

|  | triazole alanine |
| --- | --- |
| Calibration (type, number of data points) | ***Product ion (m/z = 70.0):***  Equation : Y = 14.22980\* x + 0.04992  Coefficient of correlation: r² = 99.753 |
| Calibration range | Accepted calibration range in concentration units 0.50 – 50. µg/L  Corresponding calibration range in mass ratio units for the sample (0.0020 – 0.200 mg/kg) |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Extraction | Samples were extracted and analysed within 30 days from the sampling.  All the extracts were analysed within 24 hours from their preparation, keeping them at a temperature of 5 ± 3°C until the analysis. |

Table A 126: Characteristics for the analytical method used for analytical phase of triazole lactic acid in honey residue trials

|  | triazole lactic acid |
| --- | --- |
| Calibration (type, number of data points) | ***Product ion (m/z = 70.0):***  Equation : Y = 1.06430 \* x + 0.00787  Coefficient of correlation: r² = 99.968 |
| Calibration range | Accepted calibration range in concentration units 0.50 – 50. µg/L  Corresponding calibration range in mass ratio units for the sample (0.0020 – 0.200 mg/kg) |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Extraction | Samples were extracted and analysed within 30 days from the sampling.  All the extracts were analysed within 24 hours from their preparation, keeping them at a temperature of 5 ± 3°C until the analysis. |

Table A 127: Characteristics for the Characteristics for the analytical method used for analytical phase of triazole acetic acid in honey residue trials

|  | triazole acetic acid |
| --- | --- |
| Calibration (type, number of data points) | ***Product ion (m/z = 70.0):***  Equation : Y = 1.06430 \* x + 0.00787  Coefficient of correlation: r² = 99.968 |
| Calibration range | Accepted calibration range in concentration units 0.50 – 50. µg/L  Corresponding calibration range in mass ratio units for the sample (0.0020 – 0.200 mg/kg) |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Extraction | Samples were extracted and analysed within 30 days from the sampling.  All the extracts were analysed within 24 hours from their preparation, keeping them at a temperature of 5 ± 3°C until the analysis. |

Conclusion

The analytical phase according to the analytical validation method for the quantification of triazole derivative metabolites in honey residue study was successfully validated according to Guidance Document SANTE/2020/12830 rev. 2 by definition of the calibration (linearity), limits of quantification and extraction for triazole derivative metabolites.

* + 1. Methods for post-authorization control and monitoring purposes (KCP 5.2)

No new or additional studies have been submitted.

* 1. Analytical methods for difenoconazole
     1. Methods used for the generation of pre-authorization data (KCP 5.1)
        + 1. Analytical method 1

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  In method HPLC-MS/MS detection was used.  The specificity of the method was assured by MS/MS detection. All validation parameters are in the currently required range.  The method is suitable for the intended purposes. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/01 |
| Report | Validation of the Analytical Method for the Determination of Difenoconazole and Prothioconazole residues in soil samples of Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560 coming from the Ecotoxicological tests  Garagna, D.  2021a  ChemService S.r.l. Controlli e Richerche, Novate Milanese - Italy  Report No. : CH – 0235/2021  + Amendment report No.1 to final report (Amdm1\_KCP 5.1.2/01) |
| Guideline(s): | Yes : SANTE/2020/12830, rev. 1 dated 24/02/21 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The determination of Difenoconazole residues is performed by HPLC using an external standard and MS triple quadrupole detector.  Transitions (MS/MS).  Its quantification is achieved using the calibration curve obtained by plotting standard solutions concentrations and the corresponding peak area. |

Materials and methods

***1. Materials***

***1.A. Determination of difenoconazole residues in soil samples***

The determination of difenoconazole residues in soil samples was assessed by HLPC/MS-QQQ.

|  |  |
| --- | --- |
| HPLC: | Shimadzu Technologies 8050 System |
| Column: | Zorbax Eclipse Plus C18, 3.5 µm, 100 x 4.6 mm i.d. |
| Detector | MS Triple quadrupole (Scan in MRM mode) |
| Flow rate | 0.7 mL/min |
| Volume of injection | 10 µL |
| Retention time | Approximately 10.6 minutes for difenoconazole |
| Total analysis time | 20 minutes |
| Mobile phase: | Eluent A : Water, HLPC grade  Eluent A : Demineralised water  Eluent A : Formic acid, high purity for mass spectroscopy  Eluent A : Ammonium formate, for HPLC  Eluent B : Acetonitrile, for HPLC grade |
| Mixture | |  |  |  | | --- | --- | --- | | % A | % B | Time (min) | | 45 | 55 | 12 | | 10 | 90 | 14 | | 45 | 55 | 16 | | 45 | 55 | 20 | |
| Analytical standards: | Difenoconazole PESTANAL ®  CAS No. : 119446-68-3  Batch No. : BCCD4900  Purity : 95.5 %  Expiry date: June 1, 2025 |

***2. Methods***

***2.A. Preparation of soil samples***

Soil samples were prepared in according to the guideline :

* OECD 222
* OECD 226
* OECD 232

Soil samples were extracted by solvent using the following extraction procedure :

1. Weigh in 50 mL plastic tube approximately 5 g of soil
2. Add 10 mL of Acetonitrile
3. Shake for 1 hour
4. Centrifugation at 5000 rpm for 5 minutes
5. Filter with PTFE 0.45 µm
6. If necessary, dilute with “soil extracted solvent”
7. Inject

***2.B. Determination of difenoconazole residues in soil samples***

The analytical methods for the determination of difenoconazole residues in soil samples were first validated so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were validated in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A~~7.1~~ 136: Recovery results from method validation of difenoconazole using the analytical method

| Matrix | Analyte | Fortification level  (n = x) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| -40.0 % | Difenoconazole | Low level:  27 µg/kg (mean found)  N = 5 | 105.3 | 2 % | No comments |
| -40.0 % | Difenoconazole | High level:  115.36 µg/kg (mean found)  N = 5 | 98.9 | 2 % | No comments |

Table A~~8~~ 137: Characteristics for the analytical method used for validation of difenoconazole residues for purposes of soil ecotoxicological tests

|  | Difenoconazole |
| --- | --- |
| Specificity | Untreated blank < 30 % LOQ  Result : 2.8 %  This % ratio (Blank vs LOQ) demontrates that there was no interference of the blank matrix at the retention time of the analyte. |
| Linearity / Calibration | ***Quantifier transition***  ***m/z 406.1 🡪 m/z 250.9 :***  The calibration curve (µg/kg):   * was considered as valid over 4.7 – 43.0 µg/kg.   Equation (µg/kg)  y = 79585 \* x – 23988  Correlation coefficient:  r² = 99.605  The calibration curve (µg/L):   * was considered as valid over 2.3 – 23.4 µg/L.   Equation (µg/L)  y = 144849 \* x + 13460  Correlation coefficient:  r² = 99.598  ***Quantifier 1 transition***  ***m/z 406.1 🡪 m/z 336.9 :***  The calibration curve (µg/kg):   * was considered as valid over 4.7 – 43.0 µg/kg.   Equation (µg/kg)  y = 286 \* x – 338  Correlation coefficient:  r² = 99.966  The calibration curve (µg/L):   * was considered as valid over 2.3 – 23.4 µg/L.   Equation (µg/L)  y = 520 \* x - 204  Correlation coefficient:  r² = 99.967  ***Quantifier 2 transition***  ***m/z 406.1 🡪 m/z 188 :***  The calibration curve (µg/kg):   * was considered as valid over 4.7 – 43.0 µg/kg.   Equation (µg/kg)  y = 274 \* x + 1001  Correlation coefficient:  r² = 99.319  The calibration curve (µg/L):   * was considered as valid over 2.3 – 23.4 µg/L.   Equation (µg/L)  y = 498 \* x + 1129  Correlation coefficient:  r² = 99.319 |
| Limit of determination (LOD) | LOD = 4.7 µg/kg  Lowest calibration level |
| Limit of quantification (LOQ) | LOQ = 27.5 µg/kg  Lowest fortified level |
| Stability of final extract (7 days) | % recovery = 87.0 %  (mean value of 5 replicates)  % RSD = 5 %  Range of recovery:  80.2 – 89.6 % |
| Stability of standard (3 days) | Difference = 8.3 % |

Conclusion

The analytical method for the determination of difenoconazole residues in soil samples was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, linearity, accuracy, precision, limits of determination and quantification and stability for difenoconazole residues.

* + - * 1. Analytical method 2

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  In method HPLC-MS/MS detection was used.  The specificity of the method was assured by MS/MS detection. All validation parameters are in the currently required range.  See also into the present section B7 where this validated method was employed to generation of the data in paragraphs of Appendix 2: A 2.1.3.1.1,2,3; A 2.1.5.2.1,2,3; A 2.2.3.1.1,2,3; A 2.2.5.2.1,2,3.  Independent laboratory validation ~~is ongoing and~~ is included in study plans submitted (CH-1083/2021, CH-1084/2021, CH-1082/2021, CH-1081/2021).  The studies have been submitted – relevant paragraph for evaluation details. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/02 |
| Report | Validation of an analytical method for the quantification of Difenoconazole and Prothioconazole-desthio in wheat, barley, oilseed rape and processed commodities  Longhi, D.  2021a  ChemService S.r.l. Controlli e Richerche, Novate Milanese - Italy  Report No. : 21-31 |
| Guideline(s): | Yes : SANTE/2020/12830 rev. 1 (dated 24/02/2021) ;  SANTE2017/10632 rev. 3 (dated 22/11/2017) ;  OECD ENV/JM/MONO(2007)17 ;  CEN EN 15662:2018 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The analytical method for the determination of Difenoconazole in the tested matrices (AM-GLP-STUDY-21-31) was based on the QuEChERS method (EN 15662\_2018). The instrumental determination was carried out using a HPLC-MS/MS system (high-performance liquid chromatography + triple quadrupole mass spectrometry). |

Materials and methods

***1. Materials***

***1.A. Quantification of difenoconazole in wheat, barley, oilseed rape and processed commodities***

The quantification of difenoconazole in wheat, barley, oilseed rape and processed commodities was assessed by HLPC/MS/MS.

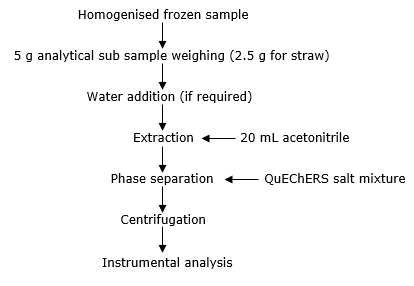
|  |  |
| --- | --- |
| HPLC: | Agilent 1290 Infinity II |
| Column: | Phenomenex Kinetix C18, 1.7 µm, 2.1 x 50 mm |
| Detector: | Agilent MS spectrometer 6470A Triple Quad |
| Column temperature: | 40 °C |
| Flow rate: | 0.6 mL/min |
| Volume of injection: | 2.5 µL |
| Retention time: | Approximatively 2.5 minutes for difenoconazole |
| Total analysis time: | 5 minutes for each run + 1 minute of post time \* 5 standard solutions |
| Divert valve: | 0 minute to waste  1.5 minutes to MS  3 minutes to waste |
| Gas temperature: | 350 °C |
| Gas flow: | 5 L/min |
| Nebulizer | 40 psi |
| Sheath gas heater: | 400 °C |
| Sheath gas flow | 12 L/min |
| Capillary: | Positive mode 3500 V  Negative mode 3000 V |
| Mobile phase: | Eluent A : Water, LC-MS grade  Eluent A : Ammonium formate  Eluent A : Formic acid  Eluent B : Methanol, LC-MS grade  Eluent B : Ammonium formate  Eluent B : Formic acid |
| Mixture | |  |  |  | | --- | --- | --- | | % A | % B | Time (min) | | 50 | 50 | 0 | | 50 | 50 | 0.5 | | 0 | 100 | 3 | |
| Analytical standards: | Difenoconazole  CAS No. : 119446-68-3  Batch No. : BCCD4900  Purity : 95.5 % (HPLC area %), with the 0.1 % of water (calculated purity of 95.4 % considering the water content |

***2. Methods***

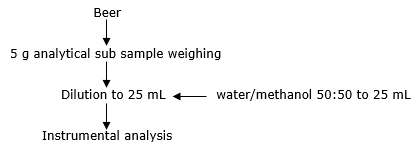
The analytical method for the quantification of difenoconazole in the tested matrices was based on the QuEChERS method (EN 15662-2018).

***2.A. Schematic diagram of the analytical method***

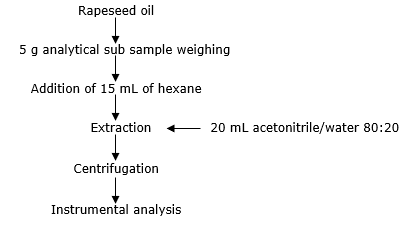
Plant matrices and processed commodities (whole plant, rapeseed seeds, wheat grain, white bread, straw)



Beer



Rapeseed oil



***2.B. Quantification of difenoconazole in wheat, barley, oilseed rape and processed commodities***

The analytical methods for the quantification of difenoconazole in wheat, barley, oilseed rape and processed commodities were first validated so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were validated in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |

Results and discussions

Method validation data can be summarised in tables below. ~~Table A.1and A2.1 to A2.7. There are for each matrix a primary test. In view of the similar results between the primary and confirmatory test, a test by an independent laboratory validation (ILV) is not required.~~

Table A~~1~~ 138: Recovery results from method validation of difenoconazole using the analytical method

| Matrix | Analyte | Fortification level  (*n* = x) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Whole Plant (Rapeseed) | Difenoconazole | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  87.3 % | ***Primary transition***:  3.1 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  87.4 % | ***Primary transition***:  6.5 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Rapeseed seeds | Difenoconazole | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  85.1 % | ***Primary transition***:  2.3 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  86.4 % | ***Primary transition***:  6.3 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Grain (wheat) | Difenoconazole | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  88.9 % | ***Primary transition***:  2.4 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  91.2 % | ***Primary transition***:  4.3 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Rapeseed oil | Difenoconazole | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  88.2 % | ***Primary transition***:  4.3 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  87.9 % | ***Primary transition***:  2.0 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| White bread (wheat) | Difenoconazole | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  100.1 % | ***Primary transition***:  7.7 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  99.5 % | ***Primary transition***:  0.54 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Beer (barley) | Difenoconazole | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  89.9 % | ***Primary transition***:  6.7 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  84.9 % | ***Primary transition***:  1.8 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Straw (wheat) | Difenoconazole | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  91.4 % | ***Primary transition***:  9.4 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  93.8 % | ***Primary transition***:  7.6 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

Table A~~2.1~~ 139: Characteristics for the analytical method used for validation of difenoconazole residues in whole plant (rapeseed)

|  | Difenoconazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Primarity transition:***  % interference mean = 7.5 |
| Calibration (type, number of data points) | ***Primarity transition:***  Equation : Y = 2393.089220 \* x + 525.166810  Coefficient of correlation: r² = 99.994148 |
| Calibration range | Accepted calibration range in concentration units 0.5 – 50.0 µg/L (from 20 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 1.6 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability (3 days) | Δ% = -5.5 |

Table A~~2.2~~ 140: Characteristics for the analytical method used for validation of difenoconazole residues in rapeseed seeds

|  | Difenoconazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Primarity transition:***  % interference mean = 9 |
| Calibration (type, number of data points) | ***Primarity transition:***  Equation : Y = 2133.411025 \* x + 406.088099  Coefficient of correlation: r² = 99.996840 |
| Calibration range | Accepted calibration range in concentration units 0.5 – 50.0 µg/L (from 20 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 5.1 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability (3 days) | Δ% = -2.5 |

Table A~~2.3~~ 141: Characteristics for the analytical method used for validation of difenoconazole residues in grain (wheat)

|  | Difenoconazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Primarity transition:***  % interference mean = 8 |
| Calibration (type, number of data points) | ***Primarity transition:***  Equation : Y = 2417.915699 \* x + 657.442734  Coefficient of correlation: r² = 99.884024 |
| Calibration range | Accepted calibration range in concentration units 0.5 – 50.0 µg/L (from 20 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = 3.9 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability (3 days) | Δ% = 3.0 |

Table A~~2.4~~ 142: Characteristics for the analytical method used for validation of difenoconazole residues in rapeseed oil

|  | Difenoconazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Primarity transition:***  % interference mean = 3.5 |
| Calibration (type, number of data points) | ***Primarity transition:***  Equation : Y = 2778.122729 \* x + 355.419442  Coefficient of correlation: r² = 99.991223 |
| Calibration range | Accepted calibration range in concentration units 0.5 – 50.0 µg/L (from 20 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 8.7 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability (3 days) | Δ% = 2.7 |

Table A~~2.5~~ 143: Characteristics for the analytical method used for validation of difenoconazole residues in white bread (wheat)

|  | Difenoconazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Primarity transition:***  % interference mean = 18 |
| Calibration (type, number of data points) | ***Primarity transition:***  Equation : Y = 2506.307430 \* x + 2064.880900  Coefficient of correlation: r² = 99.965376 |
| Calibration range | Accepted calibration range in concentration units 0.5 – 50.0 µg/L (from 20 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 9.8 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability (3 days) | Δ% = -5.2 |

Table A~~2.6~~ 144: Characteristics for the analytical method used for validation of difenoconazole residues in beer (barley)

|  | Difenoconazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Primarity transition:***  % interference mean = 21 |
| Calibration (type, number of data points) | ***Primarity transition:***  Equation : Y = 2736.071421 \* x + 1929.069427  Coefficient of correlation: r² = 99.955445 |
| Calibration range | Accepted calibration range in concentration units 0.5 – 50.0 µg/L (from 25 % of LOQ to 150 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.0025 – 0.250 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 1.7 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability (3 days) | Δ% = 0.8 |

Table A~~2.7~~ 145: Characteristics for the analytical method used for validation of difenoconazole residues in straw (wheat)

|  | Difenoconazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Primarity transition:***  % interference mean = 20.5 |
| Calibration (type, number of data points) | ***Primarity transition:***  Equation : Y = 3702.782310 \* x + 1318.496403  Coefficient of correlation: r² = 99.822494 |
| Calibration range | Accepted calibration range in concentration units 0.3 – 25.0 µg/L (from 24 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.0024 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 45.0 %  Significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.30 µg/L  (0.00240 mg/kg) |
| Stability (3 days) | Δ% = -8.4 |

Conclusion

The analytical method for the quantification of difenoconazole in wheat, barley, oilseed rape and processed commodities was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability for difenoconazole.

Independent laboratory validation

~~Not required.~~

~~The proposed ILV Rigamonti, E. 2022 / Report No. CH-1083/2021 is ongoing and will be submitted.~~

Determination of difenoconazole in straw (wheat)

|  |  |
| --- | --- |
| Comments of zRMS: | The submitted ILV of the analytical method adjusted and validated in GLP study Code GLP-STUDY-21-31 by LabAnalysis s.r.l. for the determination of Difenoconazole and Prothioconazole-desthio residues in Straw (wheat) has been accepted.  The analytical method, using the HPLC/MS/MS in MRM mode is highly specific and gave both quantification and identification data, so the confirmatory method is not necessary.  All recovery values for each analyte at both fortification levels (L.O.Q and 10 x LOQ) resulted to be in the range 70 to 120 %, with an RSD% lower than 20% therefore the analytical method can be considered suitable to quantify Difenoconazole and Prothioconazole-desthio in Straw (wheat) samples with an established L.O.Q of 0.010 mg/kg. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/16 |
| Report | Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of Difenoconazole and Prothio-desthio in Straw (wheat)  Report No.: CH-1081/2021  Nichetti, S. (2022a)  ChemService S.r.l Controlli e Ricerche - Novate Milanese (MI) - Italy |
| Guideline(s): | Yes  SANTE/2020/12830 rev. 1 dated 24/02/21: Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes. |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

Materials and methods

|  |  |
| --- | --- |
| **Principle of the method** | The Test Facility conducted an Independent Laboratory Validation (ILV) of the analytical method adjusted and validated in GLP study Code GLP-STUDY-21-31 by LabAnalysis s.r.l. for the determination of Difenoconazole residues in Straw (wheat). |

**Materials:**

|  |  |
| --- | --- |
| UHPLC/MS-QQQ: | Agilent mod. 1290, equipped with binary pump, autosampler coupled with an Agilent Jet Stream (AJS) ESI |
| Column: | Kinetex C18 100 Å, 1.7 μm, 50 x 2.1 mm i.d. |
| Detector: | MS Triple quadrupole |
| Ionisation type: | Electrospray (ESI) |
| Polarity: | Positive ion mode |
| Scan type: | MS/MS, multiple reaction monitoring |
| Drying gas temperature: | 350 °C |
| Drying gas flow: | 5 L/min |
| Nebulizer pressure: | 40 psi |
| Capillary voltage: | 3500 V |
|  |  |
| Analytical standards: | Difenoconazole  Batch No. : BCCD4900  Purity : 95.5 %  Expiry date: June 1, 2025 |
|  |  |
| Reagents: | Water, HPLC grade, obtained by the Lab Water Purification System  Methanol, HPLC grade  Acetonitrile, HPLC grade  Ammonium formate, high purity (>99%) for mass spectroscopy  Formic acid, high purity for mass spectroscopy |
|  |  |
| Matrix: | Straw (wheat)  Stored at -20°C in the dark |

**Methods:**

The analytical methods for the determination of difenoconazole in straw (wheat) were confirmed so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were confirmed in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A 146: Recovery results from independent laboratory validation of difenoconazole using the analytical method

| Matrix | Analyte | Fortification level (mg/kg) (n = 5) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Straw (wheat) | Difenoconazole  (product ion: 251.1 m/z) | At low level :  0.01 mg/kg (LOQ) | 91.3 | 9.12 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 96.8 | 3.70 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Difenoconazole  (product ion: 188.4 m/z) | At low level :  0.01 mg/kg (LOQ) | 93.9 | 10.22 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 98.2 | 6.04 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

Table A 147: Characteristics for the analytical method used for independent laboratory validation of difenoconazole residues in straw (wheat)

|  | Difenoconazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Product ion (m/z = 251.1):***  % interference mean = 0.0  ***Product ion (m/z = 188.4):***  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Product ion (m/z = 251.1):***  Equation : Y = 222 \* x + 224  Coefficient of correlation: r² = 99.675  ***Product ion (m/z = 188.4):***  Equation : Y = 18 \* x + 22  Coefficient of correlation: r² = 99.726 |
| Calibration range | Accepted calibration range in concentration units 0.29 – 23.99 µg/L  Corresponding calibration range in mass ratio units for the sample (0.0024 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = 33 %  Significant matrix effects for Difenoconazole residues in Straw (wheat) matrix were found (> ± 20%).  Therefore, the quantification should be performed using working standard solutions prepared in Straw (wheat) (matrix matched calibration). |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.30 µg/L  (0.0024 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-31 |

Conclusion

The independent laboratory validation for the quantification of difenoconazole in straw (wheat) commodities was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability.

Determination of Difenoconazole in grain (wheat)

|  |  |
| --- | --- |
| Comments of zRMS: | The submitted ILV of the analytical method adjusted and validated in GLP study Code GLP-STUDY-21-31 by LabAnalysis s.r.l. for the determination of Difenoconazole and Prothioconazole-desthio residues in Grain (wheat) has been accepted.  The analytical method, using the HPLC/MS/MS in MRM mode is highly specific and gave both quantification and identification data, so the confirmatory method is not necessary.  All recovery values for each analyte at both fortification levels (L.O.Q and 10 x LOQ) resulted to be in the range 70 to 120 %, with an RSD% lower than 20% therefore the analytical method can be considered suitable to quantify Difenoconazole and Prothioconazole-desthio in Grain (wheat) samples with an established L.O.Q of 0.010 mg/kg. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/17 |
| Report | Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of Difenoconazole and Prothio-desthio in Straw (wheat)  Report No.: CH-1082/2021  Nichetti, S. (2022b)  ChemService S.r.l Controlli e Ricerche - Novate Milanese (MI) - Italy |
| Guideline(s): | Yes  SANTE/2020/12830 rev. 1 dated 24/02/21: Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes. |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

Materials and methods

|  |  |
| --- | --- |
| **Principle of the method** | The Test Facility conducted an Independent Laboratory Validation (ILV) of the analytical method adjusted and validated in GLP study Code GLP-STUDY-21-31 by LabAnalysis s.r.l. for the determination of Difenoconazole residues in grain (wheat). |

**Materials:**

|  |  |
| --- | --- |
| UHPLC/MS-QQQ: | Agilent mod. 1290, equipped with binary pump, autosampler coupled with an Agilent Jet Stream (AJS) ESI |
| Column: | Kinetex C18 100 Å, 1.7 μm, 50 x 2.1 mm i.d. |
| Detector: | MS Triple quadrupole |
| Ionisation type: | Electrospray (ESI) |
| Polarity: | Positive ion mode |
| Scan type: | MS/MS, multiple reaction monitoring |
| Drying gas temperature: | 350 °C |
| Drying gas flow: | 5 L/min |
| Nebulizer pressure: | 40 psi |
| Capillary voltage: | 3500 V |
|  |  |
| Analytical standards: | Difenoconazole  Batch No. : BCCD4900  Purity : 95.5 %  Expiry date: June 1, 2025 |
|  |  |
| Reagents: | Water, HPLC grade, obtained by the Lab Water Purification System  Methanol, HPLC grade  Acetonitrile, HPLC grade  Ammonium formate, high purity (>99%) for mass spectroscopy  Formic acid, high purity for mass spectroscopy |
|  |  |
| Matrix: | Grain (wheat)  Stored at -20°C in the dark |

**Methods:**

The analytical methods for the determination of difenoconazole in grain (wheat) were confirmed so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were confirmed in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A 148: Recovery results from independent laboratory validation of difenoconazole using the analytical method

| Matrix | Analyte | Fortification level (mg/kg) (n = 5) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Grain (wheat) | Difenoconazole  (product ion: 251.1 m/z) | At low level :  0.01 mg/kg (LOQ) | 89.7 | 10.44 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 112.7 | 6.46 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Difenoconazole  (product ion: 188.4 m/z) | At low level :  0.01 mg/kg (LOQ) | 88.1 | 8.82 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 110.0 | 4.88 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

Table A 149: Characteristics for the analytical method used for independent laboratory validation of difenoconazole residues in grain (wheat)

|  | Difenoconazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Product ion (m/z = 251.1):***  % interference mean = 0.0  ***Product ion (m/z = 188.4):***  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Product ion (m/z = 251.1):***  Equation : Y = 253 \* x + 145  Coefficient of correlation: r² = 99.929  ***Product ion (m/z = 188.4):***  Equation : Y = 20 \* x + 22  Coefficient of correlation: r² = 99.686 |
| Calibration range | Accepted calibration range in concentration units 0.49 – 48.94 µg/L  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 3 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-31 |

Conclusion

The independent laboratory validation for the quantification of difenoconazole in grain (wheat) commodities was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability.

Determination of difenoconazole in rapeseed seeds

|  |  |
| --- | --- |
| Comments of zRMS: | The submitted ILV of the analytical method adjusted and validated in GLP study Code GLP-STUDY-21-31 by LabAnalysis s.r.l. for the determination of Difenoconazole and Prothioconazole-desthio residues in Rapeseed Seeds has been accepted.  The analytical method, using the HPLC/MS/MS in MRM mode is highly specific and gave both quantification and identification data, so the confirmatory method is not necessary.  All recovery values for each analyte at both fortification levels (L.O.Q and 10 x LOQ) resulted to be in the range 70 to 120 %, with an RSD% lower than 20% therefore the analytical method can be considered suitable to quantify Difenoconazole and Prothioconazole-desthio in Rapeseed Seeds samples with an established LOQ of 0.010 mg/kg. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/18 |
| Report | Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of Difenoconazole and Prothio-desthio in Rapeseed seeds  Report No.: CH-1083/2021  Nichetti, S. (2022c)  ChemService S.r.l Controlli e Ricerche - Novate Milanese (MI) - Italy |
| Guideline(s): | Yes  SANTE/2020/12830 rev. 1 dated 24/02/21: Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes. |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

Materials and methods

|  |  |
| --- | --- |
| **Principle of the method** | The Test Facility conducted an Independent Laboratory Validation (ILV) of the analytical method adjusted and validated in GLP study Code GLP-STUDY-21-31 by LabAnalysis s.r.l. for the determination of difenoconazole residues in rapeseed seeds. |

**Materials:**

|  |  |
| --- | --- |
| UHPLC/MS-QQQ: | Agilent mod. 1290, equipped with binary pump, autosampler coupled with an Agilent Jet Stream (AJS) ESI |
| Column: | Kinetex C18 100 Å, 1.7 μm, 50 x 2.1 mm i.d. |
| Detector: | MS Triple quadrupole |
| Ionisation type: | Electrospray (ESI) |
| Polarity: | Positive ion mode |
| Scan type: | MS/MS, multiple reaction monitoring |
| Drying gas temperature: | 350 °C |
| Drying gas flow: | 5 L/min |
| Nebulizer pressure: | 40 psi |
| Capillary voltage: | 3500 V |
|  |  |
| Analytical standards: | Difenoconazole  Batch No. : BCCD4900  Purity : 95.5 %  Expiry date: June 1, 2025 |
|  |  |
| Reagents: | Water, HPLC grade, obtained by the Lab Water Purification System  Methanol, HPLC grade  Acetonitrile, HPLC grade  Ammonium formate, high purity (>99%) for mass spectroscopy  Formic acid, high purity for mass spectroscopy |
|  |  |
| Matrix: | Rapeseed seeds  Stored at -20°C in the dark |

**Methods:**

The analytical methods for the determination of difenoconazole in rapeseed seeds were confirmed so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were confirmed in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A 150: Recovery results from independent laboratory validation of difenoconazole using the analytical method

| Matrix | Analyte | Fortification level (mg/kg) (n = 5) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Rapeseed seeds | Difenoconazole  (product ion: 251.1 m/z) | At low level :  0.01 mg/kg (LOQ) | 105.3 | 6.28 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 109.7 | 1.23 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Difenoconazole  (product ion: 188.4 m/z) | At low level :  0.01 mg/kg (LOQ) | 90.9 | 13.53 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 109.0 | 4.02 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

Table A 151: Characteristics for the analytical method used for independent laboratory validation of difenoconazole residues in rapeseed seeds

|  | Prothioconazole-desthio |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Product ion (m/z = 251.0):***  % interference mean = 0.0  ***Product ion (m/z = 188.4):***  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Product ion (m/z = 251.1):***  Equation : Y = 226 \* x + 221  Coefficient of correlation: r² = 99.922  ***Product ion (m/z = 188.4):***  Equation : Y = 18 \* x + 26  Coefficient of correlation: r² = 99.661 |
| Calibration range | Accepted calibration range in concentration units 0.49 – 48.94 µg/L  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 7 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-31 |

Conclusion

The independent laboratory validation for the quantification of difenoconazole in rapeseed seeds commodities was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability.

Determination of difenoconazole in Whole Plant (rapeseed)

|  |  |
| --- | --- |
| Comments of zRMS: | The submitted ILV of the analytical method adjusted and validated in GLP study Code GLP-STUDY-21-31 by LabAnalysis s.r.l. for the determination of Difenoconazole and Prothioconazole-desthio residues in Whole Plant (Rapeseed) has been accepted.  The analytical method, using the HPLC/MS/MS in MRM mode is highly specific and gave both quantification and identification data, so the confirmatory method is not necessary.  All recovery values for each analyte at both fortification levels (L.O.Q and 10 x LOQ) resulted to be in the range 70 to 120 %, with an RSD% lower than 20% therefore the analytical method can be considered suitable to quantify Difenoconazole and Prothioconazole-desthio in Whole Plant (Rapeseed) samples with an established LOQ of 0.010 mg/kg. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/19 |
| Report | Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of Difenoconazole and Prothio-desthio in Whole Plant (Rapeseed)  Report No.: CH-1084/2021  Nichetti, S. (2022d)  ChemService S.r.l Controlli e Ricerche - Novate Milanese (MI) - Italy |
| Guideline(s): | Yes  SANTE/2020/12830 rev. 1 dated 24/02/21: Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes. |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

Materials and methods

|  |  |
| --- | --- |
| **Principle of the method** | The Test Facility conducted an Independent Laboratory Validation (ILV) of the analytical method adjusted and validated in GLP study Code GLP-STUDY-21-31 by LabAnalysis s.r.l. for the determination of difenoconazole residues in whole plant (rapeseed). |

**Materials:**

|  |  |
| --- | --- |
| UHPLC/MS-QQQ: | Agilent mod. 1290, equipped with binary pump, autosampler coupled with an Agilent Jet Stream (AJS) ESI |
| Column: | Kinetex C18 100 Å, 1.7 μm, 50 x 2.1 mm i.d. |
| Detector: | MS Triple quadrupole |
| Ionisation type: | Electrospray (ESI) |
| Polarity: | Positive ion mode |
| Scan type: | MS/MS, multiple reaction monitoring |
| Drying gas temperature: | 350 °C |
| Drying gas flow: | 5 L/min |
| Nebulizer pressure: | 40 psi |
| Capillary voltage: | 3500 V |
|  |  |
| Analytical standards: | Difenoconazole  Batch No. : BCCD4900  Purity : 95.5 %  Expiry date: June 1, 2025 |
|  |  |
| Reagents: | Water, HPLC grade, obtained by the Lab Water Purification System  Methanol, HPLC grade  Acetonitrile, HPLC grade  Ammonium formate, high purity (>99%) for mass spectroscopy  Formic acid, high purity for mass spectroscopy |
|  |  |
| Matrix: | Whole plant (rapeseed)  Stored at -20°C in the dark |

**Methods:**

The analytical methods for the determination of difenoconazole in whole plant (rapeseed) were confirmed so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were confirmed in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A 152: Recovery results from independent laboratory validation of difenoconazole using the analytical method

| Matrix | Analyte | Fortification level (mg/kg) (n = 5) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Whole plant (Rapeseed) | Difenoconazole  (product ion: 251.1 m/z) | At low level :  0.01 mg/kg (LOQ) | 97.0 | 6.31 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 95.9 | 4.16 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Difenoconazole  (product ion: 188.4 m/z) | At low level :  0.01 mg/kg (LOQ) | 88.0 | 4.71 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 97.2 | 6.70 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

Table A 153: Characteristics for the analytical method used for independent laboratory validation of difenoconazole residues in whole plant (rapeseed)

|  | Difenoconazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Product ion (m/z = 251.1):***  % interference mean = 0.0  ***Product ion (m/z = 188.4):***  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Product ion (m/z = 251.1):***  Equation : Y = 267 \* x - 7  Coefficient of correlation: r² = 99.510  ***Product ion (m/z = 188.4):***  Equation : Y = 22 \* x - 10  Coefficient of correlation: r² = 99.314 |
| Calibration range | Accepted calibration range in concentration units 0.49 – 48.71 µg/L  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 5 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-31 |

Conclusion

The independent laboratory validation for the quantification of difenoconazole in whole plant (rapeseed) was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability.

Confirmatory method

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  In method HPLC-MS/MS detection was used. A simultaneous confirmation to the primary detection was used using the HPLC-MS/MS, monitoring additional SRM transitions.  The specificity of the method was assured by MS/MS detection. All validation parameters are in the currently required range.  See also into the present section B7 where this validated method was employed to generation of the data in paragraphs of Appendix 2: A 2.1.3.1.1,2,3; A 2.1.5.2.1,2,3; A 2.2.3.1.1,2,3; A 2.2.5.2.1,2,3. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/02 |
| Report | Validation of an analytical method for the quantification of Difenoconazole and Prothioconazole-desthio in wheat, barley, oilseed rape and processed commodities  Longhi, D.  2021a  ChemService S.r.l. Controlli e Richerche, Novate Milanese - Italy  Report No. : 21-31 |
| Guideline(s): | Yes : SANTE/2020/12830 rev. 1 (dated 24/02/2021) ;  SANTE2017/10632 rev. 3 (dated 22/11/2017) ;  OECD ENV/JM/MONO(2007)17 ;  CEN EN 15662:2018 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The confirmatory method for the determination of Difenoconazole in the tested matrices (AM-GLP-STUDY-21-31) was based on the QuEChERS method (EN 15662\_2018). The instrumental determination was carried out using a HPLC-MS/MS system (high-performance liquid chromatography + triple quadrupole mass spectrometry). |

Materials and methods

***1. Materials***

***1.A. Quantification of difenoconazole in wheat, barley, oilseed rape and processed commodities***

The quantification of difenoconazole in wheat, barley, oilseed rape and processed commodities was assessed by HLPC/MS/MS.

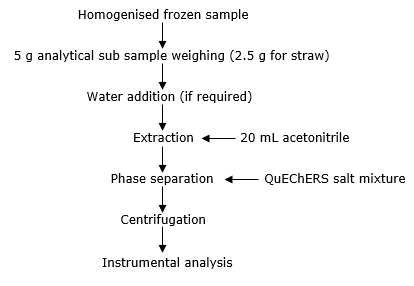
|  |  |
| --- | --- |
| HPLC: | Agilent 1290 Infinity II |
| Column: | Phenomenex Kinetix C18, 1.7 µm, 2.1 x 50 mm |
| Detector: | Agilent MS spectrometer 6470A Triple Quad |
| Column temperature: | 40 °C |
| Flow rate: | 0.6 mL/min |
| Volume of injection: | 2.5 µL |
| Retention time: | Approximatively 2.5 minutes for difenoconazole |
| Total analysis time: | 5 minutes for each run + 1 minute of post time \* 5 standard solutions |
| Divert valve: | 0 minute to waste  1.5 minutes to MS  3 minutes to waste |
| Gas temperature: | 350 °C |
| Gas flow: | 5 L/min |
| Nebulizer | 40 psi |
| Sheath gas heater: | 400 °C |
| Sheath gas flow | 12 L/min |
| Capillary: | Positive mode 3500 V  Negative mode 3000 V |
| Mobile phase: | Eluent A : Water, LC-MS grade  Eluent A : Ammonium formate  Eluent A : Formic acid  Eluent B : Methanol, LC-MS grade  Eluent B : Ammonium formate  Eluent B : Formic acid |
| Mixture | |  |  |  | | --- | --- | --- | | % A | % B | Time (min) | | 50 | 50 | 0 | | 50 | 50 | 0.5 | | 0 | 100 | 3 | |
| Analytical standards: | Difenoconazole  CAS No. : 119446-68-3  Batch No. : BCCD4900  Purity : 95.5 % (HPLC area %), with the 0.1 % of water (calculated purity of 95.4 % considering the water content |

***2. Methods***

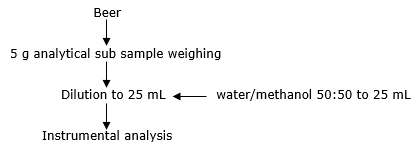
The confirmatory method for the quantification of difenoconazole in the tested matrices was based on the QuEChERS method (EN 15662-2018).

***2.A. Schematic diagram of the analytical method***

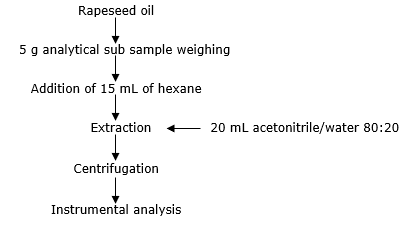
Plant matrices and processed commodities (whole plant, rapeseed seeds, wheat grain, white bread, straw)



Beer



Rapeseed oil



***2.B. Quantification of difenoconazole in wheat, barley, oilseed rape and processed commodities***

The confirmatory methods for the quantification of difenoconazole in wheat, barley, oilseed rape and processed commodities were first validated so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were validated in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |

Results and discussions

Confirmatory method validation data can be summarised in tables below ~~Table A.5 and A6.1 to A6.7~~. There are for each matrix a confirmatory test.

Table A~~5~~ 154: Recovery results from confirmatory method validation of difenoconazole using the confirmatory analytical method

| Matrix | Analyte | Fortification level  (*n* = x) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Whole Plant (Rapeseed) | Difenoconazole | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition:***  85.9 % | ***Confirmatory transition:***  4.0 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition:***  87.4 % | ***Confirmatory transition:***  7.0 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Rapeseed seeds | Difenoconazole | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition:***  86.9 % | ***Confirmatory transition:***  7.8 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition:***  86.9 % | ***Confirmatory transition:***  7.3 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Grain (wheat) | Difenoconazole | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition:***  91.4 % | ***Confirmatory transition:***  8.3 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition:***  92.5 % | ***Confirmatory transition:***  5.1 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Rapeseed oil | Difenoconazole | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition:***  90.2 % | ***Confirmatory transition:***  4.9 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition:***  87.4 % | ***Confirmatory transition:***  3.2 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| White bread (wheat) | Difenoconazole | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition:***  102.3 % | ***Confirmatory transition:***  10.2 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition:***  97.9 % | ***Confirmatory transition:***  1.8 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Beer (barley) | Difenoconazole | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition:***  85.9 % | ***Confirmatory transition:***  9.1 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition:***  85.8 % | ***Confirmatory transition:***  3.4 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Straw (wheat) | Difenoconazole | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition:***  92.8 % | ***Confirmatory transition:***  6.5 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition:***  91.2 % | ***Confirmatory transition:***  5.2 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

Table A~~6.1~~ 155: Characteristics for the confirmatory method used for validation of difenoconazole residues in whole plant (rapeseed)

|  | Difenoconazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Confirmatory transition:***  % interference mean = 8 |
| Calibration (type, number of data points) | ***Confirmatory transition:***  Equation : Y = 199.244579 \* x + 69.603090  Coefficient of correlation: r² = 99.994339 |
| Calibration range | Accepted calibration range in concentration units 0.5 – 50.0 µg/L (from 20 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 1.6 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability (3 days) | Δ% = -5.5 |

Table  ~~6.2~~ 156: Characteristics for the analytical method used for validation of difenoconazole residues in rapeseed seeds

|  | Difenoconazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Confirmatory transition:***  % interference mean = 7 |
| Calibration (type, number of data points) | ***Confirmatory transition:***  Equation : Y = 175.731294 \* x + 41.154394  Coefficient of correlation: r² = 99.970764 |
| Calibration range | Accepted calibration range in concentration units 0.5 – 50.0 µg/L (from 20 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 5.1 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability (3 days) | Δ% = -2.5 |

Table A~~6.3~~ 157: Characteristics for the confirmatory method used for validation of difenoconazole residues in grain (wheat)

|  | Difenoconazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Confirmatory transition:***  % interference mean = 8.5 |
| Calibration (type, number of data points) | ***Confirmatory transition:***  Equation : Y = 191.880245 \* x + 63.409123  Coefficient of correlation: r² = 99.933181 |
| Calibration range | Accepted calibration range in concentration units 0.5 – 50.0 µg/L (from 20 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = 3.9 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability (3 days) | Δ% = 3.0 |

Table A~~6.4~~ 158: Characteristics for the confirmatory method used for validation of difenoconazole residues in rapeseed oil

|  | Difenoconazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Confirmatory transition:***  % interference mean = 1.5 |
| Calibration (type, number of data points) | ***Confirmatory transition:***  Equation : Y = 218.069096 \* x + 19.282528  Coefficient of correlation: r² = 99.984122 |
| Calibration range | Accepted calibration range in concentration units 0.5 – 50.0 µg/L (from 20 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 8.7 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability (3 days) | Δ% = 2.7 |

Table A~~6.5~~ 159: Characteristics for the confirmatory method used for validation of difenoconazole residues in white bread (wheat)

|  | Difenoconazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Confirmatory transition:***  % interference mean = 16.5 |
| Calibration (type, number of data points) | ***Confirmatory transition:***  Equation : Y = 200.982247\* x + 149.603962  Coefficient of correlation: r² = 99.984647 |
| Calibration range | Accepted calibration range in concentration units 0.5 – 50.0 µg/L (from 20 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 9.8 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability (3 days) | Δ% = -5.2 |

Table A~~6.6~~ 160: Characteristics for the confirmatory method used for validation of difenoconazole residues in beer (barley)

|  | Difenoconazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Confirmatory transition:***  % interference mean = 19.5 |
| Calibration (type, number of data points) | ***Confirmatory transition:***  Equation : Y = 212.355822 \* x + 146.644900  Coefficient of correlation: r² = 99.985709 |
| Calibration range | Accepted calibration range in concentration units 0.5 – 50.0 µg/L (from 25 % of LOQ to 150 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.0025 – 0.250 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 1.7 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability (3 days) | Δ% = 0.8 |

Table A~~6.7~~ 161: Characteristics for the confirmatory method used for validation of difenoconazole residues in straw (wheat)

|  | Difenoconazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Confirmatory transition:***  % interference mean = 24 |
| Calibration (type, number of data points) | ***Confirmatory transition:***  Equation : Y = 282.749250 \* x + 90.678908  Coefficient of correlation: r² = 99.737378 |
| Calibration range | Accepted calibration range in concentration units 0.3 – 25.0 µg/L (from 24 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.0024 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 45.0 %  Significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.30 µg/L  (0.00240 mg/kg) |
| Stability (3 days) | Δ% = -8.4 |

Conclusion

The confirmatory method for the quantification of difenoconazole in wheat, barley, oilseed rape and processed commodities was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability for difenoconazole.

Extraction efficiency

Extraction efficiency is guided by:

* European Commission (2017): SANTE 2017/10632 rev. 3, dated 22 November 2017: Technical Guideline on the Evaluation of Extraction Efficiency of Residue Analytical Methods.
* “European Committee for Standardization (CEN) EN 15662:2018. Foods of plant origin - Multimethod for the determination of pesticide residues using GC- and LC-based analysis following acetonitrile extraction/partitioning and clean-up by dispersive SPE - Modular QuEChERS-method”.

Aliquots of 5 g of specimen (2.5 for straw) were taken from the homogenized frozen samples and put in a 50 mL screw capped centrifuge PE test tube followed by the addition of the following amounts of LC-MS grade water:

|  |  |
| --- | --- |
| **Matrix** | **Water added (mL)** |
| Whole Plant (rapeseed) | 0 |
| Rapeseed seeds | 10 |
| Wheat (grain) | 10 |
| Wheat (straw) | 10 |
| Wheat (white bread) | 10 |

Then, 20 mL of acetonitrile were added and the obtained mixture was vigorously shaken for one minute. After that, a packet of QuEChERS extraction salt (4.0 g MgSO4, 1.0 g NaCl, 1.0 g trisodium citrate dehydrate, 0.5 g disodium hydrogen citrate sesquihydrate) was added and the mixture shaken again. The separation of the organic phase was achieved by centrifugation at 4500 rpm for 5 minutes. An aliquot of about 1 mL the organic supernatant was taken, transferred in a 2 mL HPLC glass vial and analyzed with a HPLC-MS/MS system.

**Beer**

Beer was analyzed after a 5-fold dilution in a mixture of water/methanol 50:50 (about 5 g to 25 mL) and directly analyzed with a HPLC-MS/MS system.

**Rapeseed oil**

An aliquot of about 5 g of rapeseed oil was put in a 50 mL screw capped centrifuge PE test tube. Then, 15 mL of hexane were added, followed by 20 mL of a mixture of acetonitrile/water 80:20. The mixture was vigorously shaken for about one minute and then centrifuged at 4500 rpm for 5 minutes, in order to obtain 2 phases. An aliquot of the lower organic phase (acetonitrile) was taken and transferred to a 2 mL glass HPLC vial for final determination with a HPLC-MS/MS system.

* + - * 1. Analytical method 3

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  In method HPLC-MS/MS detection was used. Two transitions were monitored.  The specificity of the method was assured by MS/MS detection. All validation parameters are in the currently required range.  The method is suitable for the intended purposes. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/03 |
| Report | Validation of the Analytical Method for the Determination of Difenoconazole and Prothioconazole residues in aqueous samples of Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560 coming from the Ecotoxicological tests  Garagna, D.  2021b  ChemService S.r.l. Controlli e Richerche, Novate Milanese - Italy  Report No. : CH – 0227/2021  + Amendment report No.1 to final report (Amdm1\_KCP 5.1.2/03) |
| Guideline(s): | Yes : SANTE/2020/12830, rev. 1 dated 24/02/21 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The determination of Difenoconazole residues is performed by HPLC using an external standard and MS triple quadrupole detector.  Its quantification is achieved using the calibration curve obtained by plotting standard solutions concentrations and the corresponding peak area. |

Materials and methods

***1. Materials***

***1.A. Determination of difenoconazole residues in aqueous samples (KCP 5.2/03)***

The determination of difenoconazole residues in aqueous samples was assessed by HLPC/MS-QQQ.

|  |  |
| --- | --- |
| HPLC: | Agilent Technologies 1200 System |
| Column: | Zorbax Eclipse Plus C18, 3.5 µm, 100 x 4.6 mm i.d. |
| Detector | MS Triple quadrupole (Scan in MRM mode) |
| Mobile phase: | Water, HPLC grade  Formic acid  Ammonium formate, for HPLC  Acetonitrile, HPLC grade  QuEChERS Extract Pouch |
| Analytical standards: | Difenoconazole  CAS No. : 119446-68-3  Batch No. :  BCCD4900  Purity : 95.5 %  Expiry date: June 1, 2025 |

***2. Methods***

***2.A. Preparation of aqueous samples***

**Alga growth medium:**

It was prepared as described in Annex 3 of OECD 201, 2011 (Freshwater Alga and Cyanobacteria, Growth Inhibition Test).

The composition is as follows:

In de-ionized water (conductivity < 5 μS/cm), analytical grade, the following salts are added to a defined final nominal concentration:

* Macro-nutrients:

|  |  |
| --- | --- |
| NaHCO3 | 50.0 mg/L |
| CaCl2 x 2 H2O | 18.0 mg/L |
| NH4Cl | 15.0 mg/L |
| MgSO4 x 7 H2O | 15.0 mg/L |
| MgCl2 x 6 H2O | 12.0 mg/L |
| KH2PO4 | 1.6 mg/L |

* Trace elements:

|  |  |
| --- | --- |
| Na2EDTA x 2 H2O | 100.0 μg/L |
| FeCl3 x 6 H2O | 64.0 μg/L |
| MnCl2 x 4 H2O | 415.0 μg/L |
| H3BO3 | 185.0 μg/L |
| Na2MoO4 x 2 H2O | 7.0 μg/L |
| ZnCl2 | 3.0 μg/L |
| CoCl2 x 6 H2O | 1.5 μg/L |
| CuCl2 x 2 H2O | 0.01 μg/L |

**Reconstituted water:**

It was prepared as described in Annex 3 of OECD No. 202, 2004 (Daphnia sp., Acute Immobilization Test”).

The composition is as follows:

In de-ionized water (conductivity < 5 μS/cm), analytical grade, the following salts are added to a defined final nominal concentration:

|  |  |
| --- | --- |
| CaCl2 x 2 H2O | 2.0 mmol/L (= 294.0 mg/L) |
| MgSO4 x 7 H2O | 0.5 mmol/L (= 123.3 mg/L) |
| NaHCO3 | 0.771 mmol/L (= 64.8 mg/L) |
| KCl | 0.078 mmol/L (= 5.8 mg/L) |

**Elends M4:**

It was prepared as described in Annex 2 of OECD No. 211, 2012 (Daphnia magna, Reproduction Test). The composition is as follows:

In de-ionized water (conductivity < 5 μS/cm), analytical grade, the following salts are added to a defined final nominal concentration:

* Macro-nutrients:

|  |  |
| --- | --- |
| CaCl2 x 2 H2O | 293.8 mg/L |
| MgSO4 x 7 H2O | 123.3 mg/L |
| KCl | 5.8 mg/L |
| NaHCO3 | 64.8 mg/L |
| Na2SiO3 x 9 H2O | 10 mg/L |
| NaNO3 | 0.274 mg/L |
| KH2PO4 | 0.143 mg/L |
| K2HPO4 | 0.184 mg/L |
| Combined vitamine stock | - |

* Trace elements:

|  |  |
| --- | --- |
| H3BO3 | 2.860 mg/L |
| MnCl2 x 4 H2O | 0.361 mg/L |
| LiCl | 0.306 mg/L |
| RbCl | 0.071 mg/L |
| SrCl2 x 6 H2O | 0.152 mg/L |
| NaBr | 0.016 mg/L |
| MoNa2O4 x 2 H2O | 0.063 mg/L |
| CuCl2 x 2 H2O | 0.017 mg/L |
| ZnCl | 0.013 mg/L |
| CoCl2 x 6 H2O | 0.010 mg/L |
| KI | 0.003 mg/L |
| Na2SeO3 | 0.002 mg/L |
| NH4VO3 | 0.001 mg/L |
| Na2EDTA x 2 H2O | 2.500 mg/L |
| FeSO4 x 7 H2O | 0.996 mg/L |

***2.B. Determination of difenoconazole residues in aqueous samples (KCP 5.2/03)***

The analytical methods for the determination of difenoconazole residues in aqueous samples were first validated so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were validated in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A~~7.1~~ 162: Matric effects results from method validation of difenoconazole using the analytical method

| Matrix effect | Analyte | Slope of Algal growth medium | Slope of Reconstituted water | Slope of Elendt M4 | Comments |
| --- | --- | --- | --- | --- | --- |
| Result  < ± 20 % | Difenoconazole | -46.0 | -38.6 | 31.3 | No comments |

Table A~~7.2~~ 163: Recovery results from method validation of difenoconazole using the analytical method

| Matrix | Analyte | Fortification level  (n = x) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Reconstituted water | Difenoconazole | Low level:  7.4 µg/L  (mean found)  N = 5 | 118.1 | 2 | No comments |
| High level:  1.83 mg/L (mean found)  N = 5 | 97.6 | 9 | No comments |
| Alga growth medium | Difenoconazole | Low level:  6.5 µg/L  (mean found)  N = 5 | 110.1 | 4 | No comments |
| High level:  9.33 mg/L (mean found)  N = 5 | 111.9 | 1 | No comments |
| Elendt M4 | Difenoconazole | Low level:  0.9 µg/L  (mean found)  N = 5 | 76.8 | 5 | No comments |
| High level:  178.41 µg/L (mean found)  N = 5 | 100.8 | 4 | No comments |

Table A~~8~~ 164: Characteristics for the analytical method used for validation of difenoconazole residues for purposes of aqueous ecotoxicological tests

|  | Difenoconazole |
| --- | --- |
| Specificity | Untreated blank < 30 % LOQ  Result : 0.0 % for Alga growth medium  Result : 0.0 % for Reconstituted water  Result : 0.0 % for Elendt M4  These % ratio (Blank vs LOQ) demontrate that there was no interference of the blank matrix at the retention time of the analyte. |
| Linearity / Calibration | **Reconstituted water :**  ***Quantifier transition***  ***m/z 406.1 🡪 m/z 251 :***  The calibration curve (µg/L):   * was considered as valid over 1.9 – 191.0 µg/L.   Equation (µg/L)  y = 1317 \* x – 3688  Correlation coefficient:  r² = 99.953  ***Quantifier 1 transition***  ***m/z 406.1 🡪 m/z 337 :***  The calibration curve (µg/L):   * was considered as valid over 1.9 – 191.0 µg/L.   Equation (µg/L)  y = 204 \* x – 441  Correlation coefficient:  r² = 99.954  ***Quantifier 2 transition***  ***m/z 406.1 🡪 m/z 188 :***  The calibration curve (µg/L):   * was considered as valid over 1.9 – 191.0 µg/L.   Equation (µg/L)  y = 129 \* x -240  Correlation coefficient:  r² = 99.969  **Alga growth medium**  ***Quantifier transition***  ***m/z 406.1 🡪 m/z 251 :***  The calibration curve (µg/L):   * was considered as valid over 1.9 – 191.0 µg/L.   Equation (µg/L)  y = 1125 \* x – 1386  Correlation coefficient:  r² = 99.980  ***Quantifier 1 transition***  ***m/z 406.1 🡪 m/z 337 :***  The calibration curve (µg/L):   * was considered as valid over 1.9 – 191.0 µg/L.   Equation (µg/L)  y = 173 \* x - 2  Correlation coefficient:  r² = 99.956  ***Quantifier 2 transition***  ***m/z 406.1 🡪 m/z 188 :***  The calibration curve (µg/L):   * was considered as valid over 1.9 – 191.0 µg/L.   Equation (µg/L)  y = 109 \* x + 77  Correlation coefficient:  r² = 99.954  **Elendt M4**  ***Quantifier transition***  ***m/z 406.1 🡪 m/z 251 :***  The calibration curve (µg/L):   * was considered as valid over 0.37 – 22.92 µg/L.   Equation quadratic (µg/L)  y = -14 \* x² + 33258 \* x – 6818  Correlation coefficient:  r² = 99.783  ***Quantifier 1 transition***  ***m/z 406.1 🡪 m/z 337 :***  The calibration curve (µg/L):   * was considered as valid over 0.37 – 22.92 µg/L.   Equation quadratic (µg/L)  y = -3 \* x² + 6296 \* x – 4579  Correlation coefficient:  r² = 99.690  ***Quantifier 2 transition***  ***m/z 406.1 🡪 m/z 188 :***  The calibration curve (µg/L):   * was considered as valid over 0.37 – 22.92 µg/L.   Equation quadratic (µg/L)  y = -2 \* x² + 4495 \* x – 3816  Correlation coefficient:  r² = 99.753 |
| Limit of determination (LOD) | **Reconstituted water :**  LOD = 1.9 µg/L  Lowest calibration level  **Alga growth medium**  LOD = 1.9 µg/L  Lowest calibration level  **Elendt M4**  LOD = 0.37 µg/L  Lowest calibration level |
| Limit of quantification (LOQ) | **Reconstituted water :**  LOD = 6.3 µg/L  Lowest fortified level  **Alga growth medium**  LOD = 5.9 µg/L  Lowest fortified level  **Elendt M4**  LOD = 1.19 µg/L  Lowest fortified level |

Conclusion

The analytical method for the quantification of difenoconazole residues in aqueous samples was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability for difenoconazole.

* + - * 1. Analytical method 4

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  In method HPLC-MS/MS detection was used. Two transitions were monitored.  The specificity of the method was assured by MS/MS detection. All validation parameters are in the currently required range.  The method is suitable for the intended purposes. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/04 |
| Report | Validation of the Analytical Method for the Determination of Difenoconazole and Prothioconazole content in stock solutions of Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560 coming from the Ecotoxicological tests  Garagna, D.  2021c  ChemService S.r.l. Controlli e Richerche, Novate Milanese - Italy  Report No. : CH – 0232/2021  + Amendment report No.1 to final report (Amdm1\_KCP 5.1.2/04) |
| Guideline(s): | Yes : SANTE/2020/12830, rev. 1 dated 24/02/21 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The determination of Difenoconazole residues is performed by HPLC using an external standard and MS triple quadrupole detector.  Its quantification is achieved using the calibration curve obtained by plotting standard solutions concentrations and the corresponding peak area. |

Materials and methods

***1. Materials***

***1.A. Determination of difenoconazole content in stock solutions***

The determination of difenoconazole content in stock solutionswas assessed by HLPC/MS-QQQ.

|  |  |
| --- | --- |
| HPLC: | Shimadzu Technologies 8050 System |
| Column: | Zorbax Eclipse Plus C18, 3.5 µm, 100 x 4.6 mm i.d. |
| Detector | ESI interfaced Triple quadrupole Mass Detector |
| Mobile phase: | Water, HPLC grade  Demineralised water  Formic acid, high purity for mass spectroscopy  Ammonium formate, for HPLC  Acetonitrile, HPLC grade |
| Analytical standards: | Difenoconazole  CAS No. : 119446-68-3  Batch No. : BCCD4900  Purity : 95.5 %  Expiry date: June 1, 2025 |

***2. Methods***

***2.A. Determination of difenoconazole content in stock solutions***

The analytical methods for the determination of difenoconazole content in stock solutions were first validated so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were validated in terms of linearity, precision, accuracy, specificity, stability and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A ~~2.2.5.8.1~~ 165: Recovery results from method validation of difenoconazole content in stock solutions using the analytical method

| Matrix effect | Analyte | Fortification level  (n = x) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| -19.0% | Difenoconazole | Low level:  0.12 mg/L  (mean found)  N = 5 | 105.4 | 4 | No comments |
| -19.0% | Difenoconazole | High level:  7385.95 mg/L (mean found)  N = 5 | 104.0 | 5 | No comments |
| -19.0% | Difenoconazole | Ultra-high level:  60116.85 mg/L (mean found)  N = 5 | 112.9 | 5 | No comments |

Table A ~~2.2.5.8.2~~ 166: Characteristics for the analytical method used for validation of difenoconazole content in stock solutions for purposes of ecotoxicological tests

|  | Difenoconazole |
| --- | --- |
| Specificity | Untreated blank < 30 % LOQ  Result : 0.4 %  This % ratio (Blank vs LOQ) demontrates that there was no interference of the blank matrix at the retention time of the analyte. |
| Linearity / Calibration | ***Quantifier transition***  ***m/z 406.1 🡪 m/z 250.9 :***  The calibration curve (mg/L):   * was considered as valid over 0.02 – 1.95 mg/L.   Equation (mg/L)  y = 97434169 \* x – 1592620  Correlation coefficient:  r² = 99.991  ***Quantifier 1 transition***  ***m/z 406.1 🡪 m/z 336.9 :***  The calibration curve (mg/L):   * was considered as valid over 0.02 – 1.95 mg/L..   Equation (mg/L)  y = 16110993 \* x – 204510  Correlation coefficient:  r² = 99.990  ***Quantifier 2 transition***  ***m/z 344 🡪 m/z 188 :***  The calibration curve (mg/L):   * was considered as valid over 0.02 – 1.95 mg/L..   Equation (mg/L)  y = 15982007 \* x - 223410  Correlation coefficient:  r² = 99.991 |
| Limit of determination (LOD) | LOD = 0.02 mg/L  Lowest calibration level |
| Limit of quantification (LOQ) | LOQ = 0.11 mg/L  Lowest fortified level |
| Stability of final extract (5 days) | % recovery = 115.1 %  (mean value of 5 replicates)  % RSD = 0 %  Range of recovery:  114.6 – 115.9 % |
| Stability of standard (5 days) | Difference = -15.6 %  Not stable, the standards are prepared always freshly. |

Conclusion

The analytical method for the quantification of difenoconazole content in stock solutions was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability for difenoconazole.

* + - * 1. Analytical method 5

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  In method HPLC-MS/MS detection was used. Two transitions were monitored.  The specificity of the method was assured by MS/MS detection. All validation parameters are in the currently required range.  The method is suitable for the intended purposes.  (the method submitted as draft report in word format)  The final report of the “*Validation of the Analytical Method for the Determination of Difenoconazole and Prothioconazole content in feeding solutions of Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560 coming from the Ecotoxicological tests*” (Garagna D., 2021d – Report No. CH-0668/2021 - KCP 5.1.2/05) is available (in pdf format). |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/05 |
| Report | Validation of the Analytical Method for the Determination of Difenoconazole and Prothioconazole content in feeding solutions of Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560 coming from the Ecotoxicological tests  Garagna, D.  2021d  ChemService S.r.l. Controlli e Richerche, Novate Milanese - Italy  Report No. : CH – 0668/2021  + Amendment report No.1 to final report (Amdm1\_KCP 5.1.2/05) |
| Guideline(s): | Yes : SANTE/2020/12830, rev. 1 dated 24/02/21 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The determination of Difenoconazole residues is performed by HPLC using an external standard and MS triple quadrupole detector.  Its quantification is achieved using the calibration curve obtained by plotting standard solutions concentrations and the corresponding peak area. |

Materials and methods

***1. Materials***

***1.A. Determination of difenoconazole content in feeding solutions***

The determination of difenoconazole content in feeding solutionswas assessed by HLPC/MS-QQQ.

|  |  |
| --- | --- |
| HPLC: | Shimadzu Technologies 8050 System |
| Column: | Zorbax Eclipse Plus C18, 3.5 µm, 100 x 4.6 mm i.d. |
| Detector | ESI interfaced Triple quadrupole Mass Detector |
| Mobile phase: | Water, HPLC grade  Demineralised water  Formic acid, high purity for mass spectroscopy  Ammonium formate, for HPLC  Acetonitrile, HLPC grade |
| Analytical standards: | Difenoconazole  CAS No. : 119446-68-3  Batch No. : BCCD4900  Purity : 95.5 %  Expiry date: June 1, 2025 |

***2. Methods***

***2.A. Determination of difenoconazole content in feeding solutions***

The analytical methods for the determination of difenoconazole content in feeding solutions were first validated so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were validated in terms of linearity, precision, accuracy, specificity, stability and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A ~~2.2.5.8.1~~ 167: Recovery results from method validation of difenoconazole content in feeding solutions using the analytical method

| Matrix effect | Analyte | Fortification level  (n = x) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| 14.3% | Difenoconazole | Low level:  4.01 mg/L  (mean found)  N = 5 | 112.8 | 2 | No comments |
| 14.3% | Difenoconazole | High level:  4009.19 mg/L (mean found)  N = 5 | 113.2 | 1 | No comments |

Table A ~~2.2.5.8.2~~ 168: Characteristics for the analytical method used for validation of difenoconazole content in feeding solutions for purposes of ecotoxicological tests

|  | Difenoconazole |
| --- | --- |
| Specificity | Untreated blank < 30 % LOQ  Result : 0.6 %  This % ratio (Blank vs LOQ) demontrates that there was no interference of the blank matrix at the retention time of the analyte. |
| Linearity / Calibration | ***Quantifier transition***  ***m/z 406.1 🡪 m/z 250.9 :***  The calibration curve (mg/L):   * was considered as valid over 0.02 – 1.89 mg/L.   Equation (mg/L)  y =168863065 \* x – 6033862  Correlation coefficient:  r² = 99.934  ***Quantifier 1 transition***  ***m/z 406.1 🡪 m/z 336.9 :***  The calibration curve (mg/L):   * was considered as valid over 0.02 – 1.89 mg/L..   Equation (mg/L)  y = 29067632 \* x – 969929  Correlation coefficient:  r² = 99.927  ***Quantifier 2 transition***  ***m/z 344 🡪 m/z 188 :***  The calibration curve (mg/L):   * was considered as valid over 0.02 – 1.89 mg/L..   Equation (mg/L)  y = 28769257 \* x - 970782  Correlation coefficient:  r² = 99.928 |
| Limit of determination (LOD) | LOD = 0.02 mg/L  Lowest calibration level |
| Limit of quantification (LOQ) | LOQ = 0.11 mg/L  Lowest fortified level |

Conclusion

The analytical method for the quantification of difenoconazole content in feeding solutions was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification for difenoconazole.

* + - * 1. Analytical method 6

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  In method HPLC-MS/MS detection was used. Two transitions were monitored.  The specificity of the method was assured by MS/MS detection. All validation parameters are in the currently required range.  The method is suitable for the intended purposes. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/06 |
| Report | Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Acute Toxicity to *Daphnia magna* in a 48-hour Immobilization Test under Semi-Static Exposure  Noè F.  2021a  ChemService S.r.l. Controlli e Richerche, Novate Milanese - Italy  Report No. : CH – 0229/2021  + Amendment report No.1 to final report (Amdm1\_KCP 5.1.2/06) |
| Guideline(s): | Yes : SANTE/2020/12830, rev. 1 dated 24/02/21 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The determination of Difenoconazole residues is performed by HPLC using an external standard and MS triple quadrupole detector.  Its quantification is achieved using the calibration curve obtained by plotting standard solutions concentrations and the corresponding peak area. |

Materials and methods

***1. Materials***

The determination of difenoconazole was assessed by HLPC/MS-QQQ.

|  |  |
| --- | --- |
| HPLC: | Agilent mod. 1200 |
| Column: | Zorbax Eclipse Plus C18, 3.5 µm, 100 x 4.6 mm i.d. |
| Detector | Triple quadrupole Mass Detector |
| Scan type | MRM |
| Ion mode | ESI, positive polarity |
| Electro multiplier voltage | 300 V |
| Dry gas temperature | 300 °C |
| Dry gas flow | 7 L/min |
| Nebuliser | 40 psi |
| Dwell time | 200 msec |
| Eluent flow | 0.7 mL/min |
| Volume of injection | 5 µL |
| Retention time | Approximately 4.1 minutes |
| Total analysis time | 10 minutes + 4 minutes post time |
| Mobile phase: | Water  Reconstituted water (according to OECD No. 202, 2004 guideline)  Formic acid  Ammonium formate  Acetonitrile |
| Analytical standards: | Difenoconazole  CAS No. : 119446-68-3  Batch No. : BCCD4900  Purity : 95.5 %  Expiry date: June 1, 2025 |

***2. Methods***

The analytical method for the determination of difenoconazole in reconstituted water with *Daphnia magna* was first validated so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The method was validated in terms of linearity, precision, accuracy, specificity, limits of detection and quantification and stability.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A~~7.1~~ 169: Recovery results from method validation of difenoconazole in reconstituted water with *Daphnia magna* using the analytical method

| Matrix effect | Analyte | Fortification level | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| < ±20 % | Difenoconazole | Low level:  7.4 µg/L  (mean found) | 118.1 | 2 | No comments |
| < ±20 % | Difenoconazole | High level:  1.83 mg/L (mean found) | 97.6 | 9 | No comments |

Table A~~8~~ 170: Characteristics for the analytical method used for validation of difenoconazole in reconstituted water with *Daphnia magna* for purposes of ecotoxicological tests

|  | Difenoconazole |
| --- | --- |
| Specificity | Untreated blank < 30 % LOQ  Result : 0.0 %  This % ratio (Blank vs LOQ) demontrates that there was no interference of the blank matrix at the retention time of the analyte. |
| Linearity / Calibration | ***Quantifier transition***  ***m/z 406.1 🡪 m/z 251 :***  The calibration curve (µg/L):   * was considered as valid over 1.9– 191.0 µg/L.   Correlation coefficient:  r² = 99.953  ***Quantifier 1 transition***  ***m/z 406.1 🡪 m/z 337 :***  The calibration curve (µg/L):   * was considered as valid over 1.9– 191.0 µg/L   Correlation coefficient:  r² = 99.954  ***Quantifier 2 transition***  ***m/z 406.1 🡪 m/z 188 :***  The calibration curve (µg/L):   * was considered as valid over 1.9– 191.0 µg/L   Correlation coefficient:  r² = 99.969  ***Time 0 hours : Difenoconazole: calibration with matrix-matched standard solutions in reconstituted water***  Equation (mg/L)  y = 707 \* x – 541  Correlation coefficient:  r² = 99.921  ***Time 24 hours : Difenoconazole: calibration with matrix-matched standard solutions in reconstituted water***  Equation (mg/L)  y = 579 \* x – 203  Correlation coefficient:  r² = 99.657  ***Time 48 hours : Difenoconazole: calibration with matrix-matched standard solutions in reconstituted water***  Equation (mg/L)  y = 681 \* x – 1770  Correlation coefficient:  r² = 99.565 |
| Limit of determination (LOD) | LOD = 1.9 µg/L  Lowest calibration level |
| Limit of quantification (LOQ) | LOQ = 6.3 µg/L  Lowest fortified level |
| Stability | Samples analyzed within 24 hours (storage at 4°C) |
| Stability of standard | Freshly prepared at each analysis day |

Conclusion

The analytical method for the difenoconazole in reconstituted water with *Daphnia magna* was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and the stability for difenoconazole.

* + - * 1. Analytical method 7

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  In method HPLC-MS/MS detection was used. Two transitions were monitored.  The specificity of the method was assured by MS/MS detection. All validation parameters are in the currently required range.  The method is suitable for the intended purposes.  Since the analysis gave both quantification and identification data, a confirmatory test is not necessary. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/07 |
| Report | Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Toxicity to Green Algae *Pseudokirchneriella subcapitata* in a Growth Inhibition Study  Noè F.  2021b  ChemService S.r.l. Controlli e Richerche, Novate Milanese - Italy  Report No. : CH – 0230/2021  + Amendment report No.1 to final report (Amdm1\_KCP 5.1.2/07) |
| Guideline(s): | Yes : SANTE/2020/12830, rev. 1 dated 24/02/21 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The determination of Difenoconazole residues is performed by HPLC using an external standard and MS triple quadrupole detector.  Its quantification is achieved using the calibration curve obtained by plotting standard solutions concentrations and the corresponding peak area. |

Materials and methods

***1. Materials***

The determination of difenoconazole was assessed by HLPC/MS-QQQ.

|  |  |
| --- | --- |
| HPLC: | Agilent mod. 1200 |
| Column: | Zorbax Eclipse Plus C18, 3.5 µm, 100 x 4.6 mm i.d. |
| Detector | Triple quadrupole Mass Detector |
| Scan type | MRM |
| Ion mode | ESI, positive polarity |
| Electro multiplier voltage | 300 V |
| Dry gas temperature | 300 °C |
| Dry gas flow | 7 L/min |
| Nebuliser | 40 psi |
| Dwell time | 200 msec |
| Eluent flow | 0.7 mL/min |
| Volume of injection | 5 µL |
| Retention time | Approximately 4.1 minutes |
| Total analysis time | 10 minutes + 4 minutes post time |
| Mobile phase: | Water  Algal growth medium (according to OECD No.201, 2011 guideline)  Formic acid  Ammonium formate  Acetonitrile |
| Analytical standards: | Difenoconazole  CAS No. : 119446-68-3  Batch No. : BCCD4900  Purity : 95.5 %  Expiry date: June 1, 2025 |

***2. Methods***

The analytical method for the determination of difenoconazole in alga growth medium with Green Algae *Pseudokirchneriella subcapitata* was first validated so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The method was validated in terms of linearity, precision, accuracy, specificity, limits of detection and quantification and stability.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A~~7.1~~ 171: Recovery results from method validation of difenoconazole in alga growth medium with Green Algae *Pseudokirchneriella subcapitata* using the analytical method

| Matrix effect | Analyte | Fortification level | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| < ±20 % | Difenoconazole | Low level:  6.5 µg/L  (mean found) | 110.1 | 4 | No comments |
| < ±20 % | Difenoconazole | High level:  9.33 mg/L (mean found) | 111.9 | 1 | No comments |

Table A~~8~~ 172: Characteristics for the analytical method used for validation of difenoconazole in alga growth medium with Green Algae *Pseudokirchneriella subcapitata* for purposes of ecotoxicological tests

|  | Difenoconazole |
| --- | --- |
| Specificity | Untreated blank < 30 % LOQ  Result : 0.0 %  This % ratio (Blank vs LOQ) demontrates that there was no interference of the blank matrix at the retention time of the analyte. |
| Linearity / Calibration | ***Quantifier transition***  ***m/z 406.1 🡪 m/z 251 :***  The calibration curve (µg/L):   * was considered as valid over 1.9– 191.0 µg/L.   Correlation coefficient:  r² = 99.953  ***Quantifier 1 transition***  ***m/z 406.1 🡪 m/z 337 :***  The calibration curve (µg/L):   * was considered as valid over 1.9– 191.0 µg/L   Correlation coefficient:  r² = 99.954  ***Quantifier 2 transition***  ***m/z 406.1 🡪 m/z 188 :***  The calibration curve (µg/L):   * was considered as valid over 1.9– 191.0 µg/L   Correlation coefficient:  r² = 99.969  ***Time 0 hours : Difenoconazole: calibration with matrix-matched standard solutions in in alga growth medium***  Equation (mg/L)  y = 570 \* x – 3011  Correlation coefficient:  r² = 99.793  ***Time 72 hours : Difenoconazole: calibration with matrix-matched standard solutions in alga growth medium***  Equation (mg/L)  y = 716 \* x – 51  Correlation coefficient:  r² = 99.923 |
| Limit of determination (LOD) | LOD = 1.9 µg/L  Lowest calibration level |
| Limit of quantification (LOQ) | LOQ = 5.9 µg/L  Lowest fortified level |
| Stability | Samples analyzed within 24 hours (storage at 4°C) |
| Stability of standard | Freshly prepared at each analysis day |

Conclusion

The analytical method for the difenoconazole in alga growth medium with Green Algae *Pseudokirchneriella subcapitata* was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and the stability for difenoconazole.

* + - * 1. Analytical method 8

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  In method HPLC-MS/MS detection was used. Two transitions were monitored.  The specificity of the method was assured by MS/MS detection. All validation parameters are in the currently required range.  The method is suitable for the intended purposes.  Since the analysis gave both quantification and identification data, a confirmatory test is not necessary. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/08 |
| Report | Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Acute Toxicity to Zebrafish (*Brachydanio rerio*) in a 96-hour Study under Semi-Static Exposure  xxx.  2021c  xxx  Report No. : CH – 0228/2021  + Amendment report No.1 to final report (Amdm1\_KCP 5.1.2/08) |
| Guideline(s): | Yes : SANTE/2020/12830, rev. 1 dated 24/02/21 |
| Deviations: | Yes  Deviation 1   |  |  | | --- | --- | | Change No. 1 | The study was performed with zebrafish obtained from Model Organism Facility Department of Cellular, Computational and Integrative Biology -CIBIO University of Trento instead of Research Foundation “Edmund Mach” (S. Michele all’Adige - Italy). | | Reason of change: | Availability of organisms. | | Impact on the study: | None. | |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The determination of Difenoconazole residues is performed by HPLC using an external standard and MS triple quadrupole detector.  Its quantification is achieved using the calibration curve obtained by plotting standard solutions concentrations and the corresponding peak area. |

Materials and methods

***1. Materials***

The determination of difenoconazole was assessed by HLPC/MS-QQQ.

|  |  |
| --- | --- |
| HPLC: | Agilent mod. 1200 |
| Column: | Zorbax Eclipse Plus C18, 3.5 µm, 100 x 4.6 mm i.d. |
| Detector | Triple quadrupole Mass Detector |
| Scan type | MRM |
| Ion mode | ESI, positive polarity |
| Electro multiplier voltage | 300 V |
| Dry gas temperature | 300 °C |
| Dry gas flow | 7 L/min |
| Nebuliser | 40 psi |
| Dwell time | 200 msec |
| Eluent flow | 0.7 mL/min |
| Volume of injection | 5 µL |
| Retention time | Approximately 4.1 minutes |
| Total analysis time | 10 minutes + 4 minutes post time |
| Mobile phase: | Water  Reconstituted water (according to ISO Test Water 6341 -Fish)  Formic acid  Ammonium formate  Acetonitrile |
| Analytical standards: | Difenoconazole  CAS No. : 119446-68-3  Batch No. : BCCD4900  Purity : 95.5 %  Expiry date: June 1, 2025 |

***2. Methods***

The analytical method for the determination of difenoconazole in reconstituted water with *Brachydanio rerio* was first validated so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The method was validated in terms of linearity, precision, accuracy, specificity, limits of detection and quantification and stability.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A~~7.1~~ 173: Recovery results from method validation of difenoconazole in reconstituted water with *Brachydanio rerio* using the analytical method

| Matrix effect | Analyte | Fortification level | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| < ±20 % | Difenoconazole | Low level:  7.4 µg/L  (mean found) | 118.1 | 2 | No comments |
| < ±20 % | Difenoconazole | High level:  1.83 mg/L (mean found) | 97.6 | 9 | No comments |

Table A~~8~~ 174: Characteristics for the analytical method used for validation of difenoconazole in reconstituted water with *Brachydanio rerio* for purposes of ecotoxicological tests

|  | Difenoconazole |
| --- | --- |
| Specificity | Untreated blank < 30 % LOQ  Result : 0.0 %  This % ratio (Blank vs LOQ) demontrates that there was no interference of the blank matrix at the retention time of the analyte. |
| Linearity / Calibration | ***Quantifier transition***  ***m/z 406.1 🡪 m/z 251 :***  The calibration curve (µg/L):   * was considered as valid over 1.9– 191.0 µg/L.   Correlation coefficient:  r² = 99.953  ***Quantifier 1 transition***  ***m/z 406.1 🡪 m/z 337 :***  The calibration curve (µg/L):   * was considered as valid over 1.9– 191.0 µg/L   Correlation coefficient:  r² = 99.954  ***Quantifier 2 transition***  ***m/z 406.1 🡪 m/z 188 :***  The calibration curve (µg/L):   * was considered as valid over 1.9– 191.0 µg/L   Correlation coefficient:  r² = 99.969  ***Time 0 hours : Difenoconazole: calibration with matrix-matched standard solutions in in reconstituted water***  Equation (mg/L)  y = 1499 \* x – 74  Correlation coefficient:  r² = 99.966  ***Time 24 hours : Difenoconazole: calibration with matrix-matched standard solutions in in reconstituted water***  Equation (mg/L)  y = 1754 \* x – 501  Correlation coefficient:  r² = 99.752  ***Time 48 hours : Difenoconazole: calibration with matrix-matched standard solutions in in reconstituted water***  Equation (mg/L)  y = 2693 \* x – 9190  Correlation coefficient:  r² = 99.737  ***Time 72 hours : Difenoconazole: calibration with matrix-matched standard solutions in in reconstituted water***  Equation (mg/L)  y = 2547 \* x – 8764  Correlation coefficient:  r² = 99.485  ***Time 96 hours : Difenoconazole: calibration with matrix-matched standard solutions in in reconstituted water***  Equation (mg/L)  y = 21575 \* x – 144132  Correlation coefficient:  r² = 99.467 |
| Limit of determination (LOD) | LOD = 1.9 µg/L  Lowest calibration level |
| Limit of quantification (LOQ) | LOQ = 6.3 µg/L  Lowest fortified level |
| Stability | Samples analyzed within 24 hours (storage at 4°C) |
| Stability of standard | Freshly prepared at each analysis day |

Conclusion

The analytical method for the difenoconazole in reconstituted water with *Brachydanio rerio* was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and the stability for difenoconazole.

* + - * 1. Analytical method 9

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  In method HPLC-MS/MS detection was used. Two transitions were monitored.  The specificity of the method was assured by MS/MS detection. All validation parameters are in the currently required range.  The method is suitable for the intended purposes.  (draft in word format submitted)  The final report of the “*Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Effects on Reproduction of Earthworm Eisenia fetida in an Artificial Soil Study*” (Dini, R.., 2021a – Report No. CH-0239/2021 - KCP 5.1.2/09) is available (in pdf format). |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/09 |
| Report | Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Effects on Reproduction of Earthworm *Eisenia fetida* in an Artificial Soil Study  Dini, R.  2021a  ChemService S.r.l. Controlli e Richerche, Novate Milanese - Italy  Report No. : CH – 0239/2021 |
| Guideline(s): | Yes : SANTE/2020/12830, rev. 1 dated 24/02/21 |
| Deviations: | Yes  Deviation 1   |  |  | | --- | --- | | Change No. 1 | Deviation in Test temperature during test period. | | Reason of change: | Due to a technical error, the temperatures were recorded from 6th August instead of 5th August.. | | Impact on the study: | None, since all validity criteria were meet. | |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The determination of Difenoconazole residues is performed by HPLC using an external standard and MS triple quadrupole detector.  Its quantification is achieved using the calibration curve obtained by plotting standard solutions concentrations and the corresponding peak area. |

Materials and methods

***1. Materials***

The determination of difenoconazole was assessed by HLPC/MS-QQQ.

|  |  |
| --- | --- |
| HPLC: | Agilent mod. 1200 |
| Column: | Zorbax Eclipse Plus C18, 3.5 µm, 100 x 4.6 mm i.d. |
| Detector | Triple quadrupole Mass Detector |
| Scan type | MRM |
| Ion mode | ESI, positive polarity |
| Interface temperature | 300 °C |
| Dry gas flow | 10 L/min |
| Nebilizing gas flow | 2.9 L/min |
| Eluent flow | 0.7 mL/min |
| Volume of injection | 10 µL |
| Retention time | Approximately 10.6 minutes |
| Total analysis time | 20 minutes |
| Mobile phase: | Water  Demineralised water  Artificial soil (according to OECD No. 222, Earthworms guideline)  Formic acid  Ammonium formate  Acetonitrile |
| Analytical standards: | Difenoconazole  CAS No. : 119446-68-3  Batch No. : BCCD4900  Purity : 95.5 %  Expiry date: June 1, 2025 |

***2. Methods***

The analytical method for the determination of difenoconazole in Matrix-matched standard solutionswith *Eisenia fetida* was first validated so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The method was validated in terms of linearity, precision, accuracy, specificity, limits of detection and quantification and stability.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A~~7.1~~ 175: Recovery results from method validation of difenoconazole in Matrix-matched standard solutions with *Eisenia fetida* using the analytical method

| Matrix effect | Analyte | Fortification level | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| < ±20 % | Difenoconazole | Low level:  29.0 µg/kg  (mean found) | 105.3 | 2 | No comments |
| < ±20 % | Difenoconazole | High level:  115.36 mg/kg  (mean found) | 98.9 | 2 | No comments |

Table A~~8~~ 176: Characteristics for the analytical method used for validation of difenoconazole in Matrix-matched standard solutions with *Eisenia fetida* for purposes of ecotoxicological tests

|  | Difenoconazole |
| --- | --- |
| Specificity | Untreated blank < 30 % LOQ  Result : 2.8 %  This % ratio (Blank vs LOQ) demontrates that there was no interference of the blank matrix at the retention time of the analyte. |
| Linearity / Calibration | ***Quantifier transition***  ***m/z 406.1 🡪 m/z 250.9 :***  The calibration curve (µg/kg):   * was considered as valid over 4.7 – 43.0 µg/kg. * corresponding to range 2.3 – 23.4 µg/L   Correlation coefficient:  r² = 99.605  ***Quantifier 1 transition***  ***m/z 406.1 🡪 m/z 336.9 :***  The calibration curve (µg/kg):   * was considered as valid over 4.7 – 43.0 µg/kg. * corresponding to range 2.3 – 23.4 µg/L   Correlation coefficient:  r² = 99.966  ***Quantifier 2 transition***  ***m/z 406.1 🡪 m/z 188 :***  The calibration curve (µg/kg):   * was considered as valid over 4.7 – 43.0 µg/kg. * corresponding to range 2.3 – 23.4 µg/L   Correlation coefficient:  r² = 99.319  ***Time 0 day : Difenoconazole: calibration with matrix-matched standard solutions***  Equation (µg/kg)  y = 75410 \* x – 19504  Correlation coefficient:  r² = 99.723  ***Time 28 days : Difenoconazole: calibration with matrix-matched standard solution***  Equation (µg/kg)  y = 101261 \* x – 9393  Correlation coefficient:  r² = 99.991  ***Time 56 days : Difenoconazole: calibration with matrix-matched standard solutions***  Equation (µg/kg)  y = 61888 \* x – 67217  Correlation coefficient:  r² = 99.816 |
| Limit of determination (LOD) | LOD = 4.7 µg/kg  Lowest calibration level |
| Limit of quantification (LOQ) | LOQ = 27.5 µg/kg  Lowest fortified level |
| Stability | Recovery Mean between 70% – 120% |
| Stability (7 days) | Low level: 87.0 % |
| Stability of standard | < ±10% |
| Stability of standard (3 days) | 8.3 % |

Conclusion

The analytical method for the difenoconazole in Matrix-matched standard solutions with *Eisenia fetida* was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and the stability for difenoconazole.

* + - * 1. Analytical method 10

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  The analytical method was employed to determine the difenoconazole and prothioconazole residues in soil samples coming from the biological phase of ecotoxicological test on Collembola (*Folsomia candida*).  In the method HPLC-MS/MS detection was used. Two transitions were monitored.  The specificity of the method was assured by MS/MS detection. All validation parameters are in the currently required range.  The method is suitable for the intended purposes.  (draft in word format submitted)  The final report of the “*Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Effects on Collembolan Reproduction in an Artificial Soil Study*” (Dini, R.., 2021b – Report No. CH-0240/2021 - KCP 5.1.2/10) is available (in pdf format). |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/10 |
| Report | Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Effects on Collembolan Reproduction in an Artificial Soil Study  Dini, R.  2021b  ChemService S.r.l. Controlli e Richerche, Novate Milanese - Italy  Report No. : CH – 0240/2021 |
| Guideline(s): | Yes : SANTE/2020/12830, rev. 1 dated 24/02/21 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The determination of Difenoconazole residues is performed by HPLC using an external standard and MS triple quadrupole detector.  Its quantification is achieved using the calibration curve obtained by plotting standard solutions concentrations and the corresponding peak area. |

Materials and methods

***1. Materials***

The determination of difenoconazole was assessed by HLPC/MS-QQQ.

|  |  |
| --- | --- |
| HPLC: | Agilent mod. 1200 |
| Column: | Zorbax Eclipse Plus C18, 3.5 µm, 100 x 4.6 mm i.d. |
| Detector | Triple quadrupole Mass Detector |
| Scan type | MRM |
| Ion mode | ESI, positive polarity |
| Interface temperature | 300 °C |
| Dry gas flow | 10 L/min |
| Nebilizing gas flow | 2.9 L/min |
| Eluent flow | 0.7 mL/min |
| Volume of injection | 10 µL |
| Retention time | Approximately 10.6 minutes |
| Total analysis time | 20 minutes |
| Mobile phase: | Water, HPLC grade  Demineralised water  Artificial soil (according to OECD No. 232, 2016 guideline)  Formic acid, high purity for mass spectroscopy  Ammonium formate, high purity for mass spectroscopy  Acetonitrile, HPLC grade |
| Analytical standards: | Difenoconazole  CAS No. : 119446-68-3  Batch No. : BCCD4900  Purity : 95.5 %  Expiry date: June 1, 2025 |

***2. Methods***

The analytical method for the determination of difenoconazole in Matrix-matched standard solutionswith *Collembola* was first validated so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The method was validated in terms of linearity, precision, accuracy, specificity, limits of detection and quantification and stability.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A~~7.1~~ 177: Recovery results from method validation of difenoconazole in Matrix-matched standard solutions with *Collembola* using the analytical method

| Matrix effect | Analyte | Fortification level | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| < ±20 % | Difenoconazole | Low level:  29.0 µg/kg  (mean found) | 105.3 | 2 | No comments |
| < ±20 % | Difenoconazole | High level:  115.36 mg/kg  (mean found) | 98.9 | 2 | No comments |

Table A~~8~~ 178: Characteristics for the analytical method used for validation of difenoconazole in Matrix-matched standard solutions with *Collembola* for purposes of ecotoxicological tests

|  | Difenoconazole |
| --- | --- |
| Specificity | Untreated blank < 30 % LOQ  Result : 2.8 %  This % ratio (Blank vs LOQ) demontrates that there was no interference of the blank matrix at the retention time of the analyte. |
| Linearity / Calibration | ***Quantifier transition***  ***m/z 406.1 🡪 m/z 250.9 :***  The calibration curve (µg/kg):   * was considered as valid over 4.7 – 43.0 µg/kg. * corresponding to range 2.3 – 23.4 µg/L   Correlation coefficient:  r² = 99.605  ***Quantifier 1 transition***  ***m/z 406.1 🡪 m/z 336.9 :***  The calibration curve (µg/kg):   * was considered as valid over 4.7 – 43.0 µg/kg. * corresponding to range 2.3 – 23.4 µg/L   Correlation coefficient:  r² = 99.966  ***Quantifier 2 transition***  ***m/z 406.1 🡪 m/z 188 :***  The calibration curve (µg/kg):   * was considered as valid over 4.7 – 43.0 µg/kg. * corresponding to range 2.3 – 23.4 µg/L   Correlation coefficient:  r² = 99.319  ***Time 0 day : Difenoconazole: calibration with matrix-matched standard solutions***  Equation (µg/kg)  y = 71167 \* x + 32299  Correlation coefficient:  r² = 99.677  ***Time 13 days : Difenoconazole: calibration with matrix-matched standard solution***  Equation (µg/kg)  y = 90128 \* x + 77853  Correlation coefficient:  r² = 99.200  ***Time 28 days : Difenoconazole: calibration with matrix-matched standard solutions***  Equation (µg/kg)  y = 106834 \* x – 3525  Correlation coefficient:  r² = 99.828 |
| Limit of determination (LOD) | LOD = 4.7 µg/kg  Lowest calibration level |
| Limit of quantification (LOQ) | LOQ = 27.5 µg/kg  Lowest fortified level |
| Stability | Recovery Mean between 70% – 120% |
| Stability (7 days) | Low level: 87.0 % |
| Stability of standard | < ±10% |
| Stability of standard (3 days) | 8.3 % |

Conclusion

The analytical method for the difenoconazole in Matrix-matched standard solutions with *Collembola* was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and the stability for difenoconazole.

* + - * 1. Analytical method 11

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  For the determination of Difenoconazole and Prothioconazole residues, all samples were analysed according to the Method No. 0668/2021 validated in the GLP Study CH - 0668/2021 for the feeding solution and according to the Method No. 0232/2021 validated in the GLP Study CH - 0232/2021 for the test chemical solution.  In the method HPLC-MS/MS (LC/MS-QQQ) detection was used. Two transitions were monitored.  The specificity of the method was assured by MS/MS detection. All validation parameters are in the currently required range.  The method is suitable for the intended purposes.  (draft in word format submitted)  The final report of the “*Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Acute Oral and Contact Toxicity to adult worker bumblebees Bombus terrestris L*.” (Ponti, B., 2021a – Report No. CH-0234/2021 - KCP 5.1.2/11) is available (in pdf format). |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/11 |
| Report | Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Acute Oral and Contact Toxicity to adult worker bumblebees *Bombus terrestris* L.  Ponti, B.  2021b  ChemService S.r.l. Controlli e Richerche, Novate Milanese - Italy  Report No. : CH – 0234/2021 |
| Guideline(s): | Yes : SANTE/2020/12830, rev. 1 dated 24/02/21 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The determination of the active ingredient Difenoconazole was performed by HPLC using an external standard and a MS-QQQ detector.  The quantification of the active ingredient is achieved by calculating its concentration in the final solutions in respect to a linear calibration obtained using the working standard solutions prepared starting from the reference material. |

Materials and methods

***1. Materials***

The determination of difenoconazole was assessed by HLPC/MS-QQQ.

|  |  |
| --- | --- |
| HPLC: | Shimadzu mod. 850 |
| Column: | Zorbax Eclipse Plus C18, 3.5 µm, 100 x 4.6 mm i.d. |
| Detector | Triple quadrupole Mass Detector |
| Scan type | MRM |
| Ion mode | ESI, positive polarity |
| Interface temperature | 300 °C |
| Dry gas flow | 10 L/min |
| Nebilizing gas flow | 2.9 L/min |
| Eluent flow | 0.7 mL/min |
| Volume of injection | 10 µL |
| Retention time | Approximately 5.6 minutes |
| Total analysis time | 20 minutes |
| Mobile phase: | Water, HPLC grade  Demineralised water  Feeding solution  Formic acid, high purity for mass spectroscopy  Ammonium formate, high purity for mass spectroscopy  Acetonitrile, HPLC grade |
| Analytical standards: | Difenoconazole  CAS No. : 119446-68-3  Batch No. : BCCD4900  Purity : 95.5 %  Expiry date: June 1, 2025 |

***2. Methods***

The analytical method for the determination of difenoconazole in feeding solutionswith *Bombus terrestris* L. (oral test) was first validated so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The method was validated in terms of linearity, precision, accuracy, specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A~~7.1~~ 179: Recovery results from method validation of difenoconazole in feeding solutions with *Bombus terrestris* L. (oral test) using the analytical method

| Matrix effect | Analyte | Fortification level | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| < ±20 % | Difenoconazole | Low level:  4.01 mg/L  (mean found) | 112.8 | 2 | No comments |
| < ±20 % | Difenoconazole | High level:  4009.19 mg/L  (mean found) | 113.2 | 1 | No comments |

Table A~~8~~ 180: Characteristics for the analytical method used for validation of difenoconazole in feeding solutions with *Bombus terrestris* L. (oral test) for purposes of ecotoxicological tests

|  | Difenoconazole |
| --- | --- |
| Specificity | Untreated blank < 30 % LOQ  Result : 0.6 %  This % ratio (Blank vs LOQ) demontrates that there was no interference of the blank matrix at the retention time of the analyte. |
| Linearity / Calibration | ***Quantifier transition***  ***m/z 406.1🡪 m/z 250.9 :***  The calibration curve (mg/L):   * was considered as valid over 0.02 – 1.89 mg/L.   Correlation coefficient:  r² = 99.934  ***Quantifier 1 transition***  ***m/z 406.1🡪 m/z 336.9 :***  The calibration curve (mg/L):   * was considered as valid over 0.02 – 1.89 mg/L.   Correlation coefficient:  r² = 99.927  ***Quantifier 2 transition***  ***m/z 406.1🡪 m/z 188 :***  The calibration curve (mg/L):   * was considered as valid over 0.02 – 1.89 mg/L.   Correlation coefficient:  r² = 99.938  ***Difenoconazole – oral test (feeding solution): Linear calibration :***  Equation (mg/L)  y = 25861577 \* x – 694931  Correlation coefficient:  r² = 99.949 |
| Limit of determination (LOD) | LOD = 0.02 mg/L  Lowest calibration level |
| Limit of quantification (LOQ) | LOQ = 3.56 mg/L  Lowest fortified level |

Conclusion

The analytical method for the difenoconazole in feeding solutions with *Bombus terrestris* L. (oral test) was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects and limits of determination and quantification for difenoconazole.

* + - * 1. Analytical method 12

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  For the determination of Difenoconazole and Prothioconazole residues, all samples were analysed according to the Method No. 0668/2021 validated in the GLP Study CH - 0668/2021 for the feeding solution and according to the Method No. 0232/2021 validated in the GLP Study CH - 0232/2021 for the test chemical solution.  In the method HPLC-MS/MS (LC/MS-QQQ) detection was used. Two transitions were monitored.  The specificity of the method was assured by MS/MS detection. All validation parameters are in the currently required range.  The method is suitable for the intended purposes.  (draft in word format submitted)  The final report of the “*Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Acute Oral and Contact Toxicity to adult worker bumblebees Bombus terrestris L*.” (Ponti, B., 2021a – Report No. CH-0234/2021 - KCP 5.1.2/11) is available (in pdf format). |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/11 |
| Report | Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Acute Oral and Contact Toxicity to adult worker bumblebees *Bombus terrestris* L.  Ponti, B.  2021a  ChemService S.r.l. Controlli e Richerche, Novate Milanese - Italy  Report No. : CH – 0234/2021 |
| Guideline(s): | Yes : SANTE/2020/12830, rev. 1 dated 24/02/21 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The determination of Difenoconazole residues is performed by HPLC using an external standard and MS triple quadrupole detector.  Its quantification is achieved using the calibration curve obtained by plotting standard solutions concentrations and the corresponding peak area. |

Materials and methods

***1. Materials***

The determination of difenoconazole was assessed by HLPC/MS-QQQ.

|  |  |
| --- | --- |
| HPLC: | Shimadzu mod. 850 |
| Column: | Zorbax Eclipse Plus C18, 3.5 µm, 100 x 4.6 mm i.d. |
| Detector | Triple quadrupole Mass Detector |
| Scan type | MRM |
| Ion mode | ESI, positive polarity |
| Interface temperature | 300 °C |
| Dry gas flow | 10 L/min |
| Nebilizing gas flow | 2.9 L/min |
| Eluent flow | 0.7 mL/min |
| Volume of injection | 10 µL |
| Retention time | Approximately 5.6 minutes |
| Total analysis time | 20 minutes |
| Mobile phase: | Water, HPLC grade  Demineralised water  Feeding solution  Formic acid, high purity for mass spectroscopy  Ammonium formate, for HPLC  Acetonitrile, HPLC grade |
| Analytical standards: | Difenoconazole  CAS No. : 119446-68-3  Batch No. : BCCD4900  Purity : 95.5 %  Expiry date: June 1, 2025 |

***2. Methods***

The analytical method for the determination of difenoconazole in feeding solutionswith *Bombus terrestris* L. (contact test) was first validated so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The method was validated in terms of linearity, precision, accuracy, specificity, limits of detection and quantification and stability.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A~~7.1~~ 181: Recovery results from method validation of difenoconazole in feeding solutions with *Bombus terrestris* L. (contact test) using the analytical method

| Matrix effect | Analyte | Fortification level | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| < ±20 % | Difenoconazole | Low level:  0.12 mg/L  (mean found) | 105.4 | 4 | No comments |
| < ±20 % | Difenoconazole | High level:  7385.95 mg/L  (mean found) | 104.0 | 5 | No comments |
| < ±20 % | Difenoconazole | Ultra-High level:  60116.85 mg/L  (mean found) | 112.9 | 5 | No comments |

Table A~~8~~ 182: Characteristics for the analytical method used for validation of difenoconazole in feeding solutions with *Bombus terrestris* L. (contact test) for purposes of ecotoxicological tests

|  | Difenoconazole |
| --- | --- |
| Specificity | Untreated blank < 30 % LOQ  Result : 0.4 %  This % ratio (Blank vs LOQ) demontrates that there was no interference of the blank matrix at the retention time of the analyte. |
| Linearity / Calibration | ***Quantifier transition***  ***m/z 406.1 🡪 m/z 250.9 :***  The calibration curve (mg/L):   * was considered as valid over 0.02 – 1.95 mg/L.   Correlation coefficient:  r² = 99.991  ***Quantifier 1 transition***  ***m/z 406.1 🡪 m/z 336.9 :***  The calibration curve (mg/L):   * was considered as valid over 0.02 – 1.95 mg/L..   Correlation coefficient:  r² = 99.990  ***Quantifier 2 transition***  ***m/z 406.1 🡪 m/z 188 :***  The calibration curve (mg/L):   * was considered as valid over 0.02 – 1.95 mg/L..   Correlation coefficient:  r² = 99.991  ***Difenoconazole – contact test (feeding solution): Linear calibration :***  Equation (mg/L) :  y = 24786407 \* x – 668897  Correlation coefficient:  r² = 99.951 |
| Limit of determination (LOD) | LOD = 0.02 mg/L  Lowest calibration level |
| Limit of quantification (LOQ) | LOQ = 0.11 mg/L  Lowest fortified level |
| Stability | Recovery Mean between 70% - 120% |
| Stability (5 days) | Low level: 115.1 % |
| Stability of standard | <± 10% |
| Stability of standard (5 days) | -15.6%  Not stable, the standards are prepared always freshly |

Conclusion

The analytical method for the difenoconazole in feeding solutions with *Bombus terrestris* L. (contact test) was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability for difenoconazole.

* + - * 1. Analytical method 13

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  The method No. 0235/2021 for Difenoconazole and Prothioconazole determination in soil samples of test item, which was validated in GLP Study CH-0235/2021 was employed. In the method HPLC-MS/MS detection was used. Two transitions were monitored. Since the analysis performed by HPLC/MS/MS gave both quantification and identification data, a confirmatory test using another instrumental technique was not necessary.  The specificity of the method was assured by MS/MS detection. All validation parameters are in the currently required range.  The method is suitable for the intended purposes.  (draft in word format submitted)  The final report of the “*Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Effects on Hypoaspis (Geolaelaps) aculeifer Reproduction in an Artificial Soil Study*” (Dini, R., 2021c – Report No. CH-0241/2021 - KCP 5.1.2/12) is available (in pdf format). |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/12 |
| Report | Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Effects on *Hypoaspis (Geolaelaps) aculeifer* Reproduction in an Artificial Soil Study  Dini, R.  2021c  ChemService S.r.l. Controlli e Richerche, Novate Milanese - Italy  Report No. : CH – 0241/2021 |
| Guideline(s): | Yes : SANTE/2020/12830, rev. 1 dated 24/02/21 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The determination of Difenoconazole residues is performed by HPLC using an external standard and MS triple quadrupole detector.  Its quantification is achieved using the calibration curve obtained by plotting standard solutions concentrations and the corresponding peak area. |

Materials and methods

***1. Materials***

The determination of difenoconazole was assessed by HLPC/MS-MS.

|  |  |
| --- | --- |
| HPLC: | Shimadzu mod. 850 |
| Column: | Zorbax Eclipse Plus C18, 3.5 µm, 100 x 4.6 mm i.d. |
| Detector | Triple quadrupole Mass Detector |
| Scan type | MRM |
| Ion mode | ESI, positive polarity |
| Interface temperature | 300 °C |
| Dry gas flow | 10 L/min |
| Nebilizing gas flow | 2.9 L/min |
| Eluent flow | 0.7 mL/min |
| Volume of injection | 10 µL |
| Retention time | Approximately 10.6 minutes |
| Total analysis time | 20 minutes |
| Mobile phase: | Water, HPLC grade  Artificial soil  Formic acid, high purity for mass spectroscopy  Ammonium formate, high purity for mass spectroscopy  Acetonitrile, HPLC grade |
| Analytical standards: | Difenoconazole  CAS No. : 119446-68-3  Batch No. : BCCD4900  Purity : 95.5 %  Expiry date: June 1, 2025 |

***2. Methods***

The analytical method for the determination of difenoconazole in artificial soilwith *Hypoaspis (Geolaelaps) aculeifer* was first validated so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The method was validated in terms of linearity, precision, accuracy, specificity, limits of detection and quantification and stability.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A~~7.1~~ 183: Recovery results from method validation of difenoconazole in artificial soil with Hypoaspis (*Geolaelaps*) aculeifer using the analytical method

| Matrix effect | Analyte | Fortification level | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| < ±20 % | Difenoconazole | Low level:  28.9 mg/kg  (mean found) | 105.2 | 2 | No comments |
| < ±20 % | Difenoconazole | High level:  116.02 mg/kg  (mean found) | 99.5 | 2 | No comments |

Table A~~8~~ 184: Characteristics for the analytical method used for validation of difenoconazole in artificial soil with Hypoaspis (*Geolaelaps*) aculeifer for purposes of ecotoxicological tests

|  | Difenoconazole |
| --- | --- |
| Specificity | Untreated blank < 30 % LOQ  Result : 2.9 %  This % ratio (Blank vs LOQ) demontrates that there was no interference of the blank matrix at the retention time of the analyte. |
| Linearity / Calibration | ***Quantifier transition***  ***m/z 406.1 🡪 m/z 250.9 :***  The calibration curve (µg/kg):   * was considered as valid over 4.7 – 43.0 µg/kg. * corresponding to range 2.3 – 23.4 µg/L   Correlation coefficient:  r² = 99.592  ***Quantifier 1 transition***  ***m/z 406.1 🡪 m/z 336.9 :***  The calibration curve (µg/kg):   * was considered as valid over 4.7 – 43.0 µg/kg. * corresponding to range 2.3 – 23.4 µg/L   Correlation coefficient:  r² = 99.592  ***Quantifier 2 transition***  ***m/z 406.1 🡪 m/z 188 :***  The calibration curve (µg/kg):   * was considered as valid over 4.7 – 43.0 µg/kg. * corresponding to range 2.3 – 23.4 µg/L   Correlation coefficient:  r² = 99.592  ***Time 0 days : Difenoconazole –Linear calibration :***  Equation (µg/kg) :  y = 72255 \* x – 19374  Correlation coefficient:  r² = 99.824  ***Time 7 days : Difenoconazole –Linear calibration :***  Equation (µg/kg) :  y = 107597 \* x – 47173  Correlation coefficient:  r² = 99.947  ***Time 14 days : Difenoconazole –Linear calibration :***  Equation (µg/kg) :  y = 103926 \* x – 211071  Correlation coefficient:  r² = 99.731 |
| Limit of determination (LOD) | LOD = 4.7 µg/kg  Lowest calibration level |
| Limit of quantification (LOQ) | LOQ = 27.5 µg/kg  Lowest fortified level |
| Stability | Recovery Mean between 70% - 120% |
| Stability (5 days) | Low level: 87.0 % |
| Stability of standard | <± 10% |
| Stability of standard (5 days) | 8.3% |

Conclusion

The analytical method for the difenoconazole in artificial soilwith *Hypoaspis (Geolaelaps) aculeifer* was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability for difenoconazole.

* + - * 1. Analytical method 14

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  The method No. 0668/2021 for Difenoconazole and Prothioconazole determination in soil samples of test item, which was validated in GLP Study CH-0668/2021 was employed. In the method HPLC/MS-QQQ detection was used. Two transitions were monitored. Since the analysis performed by HPLC/MS-QQQ gave both quantification and identification data, a confirmatory test using another instrumental technique was not necessary.  The specificity of the method was assured by MS/MS detection. All validation parameters are in the currently required range.  The method is suitable for the intended purposes.  (draft in word format submitted)  The final report of the “*Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Chronic Oral Toxicity to adult worker honeybees Apis mellifera L. (10-day feeding)”* (Ponti, B., 2021b – Report No. CH-0669/2021 - KCP 5.1.2/13) is available (in pdf format). |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/13 |
| Report | Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560: Chronic Oral Toxicity to adult worker honeybees *Apis mellifera* L. (10-day feeding)  Ponti, B.  2021b  ChemService S.r.l. Controlli e Richerche, Novate Milanese - Italy  Report No. : CH – 0669/2021 |
| Guideline(s): | Yes : SANTE/2020/12830, rev. 1 dated 24/02/21 |
| Deviations: | Yes  Deviation 1   |  |  | | --- | --- | | Change No. 1 | The expiry date of the reference item is December 01, 2025 | | Reason of change: | In the study plan was wrongly reported (typing error), as expiry date, August 01, 2025 | | Impact on the study: | None. | |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The determination of the active ingredient Difenoconazole was performed by HPLC using an external standard and a MS-QQQ detector.  The quantification of the active ingredient is achieved by calculating its concentration in the final solutions in respect to a linear calibration obtained using the working standard solutions prepared starting from the reference material. |

Materials and methods

***1. Materials***

The determination of difenoconazole was assessed by HLPC/MS-QQQ.

|  |  |
| --- | --- |
| HPLC: | Shimadzu mod. 850 |
| Column: | Zorbax Eclipse Plus C18, 3.5 µm, 100 x 4.6 mm i.d. |
| Detector | Triple quadrupole Mass Detector |
| Scan type | MRM |
| Ion mode | ESI, positive polarity |
| Interface temperature | 300 °C |
| Dry gas flow | 10 L/min |
| Nebilizing gas flow | 2.9 L/min |
| Eluent flow | 0.7 mL/min |
| Volume of injection | 10 µL |
| Retention time | Approximately 7.4 minutes |
| Total analysis time | 20 minutes |
| Mobile phase: | Water, HPLC grade  Feeding solution  Formic acid, high purity for mass spectroscopy  Ammonium formate, high purity for mass spectroscopy  Acetonitrile, HPLC grade |
| Analytical standards: | Difenoconazole  CAS No. : 119446-68-3  Batch No. : BCCD4900  Purity : 95.5 %  Expiry date: June 1, 2025 |

***2. Methods***

The analytical method for the determination of difenoconazole in feeding solutionwith *Apis mellifera* L*.*was first validated so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The method was validated in terms of linearity, precision, accuracy, specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A~~7.1~~ 185: Recovery results from method validation of difenoconazole in feeding solution with *Apis mellifera* L. using the analytical method

| Matrix effect | Analyte | Fortification level | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| < ±20 % | Difenoconazole | Low level:  4.01 mg/L  (mean found) | 112.8 | 2 | No comments |
| < ±20 % | Difenoconazole | High level:  4009.19 mg/L  (mean found) | 113.2 | 1 | No comments |

Table A~~8~~ 186: Characteristics for the analytical method used for validation of difenoconazole in feeding solution with *Apis mellifera* L. for purposes of ecotoxicological tests

|  | Difenoconazole |
| --- | --- |
| Specificity | Untreated blank < 30 % LOQ  Result : 0.6 %  This % ratio (Blank vs LOQ) demontrates that there was no interference of the blank matrix at the retention time of the analyte. |
| Linearity / Calibration | ***Quantifier transition***  ***m/z 406.1 🡪 m/z 250.9 :***  The calibration curve (mg/L):   * was considered as valid over 0.02 – 1.89 mg/L.   Correlation coefficient:  r² = 99.934  ***Quantifier 1 transition***  ***m/z 406.1 🡪 m/z 336.9 :***  The calibration curve (µg/kg):   * was considered as valid over over 0.02 – 1.89 mg/L..   Correlation coefficient:  r² = 99.927  ***Quantifier 2 transition***  ***m/z 406.1 🡪 m/z 188 :***  The calibration curve (mg/L):   * was considered as valid over over 0.02 – 1.89 mg/L.   Correlation coefficient:  r² = 99.938  ***Difenoconazole content –Linear calibration :***  Equation (mg/L) :  y = 165001707 \* x – 6679423  Correlation coefficient:  r² = 99.891 |
| Limit of determination (LOD) | LOD = 0.02 mg/L  Lowest calibration level |
| Limit of quantification (LOQ) | LOQ = 3.56 mg/L  Lowest fortified level |

Conclusion

The analytical method for the difenoconazole in feeding solutionwith *Apis mellifera* L*.* was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects and limits of determination and quantification for difenoconazole.

* + - * 1. Analytical method 15

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  The analytical method for the determination of TDMs was based on the method “Quick Method for the Analysis of Highly Polar Pesticides in Food Involving Extraction with Acidified Methanol and LC- or IC-MS/MS Measurement I. - Food of Plant Origin (QuPPe-PO-Method) - Method 8 (M8)”.  The determination was carried out using a HPLC-MS/MS system (high-performance liquid chromatography + triple quadrupole mass spectrometry) equipped with a differential mobility separation device (DMS).  The specificity of the method was assured by MS/MS detection. All validation parameters are in the currently required range.  The matrix effect was not significant according to the SANTE/2020/12830 rev.1 except for triazole-alanine in whole OSR plant.  See also into the present section B7 where this validated method was employed to generation of the data in paragraphs of Appendix 2: A 2.1.3.1.1,2,3; A 2.1.5.2.1,2,3; A 2.2.3.1.1,2,3; A 2.2.5.2.1,2,3.  Independent laboratory validation ~~is ongoing and~~ is included in study plans submitted (CH-1090/2021, CH-1085/2021, CH-1087/2021, CH-1086/2021).  The studies have been submitted – relevant paragraph for evaluation details. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/14 |
| Report | Validation of an analytical method for the quantification of Triazole Derivative Metabolites (TDMs) in wheat, barley, oilseed rape and processed commodities  Longhi, D.  2021b  ChemService S.r.l. Controlli e Richerche, Novate Milanese - Italy  Report No. : 21-108 |
| Guideline(s): | Yes : SANTE/2020/12830 rev. 1 (dated 24/02/2021) ;  SANTE2017/10632 rev. 3 (dated 22/11/2017) ;  OECD ENV/JM/MONO(2007)17 ;  “Quick Method for the Analysis of Highly Polar Pesticides in Food Involving Extraction with Acidified Methanol and LC- or IC-MS/MS Measurement - Food of Plant Origin (QuPPe-PO-Method)- Method 8 (M8)”. |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The analytical method for the determination of TDMs in the tested matrices (AM-GLP-STUDY-21-108) was based on the method “Quick Method for the Analysis of Highly Polar Pesticides in Food Involving Extraction with Acidified Methanol and LC- or IC-MS/MS Measurement I. - Food of Plant Origin (QuPPe-PO-Method) - Method 8 (M8)”.  The instrumental determination was carried out using a HPLC-MS/MS system (high-performance liquid chromatography + triple quadrupole mass spectrometry) equipped with a differential mobility separation device (DMS). |

Materials and methods

***1. Materials***

***1.A. Quantification of triazole derivative metabolites in wheat, barley, oilseed rape and processed commodities***

The quantification of triazole derivative metabolites in wheat, barley, oilseed rape and processed commodities was assessed by HLPC/MS/MS.

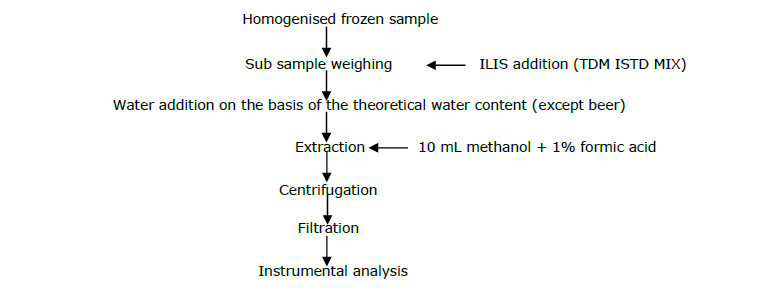
|  |  |
| --- | --- |
| HPLC: | Shimadzu LC-40 XR + spectrometer Sciex API 6500+ equipped with SelexION (Differential Mobility Separation) device |
| Column: | Thermo Hyperbare 5 μm, 2.1 x 100 mm |
| Detector: | Agilent MS spectrometer 6470A Triple Quad |
| Column temperature: | 40 °C |
| Flow rate: | 0.6 mL/min |
| Volume of injection: | 2 µL |
| Retention time: | Approximatively 2.5 minutes for difenoconazole |
| Stop time: | 10 minutes |
| Gas temperature: | 500 °C |
| Curtain Gas flow: | 30 mL/min |
| Gas flow 1: | 55 mL/min |
| Gas flow2: | 65mL/min |
| Capillary: | Positive mode 3500 V |
| Mobile phase: | A: LC-MS grade water with 1% acetic acid  B: LC-MS grade methanol with 1% acetic acid |
| Mixture-Elution: | |  |  |  | | --- | --- | --- | | % A | % B | Time (min) | | 95 | 5 | 0 | | 10 | 90 | 5 | | 10 | 90 | 6 | | 95 | 5 | 6.1 | |
| Analytical standards: | 1,2,4-triazole (1,2,4-TRZ)  CAS No. : 288-88-0  Lot: STBJ5727  Purity : 100.3% (considered 100% in the calculation)  Expiry date: December 2021  1,2,4-Triazole Alanime (TA)  CAS No. : 86362-20-1  Lot: 787796  Purity : 98.3%  Expiry date: 01/03/2024  1,2,4-Triazole lactic acid HCl (TLA)  CAS No. : 1450828-63-3  Lot: 792058  Purity : 78.5%  Expiry date: 01/11/2024  1,2,4-Triazole acetic acid (TAA)  CAS No. : 28711-29-7  Lot: BCCC0969  Purity : 95.7%  Expiry date: December 2021 |
| Isotope-labelled internal standards (ILIS): | 1,2,4-Triazole-[13C2,15N3]  CAS No. : 1261170-82-4  Lot: SL6-2012-224  Purity : 98.4%  Expiry date: 01/2023  1,2,4-Triazole Alanine [D2]  CAS No. : 2180306-38-9  Lot: 2011202L3.3  Purity : 95%  Expiry date: 20/01/2023  1,2,4-Triazole-[13C2, 15N3] Lactic Acid  CAS No. : n.d.  Lot: EFL6-2015-198A  Purity : 98.42%  Expiry date: 01/2024  1,2,4-Triazole acetic acid [13C2, 15N3]  CAS No. : n.d.  Lot: EFL6-2015-196A  Purity : 98.03%  Expiry date: 01/2024 |

***2. Methods***

The analytical method for the quantification of triazole derivatives metabolites in the tested matrices was based on the QuEChERS method (Method 8).

***2.A. Schematic diagram of the analytical method***

Plant matrices and processed commodities (rapeseed whole plant, rapeseed oil, rapeseed seeds, wheat grain, white bread (wheat), wheat straw and barley beer)



***2.B. Quantification of triazole derivative metabolites in wheat, barley, oilseed rape and processed commodities***

The analytical methods for the quantification of triazole derivatives metabolites in wheat, barley, rapeseed and processed commodities were first validated so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were validated in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |

Results and discussions

Method validation data can be summarised in tables below. ~~Table A.1and A2.1 to A2.5. There are for each matrix a primary test. In view of the similar results between the primary and confirmatory test, a test by an independent laboratory validation (ILV) is not required.~~

Table A~~1~~ 187: Recovery results from method validation of triazole derivate metabolites using the analytical method

| Matrix | Analyte | Fortification level  (*n* = x) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Whole Plant (Rapeseed) | 1,2,4-TRZ | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  87.0 % | ***Primary transition***:  7.2 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  98.7 % | ***Primary transition***:  4.1 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  98.7 % | ***Primary transition***:  2.9 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  99.5 % | ***Primary transition***:  2.3 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TLA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  101.3 % | ***Primary transition***:  1.8 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  99.8 % | ***Primary transition***:  1.5 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TAA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  100.8 % | ***Primary transition***:  1.7 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  100.0 % | ***Primary transition***:  1.3 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Rapeseed seeds | 1,2,4-TRZ | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  99.0 % | ***Primary transition***:  2.3 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  93.9% | ***Primary transition***:  4.3 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  102.0 % | ***Primary transition***:  4.6 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  112.0 % | ***Primary transition***:  1.6 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TLA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  113.0 % | ***Primary transition***:  1.7 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  106.6 % | ***Primary transition***:  0.9 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TAA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  102.9 % | ***Primary transition***:  3.2 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  99.1 % | ***Primary transition***:  1.3 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Grain (wheat) | 1,2,4-TRZ | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  87.3 % | ***Primary transition***:  8.6 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  97.7 % | ***Primary transition***:  5.4 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  91.2 % | ***Primary transition***:  6.3 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  106.5 % | ***Primary transition***:  1.5 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TLA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  104.2 % | ***Primary transition***:  3.4 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  104.6 % | ***Primary transition***:  1.9 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TAA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  100.0 % | ***Primary transition***:  4.2 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  101.9 % | ***Primary transition***:  2.1 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Straw (wheat) | 1,2,4-TRZ | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  93.9 % | ***Primary transition***:  6.6 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  95.6 % | ***Primary transition***:  2.2 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  100.0 % | ***Primary transition***:  5.2 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  99.0 % | ***Primary transition***:  3.8 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TLA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  99.5 % | ***Primary transition***:  4.8 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  99.7 % | ***Primary transition***:  1.2 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TAA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  98.9 % | ***Primary transition***:  6.7 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  101.2 % | ***Primary transition***:  1.0 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Rapeseed oil | 1,2,4-TRZ | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  94.2 % | ***Primary transition***:  8.4 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  98.4 % | ***Primary transition***:  2.5 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  100.6 % | ***Primary transition***:  1.5 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  100.0 % | ***Primary transition***:  1.5 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TLA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  100.2 % | ***Primary transition***:  2.4 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  97.7 % | ***Primary transition***:  1.2 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TAA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  103.0 % | ***Primary transition***:  1.6 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  99.4 % | ***Primary transition***:  0.8 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| White bread (wheat) | 1,2,4-TRZ | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  97.7 % | ***Primary transition***:  3.4 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  98.7 % | ***Primary transition***:  4.3 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  99.9 % | ***Primary transition***:  3.8 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  100.3 % | ***Primary transition***:  1.0 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TLA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  99.9 % | ***Primary transition***:  3.8 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  100.3 % | ***Primary transition***:  1.0 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TAA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  101.1 % | ***Primary transition***:  2.5 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  98.5 % | ***Primary transition***:  0.9 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Beer (barley) | 1,2,4-TRZ | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  102.6 % | ***Primary transition***:  3.1 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  101.6 % | ***Primary transition***:  4.7 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  104.1 % | ***Primary transition***:  12.5 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  109.4 % | ***Primary transition***:  4.8 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TLA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  97.6 % | ***Primary transition***:  3.0 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  97.5 % | ***Primary transition***:  3.7% | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TAA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Primary transition***:  100.1 % | ***Primary transition***:  17.5 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Primary transition***:  103.1 % | ***Primary transition***:  1.6 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

Table A~~2.1~~ 188: Characteristics for the analytical method used for validation of triazole derivative metabolites residues in whole plant (rapeseed)

|  | TDMs |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  **Primarity transition:**  ***1,2,4-TRZ***  % interference mean = 6  ***TA***  % interference mean = 14.5  ***TLA***  % interference mean = 4  ***TAA***  % interference mean = 0.5 |
| Calibration (type, number of data points) | **Primarity transition:**  ***1,2,4-TRZ***  Equation : Y = 1.03477 \* x + 0.07821  Coefficient of correlation: r² = 99.984  ***TA***  Equation : Y = 0.51285 \* x + 0.02730  Coefficient of correlation: r² = 99.962  ***TLA***  Equation : Y = 1.20823 \* x + 0.02456  Coefficient of correlation: r² = 99.994  ***TAA***  Equation : Y = 0.98409 \* x + 0.01569  Coefficient of correlation: r² = 99.966 |
| Calibration range | Accepted calibration range in concentration units 0.5 – 50.0 µg/L (from 20 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes : Performed comparing the analyte response of one individual standard at L4 (or L5) prepared in solvent (water/methanol 50:50 + 1% formic acid) to one prepared in blank matrix extract at the same concentration.  ***1,2,4-TRZ***  Matrix effect = 19.5 %  Not significant  ***TA***  Matrix effect = -77.5 %  Significant in accordance to the SANTE/2020/12830 rev. 1 guideline  ***TLA***  Matrix effect = -8.1 %  Not significant  ***TAA***  Matrix effect = - 2.7 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | According to SANTE/2020/12830 rev.1, the stability of the analytes in the final extracts is not required if the extracts contain an isotope-labelled internal standard (IL-IS) for quantification, since the IL-IS compensates for losses during extract storage. Since IL-IS were used, the verification of the stability was not required. Anyway, all the extracts were analysed within 24 hours from their preparation, obtaining recoveries in the fortified samples within the acceptable range of 70-120%.  In addition, since the differences between the mean responses of the analytes in the diluted solutions prepared form stored and from freshly-prepared stock solutions was < 10%, it is possible to assess that the analytes are stable in the stock solutions prepared in water for at least 78 days, if stored in the dark at 5 ± 3°C. |

Table A~~2.2~~ 189: Characteristics for the analytical method used for validation of triazole derivatives metabolites in rapeseed oil

|  | TDMs |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  **Primarity transition:**  ***1,2,4-TRZ***  % interference mean = 5  ***TA***  % interference mean = 2.5  ***TLA***  % interference mean = 0.5  ***TAA***  % interference mean = 0 |
| Calibration (type, number of data points) | **Primarity transition:**  ***1,2,4-TRZ***  Equation : Y = 0.94482 \* x + 0.03984  Coefficient of correlation: r² = 99.912  ***TA***  Equation : Y = 2.37172 \* x + 0.01580  Coefficient of correlation: r² = 99.926  ***TLA***  Equation : Y = 1.22940 \* x – 0.00528  Coefficient of correlation: r² = 99.924  ***TAA***  Equation : Y = 0.98796 \* x + 1.07526e-4  Coefficient of correlation: r² = 99.940 |
| Calibration range | Accepted calibration range in concentration units 0.5 – 50.0 µg/L (from 20 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes : Performed comparing the analyte response of one individual standard at L4 (or L5) prepared in solvent (water/methanol 50:50 + 1% formic acid) to one prepared in blank matrix extract at the same concentration.  ***1,2,4-TRZ***  Matrix effect = -2.7 %  Not significant  ***TA***  Matrix effect = -3.2 %  Not significant  ***TLA***  Matrix effect = -5.4 %  Not significant  ***TAA***  Matrix effect = -2.5 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | According to SANTE/2020/12830 rev.1, the stability of the analytes in the final extracts is not required if the extracts contain an isotope-labelled internal standard (IL-IS) for quantification, since the IL-IS compensates for losses during extract storage. Since IL-IS were used, the verification of the stability was not required. Anyway, all the extracts were analysed within 24 hours from their preparation, obtaining recoveries in the fortified samples within the acceptable range of 70-120%.  In addition, since the differences between the mean responses of the analytes in the diluted solutions prepared form stored and from freshly-prepared stock solutions was < 10%, it is possible to assess that the analytes are stable in the stock solutions prepared in water for at least 78 days, if stored in the dark at 5 ± 3°C. |

Table A~~2.3~~ 190: Characteristics for the analytical method used for validation of triazole derivative metabolites in rapeseed seeds- grain (wheat) - white bread (wheat) (calibration in solvent 0.5-50 µg/L)

|  | TDMs |
| --- | --- |
| Specificity | **RAPESEED SEEDS**  ***For, 1,2,4-TRZ, TLA and TAA*** : No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***For TA*** : Signals were detected in amounts higher than the 30% of LOQ for this matrice and were generated by the analytes and not by other interfering substanes.However, a lot of attempts were carried out by the laboratory to find samples with analytes area < 30% of LOQ, analysing a lot of commercially available commodities coming from organic farming: no samples with analytes area < 30% of LOQ were found for this matrice.  **Primarity transition:**  ***1,2,4-TRZ***  % interference mean = 8  ***TA***  % interference mean = 218.5  ***TLA***  % interference mean = 12  ***TAA***  % interference mean = 3.5  **GRAIN WHEAT**  ***For, 1,2,4-TRZ and TLA*** : No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***For TA*** ***and TAA***: Signals were detected in amounts higher than the 30% of LOQ for this matrice and were generated by the analytes and not by other interfering substanes.However, a lot of attempts were carried out by the laboratory to find samples with analytes area < 30% of LOQ, analysing a lot of commercially available commodities coming from organic farming: no samples with analytes area < 30% of LOQ were found for this matrice.  **Primarity transition:**  ***1,2,4-TRZ***  % interference mean = 28  ***TA***  % interference mean = 322.5  ***TLA***  % interference mean = 5  ***TAA***  % interference mean = 93.5  **WHITE BREAD (WHEAT)**  ***For, 1,2,4-TRZ and TLA*** : No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***For TA and TAA*** : Signals were detected in amounts higher than the 30% of LOQ for this matrice and were generated by the analytes and not by other interfering substanes.However, a lot of attempts were carried out by the laboratory to find samples with analytes area < 30% of LOQ, analysing a lot of commercially available commodities coming from organic farming: no samples with analytes area < 30% of LOQ were found for this matrice.  **Primarity transition:**  ***1,2,4-TRZ***  % interference mean = 0  ***TA***  % interference mean = 56.5  ***TLA***  % interference mean = 2  ***TAA***  % interference mean = 44.5 |
| Calibration (type, number of data points) | **Primarity transition:**  ***1,2,4-TRZ***  Equation : Y = 0.89935 \* x + 0.04423  Coefficient of correlation: r² = 99.940  ***TA***  Equation : Y = 2.76713 \* x + 0.02212  Coefficient of correlation: r² = 99.966  ***TLA***  Equation : Y = 1.02675 \* x – 9.58870e-4  Coefficient of correlation: r² = 99.898  ***TAA***  Equation : Y = 1.00664 \* x + 0.00803  Coefficient of correlation: r² = 99.996 |
| Calibration range | Accepted calibration range in concentration units 0.5 – 50.0 µg/L (from 20 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes : Performed comparing the analyte response of one individual standard at L4 (or L5) prepared in solvent (water/methanol 50:50 + 1% formic acid) to one prepared in blank matrix extract at the same concentration.  **RAPESEED SEEDS**  ***1,2,4-TRZ***  Matrix effect = -4.5 %  Not significant  ***TA***  Matrix effect = 6.0 %  Not significant  ***TLA***  Matrix effect = 6.7 %  Not significant  ***TAA***  Matrix effect = -2.1 %  Not significant  **GRAIN WHEAT**  ***1,2,4-TRZ***  Matrix effect = -7.7 %  Not significant  ***TA***  Matrix effect = -2.5 %  Not significant  ***TLA***  Matrix effect = -5.0 %  Not significant  ***TAA***  Matrix effect = -4.0 %  Not significant  **WHITE BREAD (WHEAT)**  ***1,2,4-TRZ***  Matrix effect = 3.5 %  Not significant  ***TA***  Matrix effect = 7.1 %  Not significant  ***TLA***  Matrix effect = 0.6 %  Not significant  ***TAA***  Matrix effect = -1.8 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | According to SANTE/2020/12830 rev.1, the stability of the analytes in the final extracts is not required if the extracts contain an isotope-labelled internal standard (IL-IS) for quantification, since the IL-IS compensates for losses during extract storage. Since IL-IS were used, the verification of the stability was not required. Anyway, all the extracts were analysed within 24 hours from their preparation, obtaining recoveries in the fortified samples within the acceptable range of 70-120%.  In addition, since the differences between the mean responses of the analytes in the diluted solutions prepared form stored and from freshly-prepared stock solutions was < 10%, it is possible to assess that the analytes are stable in the stock solutions prepared in water for at least 78 days, if stored in the dark at 5 ± 3°C. |

Table A~~2.4~~ 159: Characteristics for the analytical method used for validation of triazole derivative metabolites residues in straw (wheat) (calibration in solvent, 0.35 – 35 µg/L-

|  | TDMs |
| --- | --- |
| Specificity | ***For, 1,2,4-TRZ and TA*** : No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***For TLA and TAA*** : Signals were detected in amounts higher than the 30% of LOQ for this matrice and were generated by the analytes and not by other interfering substanes.However, a lot of attempts were carried out by the laboratory to find samples with analytes area < 30% of LOQ, analysing a lot of commercially available commodities coming from organic farming: no samples with analytes area < 30% of LOQ were found for this matrice. In addition no samples with analytes content < LOD, set to 20% of LOQ (28% for straw) were found. Since no matrices free from the analytes were available and the matrix effect for this matrice was negligible, it was necessary to calibrate using solvent-based standard solutions. Recoveries were necessarily evaluated subtracting the mean values measured from a duplicate analysis of the untreated samples to the values measured for the fortified ones.  **Primarity transition:**  ***1,2,4-TRZ***  % interference mean = 0  ***TA***  % interference mean = 9  ***TLA***  % interference mean = 56.5  ***TAA***  % interference mean = 126 |
| Calibration (type, number of data points) | **Primarity transition:**  ***1,2,4-TRZ***  Equation : Y = 0.97109 \* x + 0.00769  Coefficient of correlation: r² = 99.848  ***TA***  Equation : Y = 5.15827 \* x + 0.09160  Coefficient of correlation: r² = 99.988  ***TLA***  Equation : Y = 1.19902 \* x + 0.01208  Coefficient of correlation: r² = 99.956  ***TAA***  Equation : Y = 1.03861 \* x + 4.16121e-4  Coefficient of correlation: r² = 99.962 |
| Calibration range | Accepted calibration range in concentration units 0.35 – 35.0 µg/L (from 28 % of LOQ to 180 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.0028 – 0.280 mg/kg) |
| Assessment of matrix effects is presented | Yes : Performed comparing the analyte response of one individual standard at L4 (or L5) prepared in solvent (water/methanol 50:50 + 1% formic acid) to one prepared in blank matrix extract at the same concentration.  ***1,2,4-TRZ***  Matrix effect = 0.4 %  Not significant  ***TA***  Matrix effect = 1.3 %  Not significant  ***TLA***  Matrix effect = -7.9 %  Not significant  ***TAA***  Matrix effect = -3.7 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.35 µg/L  (0.0028 mg/kg) |
| Stability | According to SANTE/2020/12830 rev.1, the stability of the analytes in the final extracts is not required if the extracts contain an isotope-labelled internal standard (IL-IS) for quantification, since the IL-IS compensates for losses during extract storage. Since IL-IS were used, the verification of the stability was not required. Anyway, all the extracts were analysed within 24 hours from their preparation, obtaining recoveries in the fortified samples within the acceptable range of 70-120%.  In addition, since the differences between the mean responses of the analytes in the diluted solutions prepared form stored and from freshly-prepared stock solutions was < 10%, it is possible to assess that the analytes are stable in the stock solutions prepared in water for at least 78 days, if stored in the dark at 5 ± 3°C. |

Table A~~2.5~~ 192: Characteristics for the analytical method used for validation of triazole derivative metabolites residues in beer (barley) (calibration in solvent, 1-100 µg/L)

|  | TDMs |
| --- | --- |
| Specificity | ***For, 1,2,4-TRZ and TLA*** : No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***For TA*** ***and TAA***: Signals were detected in amounts higher than the 30% of LOQ for this matrice and were generated by the analytes and not by other interfering substanes.However, a lot of attempts were carried out by the laboratory to find samples with analytes area < 30% of LOQ, analysing a lot of commercially available commodities coming from organic farming: no samples with analytes area < 30% of LOQ were found for this matrice  **Primarity transition:**  ***1,2,4-TRZ***  % interference mean = 0  ***TA***  % interference mean = 310  ***TLA***  % interference mean = 15  ***TAA***  % interference mean = 219 |
| Calibration (type, number of data points) | **Primarity transition:**  ***1,2,4-TRZ***  Equation : Y = 0.94954 \* x + 0.00154  Coefficient of correlation: r² = 99.972  ***TA***  Equation : Y = 3.83597 \* x + 0.16176  Coefficient of correlation: r² = 99.940  ***TLA***  Equation : Y = 1.31066 \* x + 0.07797  Coefficient of correlation: r² = 99.610  ***TAA***  Equation : Y = 0.95844\* x + 0.01940  Coefficient of correlation: r² = 99.976 |
| Calibration range | Accepted calibration range in concentration units 1.00 – 100.0 µg/L (from 20 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes : Performed comparing the analyte response of one individual standard at L4 (or L5) prepared in solvent (water/methanol 50:50 + 1% formic acid) to one prepared in blank matrix extract at the same concentration.  ***1,2,4-TRZ***  Matrix effect = 5.2 %  Not significant  ***TA***  Matrix effect = 5.0 %  Not significant  ***TLA***  Matrix effect = -3.8 %  Not significant  ***TAA***  Matrix effect = 6.5 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 1 µg/L  (0.002 mg/kg) |
| Stability | According to SANTE/2020/12830 rev.1, the stability of the analytes in the final extracts is not required if the extracts contain an isotope-labelled internal standard (IL-IS) for quantification, since the IL-IS compensates for losses during extract storage. Since IL-IS were used, the verification of the stability was not required. Anyway, all the extracts were analysed within 24 hours from their preparation, obtaining recoveries in the fortified samples within the acceptable range of 70-120%.  In addition, since the differences between the mean responses of the analytes in the diluted solutions prepared form stored and from freshly-prepared stock solutions was < 10%, it is possible to assess that the analytes are stable in the stock solutions prepared in water for at least 78 days, if stored in the dark at 5 ± 3°C. |

Conclusion

The analytical method for the quantification of triazole derivative metabolites in wheat, barley, oilseed rape and processed commodities was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability for triazole derivative metabolites.

Independent laboratory validation

~~Not required.~~

Determination of Triazole Derivatives Metabolites (TMDs) in Rapeseed seeds

|  |  |
| --- | --- |
| Comments of zRMS: | The submitted ILV of the analytical method adjusted and validated in GLP study Code GLP-STUDY-21-108 by LabAnalysis s.r.l. for the determination of TDMs (1,2,4-triazole (TRZ), Triazole alanine (TA), Triazole lactic acid (TLA), Triazole acetic acid (TAA)) residues in Rapeseed seeds has been accepted.  The analytical method, using the HPLC/MS/MS in MRM mode is highly specific (2 transitions for all analytes) and gave both quantification and identification data, so the confirmatory method is not necessary.  All recovery values for each analyte at both fortification levels (LOQ and 10 x LOQ) resulted to be in the range 60-70 to 120 %, with an RSD% lower than 20-30% (SANTE/2020/12830 rev. 1) therefore the analytical method can be considered suitable to quantify TDMs residues in Rapeseed seeds samples with an established LOQ of 0.010 mg/kg. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/21 |
| Report | Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of TDM in Rapeseed seeds  Report No.: CH-1090/2021  Nichetti, S. (2022f)  ChemService S.r.l Controlli e Ricerche - Novate Milanese (MI) - Italy |
| Guideline(s): | Yes  SANTE/2020/12830 rev. 1 dated 24/02/21: Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes. |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

Materials and methods

|  |  |
| --- | --- |
| **Principle of the method** | The Test Facility conducted an Independent Laboratory Validation (ILV) of the analytical method adjusted and validated in GLP study Code GLP-STUDY-21-108 by LabAnalysis s.r.l. for the determination of triazole derivatives metabolites in rapeseed seeds: 1,2,4-triazole, triazole alanine, triazole lactic acid and triazole acetic acid. |

**Materials:**

|  |  |
| --- | --- |
| HPLC-MS/MS | Shimadzu mod. LC-40 XR, equipped with SelexION (Differential Mobility Separation) device and spectrometer Sciex API 6500 |
| Column: | THERMO LCN-412 Hypercarb, 5 μm, 100 x 2.1 mm i.d. |
| Detector: | MS Triple quadrupole equipped with a Differential Mobility Separation (DMS) device (MRM mode) |
| Ionisation type: | Electrospray (ESI) |
| Polarity: | Positive ion mode |
| Scan type: | MS/MS, multiple reaction monitoring |
| Drying gas temperature: | 500 °C |
| Curtain gas flow: | 30 mL/min |
| Nebulizer pressure: | 40 psi |
| Capillary voltage: | 3500 V |
|  |  |
| Analytical standards: | 1,2,4-Triazole  Batch No. : STBK4702  Purity : 100.0 %  Expiry date: October 1, 2023 |
|  | Triazole alanine dihydrochloride  Batch No. : 2017-0177604  Purity : 95 % (64.76% calculated as triazole alanine)  Expiry date: January 15, 2023 |
|  | Triazole lactic acid hydrochloride  Batch No. : 2019-0300703  Purity : 95 % (77.12% calculated as triazole lactic acid)  Expiry date: August 19, 2022  Triazole acetic acid  Batch No. : 2017-0130327  Purity : 95 %  Expiry date: April 11, 2023 |
|  |  |
| Reagents: | Water, HPLC grade, obtained by the Lab Water Purification System  Methanol, HPLC grade  Acetic acid, glacial  Formic acid, high purity for mass spectroscopy |
|  |  |
| Matrix: | Rapeseed seeds  Stored at -20°C in the dark |

**Methods:**

The analytical methods for the determination of triazole derivatives metabolites in rapeseed seeds were confirmed so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were confirmed in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A 193: Recovery results from independent laboratory validation of triazole derivative metabolites using the analytical method

| Matrix | Analyte | Fortification level (mg/kg) (n = 5) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Rapeseed seeds | 1,2,4-triazole  (product ion: 43.1 m/z) | At low level :  0.01 mg/kg (LOQ) | 102.7 | 3.88 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 102.3 | 3.16 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| 1,2,4-triazole  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 104.7 | 4.62 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 102.7 | 2.24 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole alanine  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 93.4 | 9.51 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 96.9 | 4.54 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole alanine  (product ion: 88.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 98.5 | 7.13 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 101.8 | 4.50 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole lactic acid  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 107.5 | 1.39 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 103.6 | 1.71 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole lactic acid  (product ion: 43.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 104.5 | 4.54 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 105.8 | 0.96 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole acetic acid  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 100.7 | 1.88 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 104.1 | 1.07 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole acetic acid  (product ion: 73.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 94.0 | 7.91 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 105.9 | 3.30 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

Table A 194: Characteristics for the analytical method used for independent laboratory validation of 1,2,4-triazole residues in rapeseed seeds

|  | 1,2,4-triazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Product ion (m/z = 43.1):***  % interference mean = 0.0  ***Product ion (m/z = 70.0):***  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Product ion (m/z = 43.1):***  Equation : Y = 0.0811 \* x – 0.0307  Coefficient of correlation: r² = 99.945  ***Product ion (m/z = 70.0):***  Equation : Y = 0.6901 \* x – 0.2197  Coefficient of correlation: r² = 99.972 |
| Calibration range | Accepted calibration range in concentration units 0.52 – 51.50 µg/L  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.200 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = 1 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-108 |

Table A 195: Characteristics for the analytical method used for independent laboratory validation of triazole alanine residues in rapeseed seeds

|  | Triazole alanine |
| --- | --- |
| Specificity | Since no matrix sample (Rapeseed seeds) with interference lower than 30 % of LOQ for all analytes was found, the evaluation of recovery for the Triazole-alanine analyte was carried out subtracting the contribute obtained from the Matrix Blank to Spike samples and the linear calibration was prepared in solvent.  ***Product ion (m/z = 88.0):***  % interference mean (low level) = 76.2  % interference mean (high level) = 26.2  ***Product ion (m/z = 70.0):***  % interference mean (low level) = 76.0  % interference mean (high level) = 25.6 |
| Calibration (type, number of data points) | ***Product ion (m/z = 88.0):***  Equation : Y = 0.1185 \* x – 0.0009  Coefficient of correlation: r² = 99.947  ***Product ion (m/z = 70.0):***  Equation : Y = 0.2044 \* x + 0.0261  Coefficient of correlation: r² = 99.991 |
| Calibration range | Accepted calibration range in concentration units 0.36 – 35.62 µg/L  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.200 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 19 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-108 |

Table A 196: Characteristics for the analytical method used for independent laboratory validation of triazole lactic acid in rapeseed seeds

|  | Triazole lactic acid |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Product ion (m/z = 43.0):***  % interference mean = 0.0  ***Product ion (m/z = 70.0):***  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Product ion (m/z = 43.0):***  Equation : Y = 0.0121 \* x + 0.0045  Coefficient of correlation: r² = 99.985  ***Product ion (m/z = 70.0):***  Equation : Y = 0.0965 \* x + 0.0262  Coefficient of correlation: r² = 99.988 |
| Calibration range | Accepted calibration range in concentration units 0.50 – 49.74 µg/L  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = 0 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-108 |

Table A 197: Characteristics for the analytical method used for independent laboratory validation of triazole acetic acid in rapeseed seeds

|  | Triazole acetic acid |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Product ion (m/z = 73.0):***  % interference mean = 0.0  ***Product ion (m/z = 70.0):***  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Product ion (m/z = 73.0):***  Equation : Y = 0.0035 \* x – 0.0010  Coefficient of correlation: r² = 99.860  ***Product ion (m/z = 70.0):***  Equation : Y = 0.0830 \* x + 0.0033  Coefficient of correlation: r² = 99.998 |
| Calibration range | Accepted calibration range in concentration units 0.51 – 50.83 µg/L  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = 1 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-108 |

Conclusion

The independent laboratory validation for the quantification of 1,2,4-triazole, triazole alanine, triazole lactic acid and triazole acetic acid in rapeseed seeds was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability.

Determination of Triazole Derivatives Metabolites (TMDs) in Whole plant (Rapeseed)

|  |  |
| --- | --- |
| Comments of zRMS: | The submitted ILV of the analytical method adjusted and validated in GLP study Code GLP-STUDY-21-108 by LabAnalysis s.r.l. for the determination of TDMs (1,2,4-triazole (TRZ), Triazole alanine (TA), Triazole lactic acid (TLA), Triazole acetic acid (TAA)) residues in Whole Plant (Rapeseed) has been accepted.  The analytical method, using the HPLC/MS/MS in MRM mode is highly specific (2 transitions for all analytes) and gave both quantification and identification data, so the confirmatory method is not necessary.  All recovery values for each analyte at both fortification levels (LOQ and 10 x LOQ) resulted to be in the range 60-70 to 120 %, with an RSD% lower than 20-30% (SANTE/2020/12830 rev. 1) therefore the analytical method can be considered suitable to quantify TDMs residues in Whole Plant (Rapeseed) samples with an established LOQ of 0.010 mg/kg.  Note: on page 15th of CH-1085/2021 it is stated: “the analytical method can be considered suitable to quantify Difenoconazole and Prothioconazole-desthio” It should be corrected. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/22 |
| Report | Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of TDM in Whole Plant (Rapeseed)  Report No.: CH-1085/2021  Nichetti, S. (2022g)  ChemService S.r.l Controlli e Ricerche - Novate Milanese (MI) - Italy |
| Guideline(s): | Yes  SANTE/2020/12830 rev. 1 dated 24/02/21: Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes. |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

Materials and methods

|  |  |
| --- | --- |
| **Principle of the method** | The Test Facility conducted an Independent Laboratory Validation (ILV) of the analytical method adjusted and validated in GLP study Code GLP-STUDY-21-108 by LabAnalysis s.r.l. for the determination of triazole derivatives metabolites in whole plant (rapeseed): 1,2,4-triazole, triazole alanine, triazole lactic acid and triazole acetic acid. |
|  |  |

**Materials:**

|  |  |
| --- | --- |
| HPLC-MS/MS | Shimadzu mod. LC-40 XR, equipped with SelexION (Differential Mobility Separation) device and spectrometer Sciex API 6500 |
| Column: | THERMO LCN-412 Hypercarb, 5 μm, 100 x 2.1 mm i.d. |
| Detector: | MS Triple quadrupole equipped with a Differential Mobility Separation (DMS) device (MRM mode) |
| Ionisation type: | Electrospray (ESI) |
| Polarity: | Positive ion mode |
| Scan type: | MS/MS, multiple reaction monitoring |
| Drying gas temperature: | 500 °C |
| Curtain gas flow: | 30 mL/min |
| Nebulizer pressure: | 40 psi |
| Capillary voltage: | 3500 V |
|  |  |
| Analytical standards: | 1,2,4-Triazole  Batch No. : STBK4702  Purity : 100.0 %  Expiry date: October 1, 2023 |
|  | Triazole alanine dihydrochloride  Batch No. : 2017-0177604  Purity : 95 % (64.76% calculated as triazole alanine)  Expiry date: January 15, 2023 |
|  | Triazole lactic acid hydrochloride  Batch No. : 2019-0300703  Purity : 95 % (77.12% calculated as triazole lactic acid)  Expiry date: August 19, 2022  Triazole acetic acid  Batch No. : 2017-0130327  Purity : 95 %  Expiry date: April 11, 2023 |
|  |  |
| Reagents: | Water, HPLC grade, obtained by the Lab Water Purification System  Methanol, HPLC grade  Acetic acid, glacial  Formic acid, high purity for mass spectroscopy |
|  |  |
| Matrix: | Whole plant (rapeseed)  Stored at -20°C in the dark |

**Methods:**

The analytical methods for the determination of triazole derivatives metabolites in whole plant (rapeseed) were confirmed so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were confirmed in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A 198: Recovery results from independent laboratory validation of triazole derivative metabolites using the analytical method

| Matrix | Analyte | Fortification level (mg/kg) (n = 5) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Whole Plant (Rapeseed) | 1,2,4-triazole  (product ion: 43.1 m/z) | At low level :  0.01 mg/kg (LOQ) | 87.5 | 5.23 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 97.1 | 3.03 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| 1,2,4-triazole  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 93.6 | 2.63 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 99.0 | 3.69 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole alanine  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 89.9 | 10.03 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 100.4 | 4.04 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole alanine  (product ion: 88.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 98.5 | 8.83 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 102.5 | 3.61 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole lactic acid  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 97.1 | 2.29 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 100.4 | 0.97 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole lactic acid  (product ion: 43.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 100.8 | 4.20 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 101.6 | 1.49 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole acetic acid  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 99.6 | 2.11 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 101.8 | 0.85 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole acetic acid  (product ion: 73.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 92.7 | 7.72 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 100.3 | 2.81 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

Table A 199: Characteristics for the analytical method used for independent laboratory validation of 1,2,4-triazole residues in whole plant (rapeseed)

|  | 1,2,4-triazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Product ion (m/z = 43.1):***  % interference mean = 0.0  ***Product ion (m/z = 70.0):***  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Product ion (m/z = 43.1):***  Equation : Y = 0.0741 \* x + 0.0238  Coefficient of correlation: r² = 99.988  ***Product ion (m/z = 70.0):***  Equation : Y = 0.6458 \* x + 0.2530  Coefficient of correlation: r² = 99.977 |
| Calibration range | Accepted calibration range in concentration units 0.53 – 52.50 µg/L  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.200 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 10 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-108 |

Table A 200: Characteristics for the analytical method used for independent laboratory validation of triazole alanine residues in whole plant (rapeseed)

|  | Triazole alanine |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Product ion (m/z = 88.0):***  % interference mean = 0.0  ***Product ion (m/z = 70.0):***  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Product ion (m/z = 88.0):***  Equation : Y = 0.0985 \* x + 0.1008  Coefficient of correlation: r² = 99.963  ***Product ion (m/z = 70.0):***  Equation : Y = 0.1743 \* x + 0.1894  Coefficient of correlation: r² = 99.982 |
| Calibration range | Accepted calibration range in concentration units 0.38 – 37.56 µg/L  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.200 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 7 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-108 |

Table A 201: Characteristics for the analytical method used for independent laboratory validation of triazole lactic acid in whole plant (rapeseed)

|  | Triazole lactic acid |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Product ion (m/z = 43.0):***  % interference mean = 0.0  ***Product ion (m/z = 70.0):***  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Product ion (m/z = 43.0):***  Equation : Y = 0.0131 \* x + 0.0028  Coefficient of correlation: r² = 99.988  ***Product ion (m/z = 70.0):***  Equation : Y = 0.1042 \* x – 0.0027  Coefficient of correlation: r² = 99.990 |
| Calibration range | Accepted calibration range in concentration units 0.48 – 48.20 µg/L  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = 3 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-108 |

Table A 202: Characteristics for the analytical method used for independent laboratory validation of triazole acetic acid in whole plant (rapeseed)

|  | Triazole acetic acid |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Product ion (m/z = 73.0):***  % interference mean = 0.0  ***Product ion (m/z = 70.0):***  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Product ion (m/z = 73.0):***  Equation : Y = 0.0037 \* x – 0.0005  Coefficient of correlation: r² = 99.960  ***Product ion (m/z = 70.0):***  Equation : Y = 0.0843 \* x + 0.0025  Coefficient of correlation: r² = 99.993 |
| Calibration range | Accepted calibration range in concentration units 0.52 – 52.25 µg/L  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = 1 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-108 |

Conclusion

The independent laboratory validation for the quantification of 1,2,4-triazole, triazole alanine, triazole lactic acid and triazole acetic acid in whole plant (rapeseed)was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability.

Determination of Triazole Derivatives Metabolites (TMDs) in Grain (wheat)

|  |  |
| --- | --- |
| Comments of zRMS: | The submitted ILV of the analytical method adjusted and validated in GLP study Code GLP-STUDY-21-108 by LabAnalysis s.r.l. for the determination of TDMs (1,2,4-triazole (TRZ), Triazole alanine (TA), Triazole lactic acid (TLA), Triazole acetic acid (TAA)) residues in Grain (wheat) has been accepted.  The analytical method, using the HPLC/MS/MS in MRM mode is highly specific (2 transitions for all analytes) and gave both quantification and identification data, so the confirmatory method is not necessary.  All recovery values for each analyte at both fortification levels (LOQ and 10 x LOQ) resulted to be in the range 60-70 to 120 %, with an RSD% lower than 20-30% (SANTE/2020/12830 rev. 1) therefore the analytical method can be considered suitable to quantify TDMs residues in Grain (wheat) samples with an established LOQ of 0.010 mg/kg. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/23 |
| Report | Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of TDM in Grain (wheat)  Report No.: CH-1087/2021  Nichetti, S. (2022h)  ChemService S.r.l Controlli e Ricerche - Novate Milanese (MI) - Italy |
| Guideline(s): | Yes  SANTE/2020/12830 rev. 1 dated 24/02/21: Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes. |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

Materials and methods

|  |  |
| --- | --- |
| **Principle of the method** | The Test Facility conducted an Independent Laboratory Validation (ILV) of the analytical method adjusted and validated in GLP study Code GLP-STUDY-21-108 by LabAnalysis s.r.l. for the determination of triazole derivatives metabolites in grain (wheat): 1,2,4-triazole, triazole alanine, triazole lactic acid and triazole acetic acid. |
|  |  |

**Materials:**

|  |  |
| --- | --- |
| HPLC-MS/MS | Shimadzu mod. LC-40 XR, equipped with SelexION (Differential Mobility Separation) device and spectrometer Sciex API 6500 |
| Column: | THERMO LCN-412 Hypercarb, 5 μm, 100 x 2.1 mm i.d. |
| Detector: | MS Triple quadrupole equipped with a Differential Mobility Separation (DMS) device (MRM mode) |
| Ionisation type: | Electrospray (ESI) |
| Polarity: | Positive ion mode |
| Scan type: | MS/MS, multiple reaction monitoring |
| Drying gas temperature: | 500 °C |
| Curtain gas flow: | 30 mL/min |
| Nebulizer pressure: | 40 psi |
| Capillary voltage: | 3500 V |
|  |  |
| Analytical standards: | 1,2,4-Triazole  Batch No. : STBK4702  Purity : 100.0 %  Expiry date: October 1, 2023 |
|  | Triazole alanine dihydrochloride  Batch No. : 2017-0177604  Purity : 95 % (64.76% calculated as triazole alanine)  Expiry date: January 15, 2023 |
|  | Triazole lactic acid hydrochloride  Batch No. : 2019-0300703  Purity : 95 % (77.12% calculated as triazole lactic acid)  Expiry date: August 19, 2022  Triazole acetic acid  Batch No. : 2017-0130327  Purity : 95 %  Expiry date: April 11, 2023 |
|  |  |
| Reagents: | Water, HPLC grade, obtained by the Lab Water Purification System  Methanol, HPLC grade  Acetic acid, glacial  Formic acid, high purity for mass spectroscopy |
|  |  |
| Matrix: | Grain (wheat)  Stored at -20°C in the dark |

**Methods:**

The analytical methods for the determination of triazole derivatives metabolites in grain (wheat) were confirmed so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were confirmed in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A 203: Recovery results from independent laboratory validation of triazole derivative metabolites using the analytical method

| Matrix | Analyte | Fortification level (mg/kg) (n = 5) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Grain (wheat) | 1,2,4-triazole  (product ion: 43.1 m/z) | At low level :  0.01 mg/kg (LOQ) | 97.6 | 3.49 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 99.7 | 3.06 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| 1,2,4-triazole  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 102.9 | 2.20 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 98.6 | 1.67 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole alanine  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 85.9 | 6.78 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 98.4 | 1.57 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole alanine  (product ion: 88.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 96.0 | 9.37 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 96.9 | 2.82 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole lactic acid  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 101.4 | 2.24 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 99.8 | 1.20 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole lactic acid  (product ion: 43.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 98.7 | 2.69 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 99.8 | 1.57 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole acetic acid  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 101.0 | 2.17 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 99.3 | 0.32 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole acetic acid  (product ion: 73.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 96.8 | 8.99 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 100.5 | 2.52 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

Table A 204: Characteristics for the analytical method used for independent laboratory validation of 1,2,4-triazole residues in grain (wheat)

|  | 1,2,4-triazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Product ion (m/z = 43.1):***  % interference mean = 0.0  ***Product ion (m/z = 70.0):***  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Product ion (m/z = 43.1):***  Equation : Y = 0.0730 \* x + 0.0348  Coefficient of correlation: r² = 99.972  ***Product ion (m/z = 70.0):***  Equation : Y = 0.6314 \* x + 0.0675  Coefficient of correlation: r² = 99.999 |
| Calibration range | Accepted calibration range in concentration units 0.53 – 52.50 µg/L  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.200 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 3 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-108 |

Table A 205: Characteristics for the analytical method used for independent laboratory validation of triazole alanine residues in grain (wheat)

|  | Triazole alanine |
| --- | --- |
| Specificity | Since no matrix sample (grain wheat) with interference lower than 30 % of LOQ for all analytes was found, the evaluation of recovery for the Triazole-alanine analyte was carried out subtracting the contribute obtained from the Matrix Blank to Spike samples and the linear calibration was prepared in solvent.  ***Product ion (m/z = 88.0):***  % interference mean (low level) = 53.4  % interference mean (high level) = 11.0  ***Product ion (m/z = 70.0):***  % interference mean (low level) = 55.6  % interference mean (high level) = 11.2 |
| Calibration (type, number of data points) | ***Product ion (m/z = 88.0):***  Equation : Y = 0.1175 \* x – 0.0022  Coefficient of correlation: r² = 99.942  ***Product ion (m/z = 70.0):***  Equation : Y = 0.2101 \* x – 0.0264  Coefficient of correlation: r² = 99.870 |
| Calibration range | Accepted calibration range in concentration units 0.39 – 39.18 µg/L  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.200 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = 0 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-108 |

Table A 206: Characteristics for the analytical method used for independent laboratory validation of triazole lactic acid in grain (wheat)

|  | Triazole lactic acid |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Product ion (m/z = 43.0):***  % interference mean = 0.0  ***Product ion (m/z = 70.0):***  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Product ion (m/z = 43.0):***  Equation : Y = 0.0124 \* x – 0.0010  Coefficient of correlation: r² = 99.999  ***Product ion (m/z = 70.0):***  Equation : Y = 0.0950 \* x + 0.0052  Coefficient of correlation: r² = 99.997 |
| Calibration range | Accepted calibration range in concentration units 0.48 – 48.20 µg/L  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 1 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-108 |

Table A 207: Characteristics for the analytical method used for independent laboratory validation of triazole acetic acid in grain (wheat)

|  | Triazole acetic acid |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Product ion (m/z = 73.0):***  % interference mean (low level) = 23.3  % interference mean (high level) = 3.2  ***Product ion (m/z = 70.0):***  % interference mean (low level) = 20.1  % interference mean (high level) = 2.6 |
| Calibration (type, number of data points) | ***Product ion (m/z = 73.0):***  Equation : Y = 0.0036 \* x – 0.0003  Coefficient of correlation: r² = 99.993  ***Product ion (m/z = 70.0):***  Equation : Y = 0.0851 \* x – 0.0010  Coefficient of correlation: r² = 99.992 |
| Calibration range | Accepted calibration range in concentration units 0.50 – 50.35 µg/L  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 3 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-108 |

Conclusion

The independent laboratory validation for the quantification of 1,2,4-triazole, triazole alanine, triazole lactic acid and triazole acetic acid in grain (wheat) was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability.

Determination of Triazole Derivatives Metabolites (TMDs) in ~~Grain~~ straw (wheat)

|  |  |
| --- | --- |
| Comments of zRMS: | he submitted ILV of the analytical method adjusted and validated in GLP study Code GLP-STUDY-21-108 by LabAnalysis s.r.l. for the determination of TDMs (1,2,4-triazole (TRZ), Triazole alanine (TA), Triazole lactic acid (TLA), Triazole acetic acid (TAA)) residues in Straw (wheat) has been accepted.  The analytical method, using the HPLC/MS/MS in MRM mode is highly specific (2 transitions for all analytes) and gave both quantification and identification data, so the confirmatory method is not necessary.  All recovery values for each analyte at both fortification levels (LOQ and 10 x LOQ) resulted to be in the range 60-70 to 120 %, with an RSD% lower than 20-30% (SANTE/2020/12830 rev. 1) therefore the analytical method can be considered suitable to quantify TDMs residues in Straw (wheat) samples with an established LOQ of 0.010 mg/kg |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/24 |
| Report | Independent Laboratory Validation (ILV) of the Analytical Method for the Determination of TDM in Straw (wheat)  Report No.: CH-1086/2021  Nichetti, S. (2022i)  ChemService S.r.l Controlli e Ricerche - Novate Milanese (MI) - Italy |
| Guideline(s): | Yes  SANTE/2020/12830 rev. 1 dated 24/02/21: Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes. |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

Materials and methods

|  |  |
| --- | --- |
| **Principle of the method** | The Test Facility conducted an Independent Laboratory Validation (ILV) of the analytical method adjusted and validated in GLP study Code GLP-STUDY-21-108 by LabAnalysis s.r.l. for the determination of triazole derivatives metabolites in straw (wheat): 1,2,4-triazole, triazole alanine, triazole lactic acid and triazole acetic acid. |
|  |  |

**Materials:**

|  |  |
| --- | --- |
| HPLC-MS/MS | Shimadzu mod. LC-40 XR, equipped with SelexION (Differential Mobility Separation) device and spectrometer Sciex API 6500 |
| Column: | THERMO LCN-412 Hypercarb, 5 μm, 100 x 2.1 mm i.d. |
| Detector: | MS Triple quadrupole equipped with a Differential Mobility Separation (DMS) device (MRM mode) |
| Ionisation type: | Electrospray (ESI) |
| Polarity: | Positive ion mode |
| Scan type: | MS/MS, multiple reaction monitoring |
| Drying gas temperature: | 500 °C |
| Curtain gas flow: | 30 mL/min |
| Nebulizer pressure: | 40 psi |
| Capillary voltage: | 3500 V |
|  |  |
| Analytical standards: | 1,2,4-Triazole  Batch No. : STBK4702  Purity : 100.0 %  Expiry date: October 1, 2023 |
|  | Triazole alanine dihydrochloride  Batch No. : 2017-0177604  Purity : 95 % (64.76% calculated as triazole alanine)  Expiry date: January 15, 2023 |
|  | Triazole lactic acid hydrochloride  Batch No. : 2019-0300703  Purity : 95 % (77.12% calculated as triazole lactic acid)  Expiry date: August 19, 2022  Triazole acetic acid  Batch No. : 2017-0130327  Purity : 95 %  Expiry date: April 11, 2023 |
|  |  |
| Reagents: | Water, HPLC grade, obtained by the Lab Water Purification System  Methanol, HPLC grade  Acetic acid, glacial  Formic acid, high purity for mass spectroscopy |
|  |  |
| Matrix: | Straw (wheat)  Stored at -20°C in the dark |

**Methods:**

The analytical methods for the determination of triazole derivatives metabolites in straw (wheat) were confirmed so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were confirmed in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A 208: Recovery results from independent laboratory validation of triazole derivative metabolites using the analytical method

| Matrix | Analyte | Fortification level (mg/kg) (n = 5) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Straw (wheat) | 1,2,4-triazole  (product ion: 43.1 m/z) | At low level :  0.01 mg/kg (LOQ) | 103.2 | 5.11 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 106.8 | 4.66 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| 1,2,4-triazole  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 110.6 | 3.36 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 106.9 | 1.06 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole alanine  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 96.2 | 9.55 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 102.3 | 5.19 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole alanine  (product ion: 88.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 99.2 | 11.53 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 104.5 | 5.47 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole lactic acid  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 90.6 | 2.90 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 105.3 | 2.46 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole lactic acid  (product ion: 43.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 97.6 | 5.71 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 106.6 | 2.01 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole acetic acid  (product ion: 70.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 117.9 | 1.67 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 110.8 | 1.48 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Triazole acetic acid  (product ion: 73.0 m/z) | At low level :  0.01 mg/kg (LOQ) | 112.2 | 6.30 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 103.8 | 6.04 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

Table A 209: Characteristics for the analytical method used for independent laboratory validation of 1,2,4-triazole residues in straw (wheat)

|  | 1,2,4-triazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***Product ion (m/z = 43.1):***  % interference mean = 0.0  ***Product ion (m/z = 70.0):***  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Product ion (m/z = 43.1):***  Equation : Y = 0.0783 \* x + 0.0279  Coefficient of correlation: r² = 99.950  ***Product ion (m/z = 70.0):***  Equation : Y = 0.6773 \* x + 0.1250  Coefficient of correlation: r² = 99.969 |
| Calibration range | Accepted calibration range in concentration units 0.35 – 34.65 µg/L  Corresponding calibration range in mass ratio units for the sample (0.0028 – 0.280 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 13 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.35 µg/L  (0.0028 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-108 |

Table A 210: Characteristics for the analytical method used for independent laboratory validation of triazole alanine residues in straw (wheat)

|  | Triazole alanine |
| --- | --- |
| Specificity | Since no matrix sample (straw wheat) with interference lower than 30 % of LOQ for all analytes was found, the evaluation of recovery for the Triazole-alanine analyte was carried out subtracting the contribute obtained from the Matrix Blank to Spike samples and the linear calibration was prepared in solvent.  ***Product ion (m/z = 88.0):***  % interference mean (low level) = 82.9  % interference mean (high level) = 36.1  ***Product ion (m/z = 70.0):***  % interference mean (low level) = 83.4  % interference mean (high level) = 35.4 |
| Calibration (type, number of data points) | ***Product ion (m/z = 88.0):***  Equation : Y = 0.1492 \* x + 0.0848  Coefficient of correlation: r² = 99.867  ***Product ion (m/z = 70.0):***  Equation : Y = 0.2649 \* x + 0.1264  Coefficient of correlation: r² = 99.837 |
| Calibration range | Accepted calibration range in concentration units 0.28 – 27.88 µg/L  Corresponding calibration range in mass ratio units for the sample (0.0028 – 0.280 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 4 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.35 µg/L  (0.0028 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-108 |

Table A 211: Characteristics for the analytical method used for independent laboratory validation of triazole lactic acid in straw (wheat)

|  | Triazole lactic acid |
| --- | --- |
| Specificity | Since no matrix sample (straw wheat) with interference lower than 30 % of LOQ for all analytes was found, the evaluation of recovery for the Triazole lactic acid analyte was carried out subtracting the contribute obtained from the Matrix Blank to Spike samples and the linear calibration was prepared in solvent.  ***Product ion (m/z = 43.0):***  % interference mean (low level) = 83.1  % interference mean (high level) = 30.4  ***Product ion (m/z = 70.0):***  % interference mean (low level) = 84.8  % interference mean (high level) = 30.6 |
| Calibration (type, number of data points) | ***Product ion (m/z = 43.0):***  Equation : Y = 0.0123 \* x + 0.0024  Coefficient of correlation: r² = 99.979  ***Product ion (m/z = 70.0):***  Equation : Y = 0.1003 \* x + 0.0139  Coefficient of correlation: r² = 99.965 |
| Calibration range | Accepted calibration range in concentration units 0.33 – 33.20 µg/L  Corresponding calibration range in mass ratio units for the sample (0.0028 – 0.280 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = 5 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.35 µg/L  (0.0028 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-108 |

Table A 212: Characteristics for the analytical method used for independent laboratory validation of triazole acetic acid in straw (wheat)

|  | Triazole acetic acid |
| --- | --- |
| Specificity | Since no matrix sample (straw wheat) with interference lower than 30 % of LOQ for all analytes was found, the evaluation of recovery for the Triazole acetic acid analyte was carried out subtracting the contribute obtained from the Matrix Blank to Spike samples and the linear calibration was prepared in solvent.  ***Product ion (m/z = 73.0):***  % interference mean (low level) = 36.6  % interference mean (high level) = 6.0  ***Product ion (m/z = 70.0):***  % interference mean (low level) = 34.8  % interference mean (high level) = 5.2 |
| Calibration (type, number of data points) | ***Product ion (m/z = 73.0):***  Equation : Y = 0.0036 \* x + 0.0004  Coefficient of correlation: r² = 99.974  ***Product ion (m/z = 70.0):***  Equation : Y = 0.0863 \* x + 0.0143  Coefficient of correlation: r² = 99.953 |
| Calibration range | Accepted calibration range in concentration units 0.34 – 34.25 µg/L  Corresponding calibration range in mass ratio units for the sample (0.0028 – 0.280 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 3 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.35 µg/L  (0.0028 mg/kg) |
| Stability | The stability of stock, fortification and calibration solutions were not assessed since solutions were analysed within 24 hours after preparation.  The stability of final extracts/ dilutions was not assessed since extracts/ dilutions were analysed within 24 hours after preparation. |
| Extraction efficiency | The extraction efficiency was demonstrated following the extraction procedure reported, and already judged acceptable in the GLP study Code GLP-STUDY-21-108 |

Conclusion

The independent laboratory validation for the quantification of 1,2,4-triazole, triazole alanine, triazole lactic acid and triazole acetic acid in straw (wheat) was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability.

Confirmatory method

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  The analytical method for the determination of TDMs was based on the method “Quick Method for the Analysis of Highly Polar Pesticides in Food Involving Extraction with Acidified Methanol and LC- or IC-MS/MS Measurement I. - Food of Plant Origin (QuPPe-PO-Method) - Method 8 (M8)”.  The determination was carried out using a HPLC-MS/MS system (high-performance liquid chromatography + triple quadrupole mass spectrometry) equipped with a differential mobility separation device (DMS).  As two mass transitions were validated, the confirmatory method is not required. Duplication of the study description within the report is not necessary (this happens several times).  The specificity of the method was assured by MS/MS detection. All validation parameters are in the currently required range.  The matrix effect was not significant according to the SANTE/2020/12830 rev.1 except for triazole-alanine in whole OSR plant.  See also into the present section B7 where this validated method was employed to generation of the data in paragraphs of Appendix 2: A 2.1.3.1.1,2,3; A 2.1.5.2.1,2,3; A 2.2.3.1.1,2,3; A 2.2.5.2.1,2,3. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/14 |
| Report | Validation of an analytical method for the quantification of Triazole Derivative Metabolites (TDMs) in wheat, barley, oilseed rape and processed commodities  Longhi, D.  2021b  ChemService S.r.l. Controlli e Richerche, Novate Milanese - Italy  Report No. : 21-108 |
| Guideline(s): | Yes : SANTE/2020/12830 rev. 1 (dated 24/02/2021) ;  SANTE2017/10632 rev. 3 (dated 22/11/2017) ;  OECD ENV/JM/MONO(2007)17 ;  “Quick Method for the Analysis of Highly Polar Pesticides in Food Involving Extraction with Acidified Methanol and LC- or IC-MS/MS Measurement - Food of Plant Origin (QuPPe-PO-Method)- Method 8 (M8)”. |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |
| **Principle of the method** | The confirmatory method for the determination of TDMs in the tested matrices (AM-GLP-STUDY-21-108) was based on the method “Quick Method for the Analysis of Highly Polar Pesticides in Food Involving Extraction with Acidified Methanol and LC- or IC-MS/MS Measurement I. - Food of Plant Origin (QuPPe-PO-Method) - Method 8 (M8)”.  The instrumental determination was carried out using a HPLC-MS/MS system (high-performance liquid chromatography + triple quadrupole mass spectrometry) equipped with a differential mobility separation device (DMS). |

Materials and methods

***1. Materials***

***1.A. Quantification of triazole derivative metabolites in wheat, barley, oilseed rape and processed commodities***

The quantification of triazole derivative metabolites in wheat, barley, oilseed rape and processed commodities was assessed by HLPC/MS/MS.

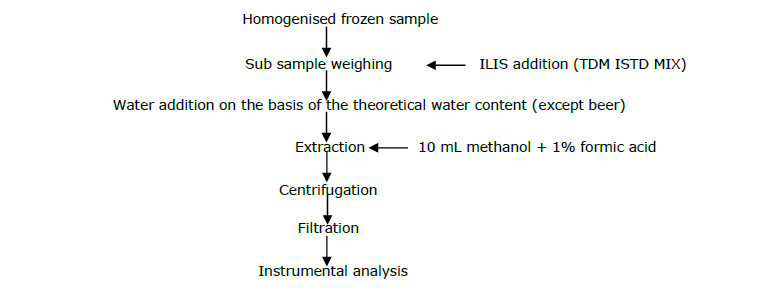
|  |  |
| --- | --- |
| HPLC: | Shimadzu LC-40 XR + spectrometer Sciex API 6500+ equipped with SelexION (Differential Mobility Separation) device |
| Column: | Thermo Hyperbare 5 μm, 2.1 x 100 mm |
| Detector: | Agilent MS spectrometer 6470A Triple Quad |
| Column temperature: | 40 °C |
| Flow rate: | 0.6 mL/min |
| Volume of injection: | 2 µL |
| Retention time: | Approximatively 2.5 minutes for difenoconazole |
| Stop time: | 10 minutes |
| Gas temperature: | 500 °C |
| Curtain Gas flow: | 30 mL/min |
| Gas flow 1: | 55 mL/min |
| Gas flow2: | 65mL/min |
| Capillary: | Positive mode 3500 V |
| Mobile phase: | A: LC-MS grade water with 1% acetic acid  B: LC-MS grade methanol with 1% acetic acid |
| Mixture-Elution: | |  |  |  | | --- | --- | --- | | % A | % B | Time (min) | | 95 | 5 | 0 | | 10 | 90 | 5 | | 10 | 90 | 6 | | 95 | 5 | 6.1 | |
| Analytical standards: | 1,2,4-triazole (1,2,4-TRZ)  CAS No. : 288-88-0  Lot: STBJ5727  Purity : 100.3% (considered 100% in the calculation)  Expiry date: December 2021  1,2,4-Triazole Alanime (TA)  CAS No. : 86362-20-1  Lot: 787796  Purity : 98.3%  Expiry date: 01/03/2024  1,2,4-Triazole lactic acid HCl (TLA)  CAS No. : 1450828-63-3  Lot: 792058  Purity : 78.5%  Expiry date: 01/11/2024  1,2,4-Triazole acetic acid (TAA)  CAS No. : 28711-29-7  Lot: BCCC0969  Purity : 95.7%  Expiry date: December 2021 |
| Isotope-labelled internal standards (ILIS): | 1,2,4-Triazole-[13C2,15N3]  CAS No. : 1261170-82-4  Lot: SL6-2012-224  Purity : 98.4%  Expiry date: 01/2023  1,2,4-Triazole Alanine [D2]  CAS No. : 2180306-38-9  Lot: 2011202L3.3  Purity : 95%  Expiry date: 20/01/2023  1,2,4-Triazole-[13C2, 15N3] Lactic Acid  CAS No. : n.d.  Lot: EFL6-2015-198A  Purity : 98.42%  Expiry date: 01/2024  1,2,4-Triazole acetic acid [13C2, 15N3]  CAS No. : n.d.  Lot: EFL6-2015-196A  Purity : 98.03%  Expiry date: 01/2024 |

***2. Methods***

The analytical method for the quantification of triazole derivatives metabolites in the tested matrices was based on the QuEChERS method (Method 8).

***2.A. Schematic diagram of the analytical method***

Plant matrices and processed commodities (rapeseed whole plant, rapeseed oil, rapeseed seeds, wheat grain, white bread (wheat), wheat straw and barley beer)



***2.B. Quantification of triazole derivative metabolites in wheat, barley, oilseed rape and processed commodities***

The analytical methods for the quantification of triazole derivatives metabolites in wheat, barley, rapeseed and processed commodities were first validated so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were validated in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |

Results and discussions

Confirmatory method validation data can be summarised in tables below. ~~Table A.1and A2.1 to A2.5. There are for each matrix a primary test. In view of the similar results between the primary and confirmatory test, a test by an independent laboratory validation (ILV) is not required.~~

Table A~~1~~ 213: Recovery results from method validation of triazole derivate metabolites using the analytical method

| Matrix | Analyte | Fortification level  (*n* = x) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Whole Plant (Rapeseed) | 1,2,4-TRZ | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  92.3 % | ***Confirmatory transition***:  7.7 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  114.3 % | ***Confirmatory transition***:  3.0 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  97.8 % | ***Confirmatory transition***:  4.8 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  100.9 % | ***Confirmatory transition***:  1.3 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TLA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  99.7 % | ***Confirmatory transition***:  4.3 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  101.1 % | ***Confirmatory transition***:  1.0 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TAA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  99.9 % | ***Confirmatory transition***:  1.6 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  97.5 % | ***Confirmatory transition***:  1.6 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Rapeseed seeds | 1,2,4-TRZ | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  95.9 % | ***Confirmatory transition***:  5.0 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  92.0 % | ***Confirmatory transition***:  4.5 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  102.2 % | ***Confirmatory transition***:  6.3 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  112.1 % | ***Confirmatory transition***:  3.5 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TLA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  112.5 % | ***Confirmatory transition***:  3.3 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  107.1 % | ***Confirmatory transition***:  0.88 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TAA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  101.6 % | ***Confirmatory transition***:  5.0 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  98.6 % | ***Confirmatory transition***:  2.3 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Grain (wheat) | 1,2,4-TRZ | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  90.1 % | ***Confirmatory transition***:  3.7 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  97.7 % | ***Confirmatory transition***:  4.0 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  89.5 % | ***Confirmatory transition***:  13.1 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  107.8 % | ***Confirmatory transition***:  1.8 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TLA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  103.4 % | ***Confirmatory transition***:  6.2 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  106.5 % | ***Confirmatory transition***:  1.1 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TAA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  92.7 % | ***Confirmatory transition***:  15.6 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  102.4 % | ***Confirmatory transition***:  2.9 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Straw (wheat) | 1,2,4-TRZ | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  96.4 % | ***Confirmatory transition***:  4.0 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  99.2 % | ***Confirmatory transition***:  2.3 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  98.2 % | ***Confirmatory transition***:  6.9 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  102.1 % | ***Confirmatory transition***:  4.9 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TLA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  99.4 % | ***Confirmatory transition***:  6.6 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  96.3 % | ***Confirmatory transition***:  1.5 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TAA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  98.7 % | ***Confirmatory transition***:  5.3 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  98.3 % | ***Confirmatory transition***:  1.6 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Rapeseed oil | 1,2,4-TRZ | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  98.4 % | ***Confirmatory transition***:  5.7 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  97.9 % | ***Confirmatory transition***:  1.8 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  99.4 % | ***Confirmatory transition***:  1.7 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  100.0 % | ***Confirmatory transition***:  1.0 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TLA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  99.2 % | ***Confirmatory transition***:  1.9 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  101.0 % | ***Confirmatory transition***:  2.0 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TAA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  102.8 % | ***Confirmatory transition***:  1.4 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  98.1 % | ***Confirmatory transition***:  2.9 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| White bread (wheat) | 1,2,4-TRZ | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  94.4 % | ***Confirmatory transition***:  3.7 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  99.6 % | ***Confirmatory transition***:  1.9 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  105.7 % | ***Confirmatory transition***:  6.9 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  101.3 % | ***Confirmatory transition***:  2.7 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TLA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  99.6 % | ***Confirmatory transition***:  5.8 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  99.8 % | ***Confirmatory transition***:  2.1 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TAA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  100.9 % | ***Confirmatory transition***:  3.4 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  99.6 % | ***Confirmatory transition***:  2.3 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Beer (barley) | 1,2,4-TRZ | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  102.8 % | ***Confirmatory transition***:  2.0 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  101.9 % | ***Confirmatory transition***:  2.8 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  103.5 % | ***Confirmatory transition***:  10.3 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  111.3 % | ***Confirmatory transition***:  4.5 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TLA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  101.4 % | ***Confirmatory transition***:  5.2 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  98.0 % | ***Confirmatory transition***:  4.2 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| TAA | At low level :  0.01 mg/kg (LOQ)  N= 5 | ***Confirmatory transition***:  93.0 % | ***Confirmatory transition***:  10.3 % | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ)  N= 5 | ***Confirmatory transition***:  102.1 % | ***Confirmatory transition***:  2.0 % | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

Table A~~2.1~~ 214: Characteristics for the confirmatory method used for validation of triazole derivative metabolites residues in whole plant (rapeseed)

|  | TDMs |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  **Confirmatory transition:**  ***1,2,4-TRZ***  % interference mean = 7  ***TA***  % interference mean = 16.5  ***TLA***  % interference mean = 11.5  ***TAA***  % interference mean = 2.5 |
| Calibration (type, number of data points) | **Confirmatory transition:**  ***1,2,4-TRZ***  Equation : Y = 8.97514 \* x + 0.73213  Coefficient of correlation: r² = 99.972  ***TA***  Equation : Y = 0.28175 \* x + 0.01482  Coefficient of correlation: r² = 99.928  ***TLA***  Equation : Y = 0.14667 \* x + 0.00442  Coefficient of correlation: r² = 99.956  ***TAA***  Equation : Y = 0.04289 \* x + 7.35409e-4  Coefficient of correlation: r² = 99.816 |
| Calibration range | Accepted calibration range in concentration units 0.5 – 50.0 µg/L (from 20 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes : Performed comparing the analyte response of one individual standard at L4 (or L5) prepared in solvent (water/methanol 50:50 + 1% formic acid) to one prepared in blank matrix extract at the same concentration.  ***1,2,4-TRZ***  Matrix effect = 19.5 %  Not significant  ***TA***  Matrix effect = -77.5 %  Significant in accordance to the SANTE/2020/12830 rev. 1 guideline  ***TLA***  Matrix effect = -8.1 %  Not significant  ***TAA***  Matrix effect = - 2.7 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | According to SANTE/2020/12830 rev.1, the stability of the analytes in the final extracts is not required if the extracts contain an isotope-labelled internal standard (IL-IS) for quantification, since the IL-IS compensates for losses during extract storage. Since IL-IS were used, the verification of the stability was not required. Anyway, all the extracts were analysed within 24 hours from their preparation, obtaining recoveries in the fortified samples within the acceptable range of 70-120%.  In addition, since the differences between the mean responses of the analytes in the diluted solutions prepared form stored and from freshly-prepared stock solutions was < 10%, it is possible to assess that the analytes are stable in the stock solutions prepared in water for at least 78 days, if stored in the dark at 5 ± 3°C. |

Table A~~2.2~~ 215: Characteristics for the confirmatory method used for validation of triazole derivatives metabolites in rapeseed oil

|  | TDMs |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  **Confirmatory transition:**  ***1,2,4-TRZ***  % interference mean = 5  ***TA***  % interference mean = 3  ***TLA***  % interference mean = 1  ***TAA***  % interference mean = 1 |
| Calibration (type, number of data points) | **Confirmatory transition:**  ***1,2,4-TRZ***  Equation : Y = 8.86439 \* x + 0.32346  Coefficient of correlation: r² = 99.846  ***TA***  Equation : Y = 1.29734 \* x + 0.00401  Coefficient of correlation: r² = 99.992  ***TLA***  Equation : Y = 0.14024 \* x – 8.85909e-4  Coefficient of correlation: r² = 99.868  ***TAA***  Equation : Y = 0.04240 \* x + 1.6442e-4  Coefficient of correlation: r² = 99.966 |
| Calibration range | Accepted calibration range in concentration units 0.5 – 50.0 µg/L (from 20 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes : Performed comparing the analyte response of one individual standard at L4 (or L5) prepared in solvent (water/methanol 50:50 + 1% formic acid) to one prepared in blank matrix extract at the same concentration.  ***1,2,4-TRZ***  Matrix effect = -2.7 %  Not significant  ***TA***  Matrix effect = -3.2 %  Not significant  ***TLA***  Matrix effect = -5.4 %  Not significant  ***TAA***  Matrix effect = -2.5 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | According to SANTE/2020/12830 rev.1, the stability of the analytes in the final extracts is not required if the extracts contain an isotope-labelled internal standard (IL-IS) for quantification, since the IL-IS compensates for losses during extract storage. Since IL-IS were used, the verification of the stability was not required. Anyway, all the extracts were analysed within 24 hours from their preparation, obtaining recoveries in the fortified samples within the acceptable range of 70-120%.  In addition, since the differences between the mean responses of the analytes in the diluted solutions prepared form stored and from freshly-prepared stock solutions was < 10%, it is possible to assess that the analytes are stable in the stock solutions prepared in water for at least 78 days, if stored in the dark at 5 ± 3°C. |

Table A~~2.3~~ 216: Characteristics for the confirmatory method used for validation of triazole derivative metabolites in rapeseed seeds- grain (wheat) - white bread (wheat) (calibration in solvent 0.5-50 µg/L)

|  | TDMs |
| --- | --- |
| Specificity | **RAPESEED SEEDS**  ***For, 1,2,4-TRZ, TLA and TAA*** : No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***For TA*** : Signals were detected in amounts higher than the 30% of LOQ for this matrice and were generated by the analytes and not by other interfering substanes.However, a lot of attempts were carried out by the laboratory to find samples with analytes area < 30% of LOQ, analysing a lot of commercially available commodities coming from organic farming: no samples with analytes area < 30% of LOQ were found for this matrice.  **Confirmatory transition:**  ***1,2,4-TRZ***  % interference mean = 0  ***TA***  % interference mean = 239.5  ***TLA***  % interference mean = 16  ***TAA***  % interference mean = 0.5  **GRAIN WHEAT**  ***For, 1,2,4-TRZ and TLA*** : No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***For TA*** ***and TAA***: Signals were detected in amounts higher than the 30% of LOQ for this matrice and were generated by the analytes and not by other interfering substanes.However, a lot of attempts were carried out by the laboratory to find samples with analytes area < 30% of LOQ, analysing a lot of commercially available commodities coming from organic farming: no samples with analytes area < 30% of LOQ were found for this matrice.  **Confirmatory transition:**  ***1,2,4-TRZ***  % interference mean = 25  ***TA***  % interference mean = 227  ***TLA***  % interference mean = 9  ***TAA***  % interference mean = 103.5  **WHITE BREAD (WHEAT)**  ***For, 1,2,4-TRZ and TLA*** : No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***For TA and TAA*** : Signals were detected in amounts higher than the 30% of LOQ for this matrice and were generated by the analytes and not by other interfering substanes.However, a lot of attempts were carried out by the laboratory to find samples with analytes area < 30% of LOQ, analysing a lot of commercially available commodities coming from organic farming: no samples with analytes area < 30% of LOQ were found for this matrice.  **Confirmatory transition:**  ***1,2,4-TRZ***  % interference mean = 0  ***TA***  % interference mean = 57  ***TLA***  % interference mean = 3  ***TAA***  % interference mean = 51.5 |
| Calibration (type, number of data points) | **Confirmatory transition:**  ***1,2,4-TRZ***  Equation : Y = 7.61329 \* x + 0.39044  Coefficient of correlation: r² = 99.942  ***TA***  Equation : Y = 1.53819 \* x + 0.00827  Coefficient of correlation: r² = 99.934  ***TLA***  Equation : Y = 0.12896 \* x + 2.39631e-4  Coefficient of correlation: r² = 99.930  ***TAA***  Equation : Y = 0.04310 \* x + 5.90837e-4  Coefficient of correlation: r² = 99.964 |
| Calibration range | Accepted calibration range in concentration units 0.5 – 50.0 µg/L (from 20 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes : Performed comparing the analyte response of one individual standard at L4 (or L5) prepared in solvent (water/methanol 50:50 + 1% formic acid) to one prepared in blank matrix extract at the same concentration.  **RAPESEED SEEDS**  ***1,2,4-TRZ***  Matrix effect = -4.5 %  Not significant  ***TA***  Matrix effect = 6.0 %  Not significant  ***TLA***  Matrix effect = 6.7 %  Not significant  ***TAA***  Matrix effect = -2.1 %  Not significant  **GRAIN WHEAT**  ***1,2,4-TRZ***  Matrix effect = -7.7 %  Not significant  ***TA***  Matrix effect = -2.5 %  Not significant  ***TLA***  Matrix effect = -5.0 %  Not significant  ***TAA***  Matrix effect = -4.0 %  Not significant  **WHITE BREAD (WHEAT)**  ***1,2,4-TRZ***  Matrix effect = 3.5 %  Not significant  ***TA***  Matrix effect = 7.1 %  Not significant  ***TLA***  Matrix effect = 0.6 %  Not significant  ***TAA***  Matrix effect = -1.8 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.50 µg/L  (0.002 mg/kg) |
| Stability | According to SANTE/2020/12830 rev.1, the stability of the analytes in the final extracts is not required if the extracts contain an isotope-labelled internal standard (IL-IS) for quantification, since the IL-IS compensates for losses during extract storage. Since IL-IS were used, the verification of the stability was not required. Anyway, all the extracts were analysed within 24 hours from their preparation, obtaining recoveries in the fortified samples within the acceptable range of 70-120%.  In addition, since the differences between the mean responses of the analytes in the diluted solutions prepared form stored and from freshly-prepared stock solutions was < 10%, it is possible to assess that the analytes are stable in the stock solutions prepared in water for at least 78 days, if stored in the dark at 5 ± 3°C. |

Table A~~2.4~~ 217: Characteristics for the confirmatory method used for validation of triazole derivative metabolites residues in straw (wheat) (calibration in solvent, 0.35 – 35 µg/L-

|  | TDMs |
| --- | --- |
| Specificity | ***For, 1,2,4-TRZ and TA*** : No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***For TLA and TAA*** : Signals were detected in amounts higher than the 30% of LOQ for this matrice and were generated by the analytes and not by other interfering substanes.However, a lot of attempts were carried out by the laboratory to find samples with analytes area < 30% of LOQ, analysing a lot of commercially available commodities coming from organic farming: no samples with analytes area < 30% of LOQ were found for this matrice. In addition no samples with analytes content < LOD, set to 20% of LOQ (28% for straw) were found. Since no matrices free from the analytes were available and the matrix effect for this matrice was negligible, it was necessary to calibrate using solvent-based standard solutions. Recoveries were necessarily evaluated subtracting the mean values measured from a duplicate analysis of the untreated samples to the values measured for the fortified ones.  **Confirmatory transition:**  ***1,2,4-TRZ***  % interference mean = 0  ***TA***  % interference mean = 9  ***TLA***  % interference mean = 55.5  ***TAA***  % interference mean = 135.5 |
| Calibration (type, number of data points) | **Confirmatory transition:**  ***1,2,4-TRZ***  Equation : Y = 8.30803 \* x + 0.07988  Coefficient of correlation: r² = 99.928  ***TA***  Equation : Y = 2.92033 \* x + 0.05126  Coefficient of correlation: r² = 99.918  ***TLA***  Equation : Y = 0.15019 \* x + 2.79134e-4  Coefficient of correlation: r² = 99.938  ***TAA***  Equation : Y = 0.04473 \* x + 1.75957e-4  Coefficient of correlation: r² = 99.968 |
| Calibration range | Accepted calibration range in concentration units 0.35 – 35.0 µg/L (from 28 % of LOQ to 180 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.0028 – 0.280 mg/kg) |
| Assessment of matrix effects is presented | Yes : Performed comparing the analyte response of one individual standard at L4 (or L5) prepared in solvent (water/methanol 50:50 + 1% formic acid) to one prepared in blank matrix extract at the same concentration.  ***1,2,4-TRZ***  Matrix effect = 0.4 %  Not significant  ***TA***  Matrix effect = 1.3 %  Not significant  ***TLA***  Matrix effect = -7.9 %  Not significant  ***TAA***  Matrix effect = -3.7 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.35 µg/L  (0.0028 mg/kg) |
| Stability | According to SANTE/2020/12830 rev.1, the stability of the analytes in the final extracts is not required if the extracts contain an isotope-labelled internal standard (IL-IS) for quantification, since the IL-IS compensates for losses during extract storage. Since IL-IS were used, the verification of the stability was not required. Anyway, all the extracts were analysed within 24 hours from their preparation, obtaining recoveries in the fortified samples within the acceptable range of 70-120%.  In addition, since the differences between the mean responses of the analytes in the diluted solutions prepared form stored and from freshly-prepared stock solutions was < 10%, it is possible to assess that the analytes are stable in the stock solutions prepared in water for at least 78 days, if stored in the dark at 5 ± 3°C. |

Table A~~2.5~~ 218: Characteristics for the confirmatory method used for validation of triazole derivative metabolites residues in beer (barley) (calibration in solvent, 1-100 µg/L)

|  | TDMs |
| --- | --- |
| Specificity | ***For, 1,2,4-TRZ and TLA*** : No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  ***For TA*** ***and TAA***: Signals were detected in amounts higher than the 30% of LOQ for this matrice and were generated by the analytes and not by other interfering substanes.However, a lot of attempts were carried out by the laboratory to find samples with analytes area < 30% of LOQ, analysing a lot of commercially available commodities coming from organic farming: no samples with analytes area < 30% of LOQ were found for this matrice  **Confirmatory transition:**  ***1,2,4-TRZ***  % interference mean = 0  ***TA***  % interference mean = 314  ***TLA***  % interference mean = 16.5  ***TAA***  % interference mean = 217.5 |
| Calibration (type, number of data points) | **Confirmatory transition:**  ***1,2,4-TRZ***  Equation : Y = 8.33313 \* x + 0.11477  Coefficient of correlation: r² = 99.932  ***TA***  Equation : Y = 2.15353 \* x + 0.11609  Coefficient of correlation: r² = 99.912  ***TLA***  Equation : Y = 0.15499 \* x + 0.00652  Coefficient of correlation: r² = 99.487  ***TAA***  Equation : Y = 0.04053 \* x + 0.00126  Coefficient of correlation: r² = 99.906 |
| Calibration range | Accepted calibration range in concentration units 1.00 – 100.0 µg/L (from 20 % of LOQ to 100 % above 10\*LOQ)  Corresponding calibration range in mass ratio units for the sample (0.002 – 0.2 mg/kg) |
| Assessment of matrix effects is presented | Yes : Performed comparing the analyte response of one individual standard at L4 (or L5) prepared in solvent (water/methanol 50:50 + 1% formic acid) to one prepared in blank matrix extract at the same concentration.  ***1,2,4-TRZ***  Matrix effect = 5.2 %  Not significant  ***TA***  Matrix effect = 5.0 %  Not significant  ***TLA***  Matrix effect = -3.8 %  Not significant  ***TAA***  Matrix effect = 6.5 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 1 µg/L  (0.002 mg/kg) |
| Stability | According to SANTE/2020/12830 rev.1, the stability of the analytes in the final extracts is not required if the extracts contain an isotope-labelled internal standard (IL-IS) for quantification, since the IL-IS compensates for losses during extract storage. Since IL-IS were used, the verification of the stability was not required. Anyway, all the extracts were analysed within 24 hours from their preparation, obtaining recoveries in the fortified samples within the acceptable range of 70-120%.  In addition, since the differences between the mean responses of the analytes in the diluted solutions prepared form stored and from freshly-prepared stock solutions was < 10%, it is possible to assess that the analytes are stable in the stock solutions prepared in water for at least 78 days, if stored in the dark at 5 ± 3°C. |

Conclusion

The confirmatory method for the quantification of triazole derivatives metabolites in wheat, barley, oilseed rape and processed commodities was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability for triazole derivatives metabolites.

Extraction efficiency

Extraction efficiency is guided by:

* European Commission (2017): SANTE 2017/10632 rev. 3, dated 22 November 2017: Technical Guideline on the Evaluation of Extraction Efficiency of Residue Analytical Methods.

An appropriate aliquot of each specimen was taken from the homogenised frozen samples and put in a 50 mL screw capped centrifuge PE test tube followed by the addition of 100 μL of the internal standard solution TDM ISTD MIX (2 mg/L of each internal standard) and by the following amounts of deionized water (added on the basis of QuPPe-PO-Method and considering the theoretical water content of each matrix):

|  |  |  |
| --- | --- | --- |
| **Matrix** | **Sample weight (g)** | **Water added (mL)** |
| Whole Plant (rapeseed) | 5 | 5 |
| Rapeseed seeds | 5 | 10 |
| Wheat (grain) | 5 | 10 |
| Wheat (straw) | 2.5 | 10 |
| Rapeseed oil | 5 | 10 |
| Wheat (white bread) | 5 | 10 |
| Beer (barley) | 10 | 0 |

Then, 10 mL of 1% formic acid in methanol were added and the obtained mixture was vigorously shaken for 3 minutes. The volume of the final extract is considered to be 20 mL: little variation due to the actual water content of each sample are corrected by the presence of the internal standard, that is added to produce a concentration in the final extract nominally of 10 μg/L of each compound.

The separation of the liquid phase from the solid one was achieved by centrifugation at 5000 rpm for 5 minutes. An aliquot of about 1 mL the supernatant was taken, filtered with a 0.45 μm PVDF filter and transferred in a 2 mL HPLC glass vial for the final analysis with a HPLC-DMS-MS/MS system.

* + - * 1. Analytical method 16

|  |  |
| --- | --- |
| Comments of zRMS: | The analytical method validation has been accepted.  The LC-MS/MS method was applied in tunnel test. 2 transitions were monitored. The confirmatory method is not necessary. The validation parameters are in the required range. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/26 |
| Report | Difenoconazole 130 g/L + Prothioconazole 250 g/L EC – IN233C1560 Validation of the Analytical Method for the Determination of Difenoconazole and Prothioconazole Residues in Pollen and Nectar from Ecotoxicological Study  Report No.: CH-0223/2022  Garagna, D. (2022b)  ChemService S.r.l Controlli e Ricerche - Novate Milanese (MI) - Italy |
| Guideline(s): | Yes  SANTE/2020/12830 rev. 1 dated 24/02/21: Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes. |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

Materials and methods

|  |  |
| --- | --- |
| **Principle of the method** | The analytical phase was conducted to determine Difenoconazole concentrations in samples coming from the biological phase of the ecotoxicological test on Honeybee *Apis Mellifera L*. |

**Materials:**

|  |  |
| --- | --- |
| UHPLC/MS-QQQ: | Shimadzu Technologies, mod. 8050, equipped with binary pump, autosampler, coupled with an ESI |
| Column: | Zorbax Eclipse Plus C18, 3.5 µm, 100 x 4.6 mm i.d. |
| Detector: | Triple Quadrupole Mass Detector |
| Ionisation type: | Electrospray (ESI) |
| Polarity: | Positive ion mode |
| Eluent: | A = Water / Formic acid 0.1% / Ammonium formate 10 mM  B = Acetonitrile |
| Eluent flow: | 0.7 mL/min |
| Elution mode: | Gradient condition   |  |  |  | | --- | --- | --- | | % A | % B | Time (min) | | 40 | 60 | 0 | | Waste on *during the samples analysis the matrix is send to waste, during wash analysis not.* | | 4.5 | | 40 | 60 | 8 | | 10 | 90 | 8.1 | | Waste off *during the samples analysis the matrix is send to waste, during wash analysis not* | | 9 | | 10 | 90 | 12 | | 40 | 60 | 16 | | 40 | 60 | 18 | |
| Volume of injection: | 10 µL |
| Retention time: | Approximately 5.3 minutes for a total analysis time of 18 minutes |
| Scan type: | Multiple reaction monitoring |
| Interface temperature: | 300 °C |
| DL temperature: | 250 °C |
| Heat Block: | 30 °C |
| Drying gas flow: | 10 L/min |
| Nebulizer Gas Flow: | 2.9 L/min |
| Heating Gas Flow: | 5 L/min |
|  |  |
| Analytical standards: | Difenoconazole  Batch No. : BCCD4900  Purity : 95.5 %  Expiry date: June 1, 2025 |
|  |  |
| Reagents: | Water, HPLC grade  Acetonitrile, HPLC grade  Ammonium formate  Formic acid  QuEChERS Extraction Salt Packet |
|  |  |

**Methods:**

The analytical methods for the determination of difenoconazole in pollen and in nectar were confirmed so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were confirmed in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A 219: Recovery results from validation method of difenoconazole using the analytical method

| Matrix | Analyte | Fortification level (mg/kg) (n = 5) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Pollen | Difenoconazole | At low level :  30.0 µg/kg (LOQ) | 74.9 | 3.0 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  300 µg/kg (10\*LOQ) | 77.7 | 6.0 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Nectar | Difenoconazole | At low level :  30.0 µg/kg (LOQ) | 73.9 | 3.0 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  300 µg/kg (10\*LOQ) | 92.7 | 8.0 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

Table A 220: Characteristics for the analytical method used for validation method of difenoconazole residues in pollen

|  | Difenoconazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Quantifier transition m/z 406.1 🡪 m/z 250.9***  Equation (µg/L) : Y = 205847 \* x – 7749  Equation (µg/kg) : Y = 10292 \* x – 7749  Coefficient of correlation: r² = 99.803  ***Quantifier 1 transition m/z 406.1 🡪 m/z 336.9***  Equation (µg/L) : Y = 37408 \* x – 105355  Equation (µg/kg) : Y = 1870 \* x – 10535  Coefficient of correlation: r² = 99.931  ***Quantifier 2 transition m/z 406.1 🡪 m/z 188***  Equation (µg/L) : Y = 35068 \* x – 3978  Equation (µg/kg) : Y = 1753 \* x – 3978  Coefficient of correlation: r² = 99.869 |
| Calibration range | Accepted calibration range in concentration units 0.9 – 47.3 µg/L  Corresponding calibration range in mass ratio units for the sample (18.9 – 945.5 µg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = 13.1 %  Not significant matrix effects |
| Limit of quantification (LOQ) | LOQ = 30.0 µg/kg |
| Limit of determination (LOD) | LOD = 0.9 µg/L |
| Stability | Analysis performed within 24 hours from preparation; stability check not performed.  Standard prepared freshly; stability check not performed. |
| Residue amount | Residue results calculated as values lower than the LOD are classified as not detected (n.d.).  Residue results calculated as higher than the limit of detection but less than limit of quantification, are designated as < LOQ. |

Table A 221: Characteristics for the analytical method used for validation method of difenoconazole residues in nectar

|  | Difenoconazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Quantifier transition m/z 406.1 🡪 m/z 250.9***  Equation (µg/L) : Y = 173482 \* x – 23469  Equation (µg/kg) : Y = 8674 \* x – 23469  Coefficient of correlation: r² = 99.934  ***Quantifier 1 transition m/z 406.1 🡪 m/z 336.9***  Equation (µg/L) : Y = 29619 \* x + 1017  Equation (µg/kg) : Y = 1481 \* x + 1017  Coefficient of correlation: r² = 99.981  ***Quantifier 2 transition m/z 406.1 🡪 m/z 188***  Equation (µg/L) : Y = 28699 \* x + 73  Equation (µg/kg) : Y = 1435 \* x + 73  Coefficient of correlation: r² = 99.986 |
| Calibration range | Accepted calibration range in concentration units 0.9 – 47.3 µg/L  Corresponding calibration range in mass ratio units for the sample (18.9 – 945.5 µg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 3 %  Not significant matrix effects |
| Limit of quantification (LOQ) | LOQ = 30.0 µg/kg |
| Limit of determination (LOD) | LOD = 0.9 µg/L |
| Stability | Analysis performed within 24 hours from preparation; stability check not performed.  Standard prepared freshly; stability check not performed. |
| Residue amount | Residue results calculated as values lower than the LOD are classified as not detected (n.d.).  Residue results calculated as higher than the limit of detection but less than limit of quantification, are designated as < LOQ. |

Conclusion

The validation method for the quantification of difenoconazole in pollen and nectar was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability.

* + - * 1. Analytical method 17

|  |  |
| --- | --- |
| Comments of zRMS: | The analytical method validation has been accepted.  The LC-MS/MS method 0223/2022 (see previous study) was applied in tunnel test. 2 transitions were monitored. The confirmatory method is not necessary. The validation parameters are in the required range. |

|  |  |
| --- | --- |
| Reference: | KCP 5.1.2/27 |
| Report | Difenoconazole 130 g/L + Prothioconazole 250 g/L EC- IN233C1560: Effects on Honey Bee Brood (Apis Mellifera L.) under Semi-Field Conditions – Tunnel Test (Analytical Phase)  Report No.: 168191033  Test site study Report No.: CH-0695/2022  Garagna, D. (2022c)  Ibacom GmBH, Rossdorf - Germany |
| Guideline(s): | Yes  SANTE/2020/12830 rev. 1 dated 24/02/21: Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes. |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

Materials and methods

|  |  |
| --- | --- |
| **Principle of the method** | The analytical phase was conducted to determine Difenoconazole concentrations in samples coming from the biological phase of the ecotoxicological test on Honeybee *Apis Mellifera L*. |

**Materials:**

|  |  |
| --- | --- |
| UHPLC/MS-QQQ: | Shimadzu Technologies, mod. 8050, equipped with binary pump, autosampler, coupled with an ESI |
| Column: | Zorbax Eclipse Plus C18, 3.5 µm, 100 x 4.6 mm i.d. |
| Detector: | Triple Quadrupole Mass Detector |
| Ionisation type: | Electrospray (ESI) |
| Polarity: | Positive ion mode |
| Eluent: | A = Water / Formic acid 0.1% / Ammonium formate 10 mM  B = Acetonitrile |
| Eluent flow: | 0.7 mL/min |
| Elution mode: | Gradient condition   |  |  |  | | --- | --- | --- | | % A | % B | Time (min) | | 40 | 60 | 0 | | Waste on *during the samples analysis the matrix is send to waste, during wash analysis not.* | | 4.5 | | 40 | 60 | 8 | | 10 | 90 | 8.1 | | Waste off *during the samples analysis the matrix is send to waste, during wash analysis not* | | 9 | | 10 | 90 | 12 | | 40 | 60 | 16 | | 40 | 60 | 18 | |
| Volume of injection: | 10 µL |
| Retention time: | Approximately 5.3 minutes for a total analysis time of 18 minutes |
| Scan type: | Multiple reaction monitoring |
| Interface temperature: | 300 °C |
| DL temperature: | 250 °C |
| Heat Block: | 30 °C |
| Drying gas flow: | 10 L/min |
| Nebulizer Gas Flow: | 2.9 L/min |
| Heating Gas Flow: | 5 L/min |
|  |  |
| Analytical standards: | Difenoconazole  Batch No. : BCCD4900  Purity : 95.5 %  Expiry date: June 1, 2025 |
|  |  |
| Reagents: | Water, HPLC grade  Acetonitrile, HPLC grade  Ammonium formate  Formic acid  QuEChERS Extraction Salt Packet |
|  |  |

**Methods:**

The analytical methods for the determination of difenoconazole in pollen and in nectar were confirmed so as to comply with the Guidance Document SANTE/2020/12830 rev.1 (dated 24/02/21). The methods were confirmed in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A 222: Recovery results from validation method of difenoconazole using the analytical method

| Matrix | Analyte | Fortification level (mg/kg) (n = 5) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Pollen | Difenoconazole | At low level :  30.0 µg/kg (LOQ) | 74.9 | 3.0 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  300 µg/kg (10\*LOQ) | 77.7 | 6.0 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Nectar | Difenoconazole | At low level :  30.0 µg/kg (LOQ) | 73.9 | 3.0 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| At high level:  300 µg/kg (10\*LOQ) | 92.7 | 8.0 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

Table A 223: Characteristics for the analytical method used for validation method of difenoconazole residues in pollen

|  | Difenoconazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  % interference mean = 0.0 |
| Calibration (type, number of data points) | ***Pollen***  Equation : Y = 351143 \* x – 23893  Coefficient of correlation: r² = 99.678  ***Pollen (diluted samples)***  Equation : Y = 211382 \* x – 111379  Coefficient of correlation: r² = 99.910 |
| Calibration range | Accepted calibration range in concentration units 0.9 – 47.3 µg/L  Corresponding calibration range in mass ratio units for the sample (0.01 – 0.47 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = 13.1 %  Not significant matrix effects |
| Limit of quantification (LOQ) | LOQ = 30.0 µg/kg |
| Limit of determination (LOD) | LOD = 0.9 µg/L |
| Stability | Analysis performed within 24 hours from preparation; stability check not performed.  Standard prepared freshly; stability check not performed. |
| Residue amount | Residue results calculated as values lower than the LOD are classified as not detected (n.d.).  Residue results calculated as higher than the limit of detection but less than limit of quantification, are designated as < LOQ. |

Table A 224: Characteristics for the analytical method used for validation method of difenoconazole residues in nectar

|  | Difenoconazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 1 (dated 24/02/2021).  % interference mean = 0.0 |
| Calibration (type, number of data points) | Equation : Y = 153504 \* x – 3410  Coefficient of correlation: r² = 99.944 |
| Calibration range | Accepted calibration range in concentration units 0.9 – 47.3 µg/L  Corresponding calibration range in mass ratio units for the sample (0.01 – 0.47 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 3 %  Not significant matrix effects |
| Limit of quantification (LOQ) | LOQ = 30.0 µg/kg |
| Limit of determination (LOD) | LOD = 0.9 µg/L |
| Stability | Analysis performed within 24 hours from preparation; stability check not performed.  Standard prepared freshly; stability check not performed. |
| Residue amount | Residue results calculated as values lower than the LOD are classified as not detected (n.d.).  Residue results calculated as higher than the limit of detection but less than limit of quantification, are designated as < LOQ. |

Conclusion

The validation method for the quantification of difenoconazole in pollen and nectar was successfully validated according to Guidance Document SANTE/2020/12830 rev. 1 (dated 24/02/2021) by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability.

* + - * 1. Analytical method 18

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  The LC-MS/MS detection were used. 2 transitions were monitored. The specificity of the method was assured by MS/MS detection. The limit of quantification (LOQ) was set to 0.01 mg/kg. All validation parameters are in the currently required range |

|  |  |
| --- | --- |
| Reference: | KCP 5.3.2.8/01 |
| Report | Validation of an analytical method for the quantification of Difenoconazole, Prothioconazole and Prothioconazole-desthio in honey  Report No.: LBN-0092-2023  Longhi, D. 2023a  LabAnalysis s.r.l., Casanova Lonati (PV) - Italy |
| Guideline(s): | Yes  SANTE/2020/12830 rev. 2: Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes. |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

Materials and methods

|  |  |
| --- | --- |
| **Principle of the method** | The analytical method was based on the method “European Committee for Standardisation (CEN) EN 15662:2018. Foods of plant origin - Multimethod for the determination of pesticide residues using GC- and LC-based analysis following acetonitrile extraction/partitioning and clean-up by dispersive SPE - Modular QuEChERS-method”.  The analytical method was based on an instrumental determination using a HPLC-MS/MS (high-performance liquid chromatography + triple-quadrupole mass spectrometry).  The limit of quantification (LOQ) was set to 0.01 mg/kg.  The analytical method was validated under GLP compliance according to SANTE/2020/12830 Rev.2. |

**Materials:**

|  |  |
| --- | --- |
| HPLC-MS/MS | Agilent HPLC 1290 Infinity II + Agilent MS spectrometer 6470A Triple Quad |
| Column: | Phenomenex Kinetex C18, 1.7 μm, 2.1 x 50 mm |
| Column temperature: | 40°C |
| Flow: | 0.6 mL/min |
| Injection volume: | 2.5 µL |
| Mobile phase:  Elution: | A = LC-MS grade water with 0.2% formic acid and 5 mM ammonium formate  B = LC-MS grade methanol with 0.2% formic acid and 5 mM ammonium formate   |  |  |  | | --- | --- | --- | | Time (min) | % A | % B | | 0 | 70 | 30 | | 0.5 | 70 | 30 | | 3.0 | 0 | 100 | |
| Stop time: | 5 min |
| Post time: | 1 min |
| Divert value: | 0 min. to waste, 2 min to MS, 3.5 min. to waste |
| Source type: | ESI |
| Gas temperature: | 350°C |
| Gas flow: | 8 L/min |
| Nebulizer: | 40 psi |
| Sheath gas heater: | 400°C |
| Sheath gas flow: | 12 L/min |
| Capillary: | Positive mode 3500V  Negative mode 3000V |
| Vcharging: | 0 |
| Acquiring mode: | ESI positive and ESI negative, MRM (multi-reaction monitoring) |

|  |  |
| --- | --- |
| Analytical standards: | Difenoconazole  CAS No.: 119449-68-3  Batch No.: BCCD4900  Purity : 95.5% with 0.1% water (purity corrected for the water content: 95.4 %)  Expiry date: June, 2025 |
| Reagents: | Water, LC-MS grade  Cysteine HCl  Formic acid  Acetonitrile  Unbuffered QuEChERS Extraction Salt Packet  Ammonium formate  Methanol |
| Buffer,  reference standard: | Buffer, pH 4.00 ± 0.01 (25°C)  Batch No.: MKCR2269  Product No.: B5020  Certified value (pH); 4.01  Expiry date: January, 2024  Buffer, pH 7.00 ± 0.01 (25°C)  Batch No.: MKCR0856  Product No.: B4770  Certified value (pH); 7.00  Expiry date: December, 2023 |
| Matrix: | Honey (multiflower origin) purchased in a local market Esselunga, Broni (PV), Italy  Storage: frozen  Measured pH-value: 4.2 according to method CIPC MT 75.3 |

**Methods:**

The analytical method for the quantification of difenoconazole in the honey are presented below.

***2.A. Schematic diagram of the analytical method***

***Une image contenant texte, capture d’écran, Police, ligne

Description générée automatiquement***

***2.B. Quantification of difenoconazole in honey***

The analytical methods for the determination of difenoconazole were confirmed so as to comply with the Guidance Document SANTE/2020/12830 rev.2. The methods were confirmed in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A 225: Recovery results from validation method of difenoconazole using the analytical method

| Matrix | Analyte | Fortification level (mg/kg) (n = 5) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Honey | Difenoconazole  (product ion: 251.1 m/z) | At low level :  0.01 mg/kg (LOQ) | 98.7 | 5.7 | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 100 | 2.8 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

Table A 226: Characteristics for the analytical method used for validation method of difenoconazole residues in honey

|  | difenoconazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 2.  ***Product ion (m/z = 251.1):***  % interference mean = 4.0 |
| Calibration (type, number of data points) | ***Product ion (m/z = 251.1):***  Equation : Y = 2936.158916 \* x + 265.995556  Coefficient of correlation: r² = 99.91 |
| Calibration range | Accepted calibration range in concentration units 0.50 – 50. µg/L  Corresponding calibration range in mass ratio units for the sample (0.0020 – 0.200 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 7.93 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.5 µg/L  (0.002 mg/kg)  228.5 S/N at LOD level |
| Stability | Λ% = 1.5 |
| Stability of standard | % difference = - 3.5%  Since the differences between the mean responses of the analytes in the solutions prepared form stored and from freshly-prepared stock solutions was < 10%, it is possible to assess that the analyte difenoconazole is stable in the stock solutions prepared in acetonitrile for 55 days, if stored in the dark at 5 ± 3°C. |

Conclusion

The analytical method for the quantification of difenoconazole residues in honey was successfully validated according to Guidance Document SANTE/2020/12830 rev. 2 by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability for difenoconazole.

Confirmatory method

|  |  |
| --- | --- |
| Comments of zRMS: | The method validation has been accepted.  The LC-MS/MS detection were used. 2 transitions were monitored. The specificity of the method was assured by MS/MS detection. The limit of quantification (LOQ) was set to 0.01 mg/kg. All validation parameters are in the currently required range |

|  |  |
| --- | --- |
| Reference: | KCP 5.3.2.8/01 |
| Report | Validation of an analytical method for the quantification of Difenoconazole, Prothioconazole and Prothioconazole-desthio in honey  Report No.: LBN-0092-2023  Longhi, D. 2023a  LabAnalysis s.r.l., Casanova Lonati (PV) - Italy |
| Guideline(s): | Yes  SANTE/2020/12830 rev. 2: Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes. |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

Materials and methods

|  |  |
| --- | --- |
| **Principle of the method** | The analytical method was based on the method “European Committee for Standardisation (CEN) EN 15662:2018. Foods of plant origin - Multimethod for the determination of pesticide residues using GC- and LC-based analysis following acetonitrile extraction/partitioning and clean-up by dispersive SPE - Modular QuEChERS-method”.  The analytical method was based on an instrumental determination using a HPLC-MS/MS (high-performance liquid chromatography + triple-quadrupole mass spectrometry).  The limit of quantification (LOQ) was set to 0.01 mg/kg.  The analytical method was validated under GLP compliance according to SANTE/2020/12830 Rev.2. |

**Materials:**

|  |  |
| --- | --- |
| HPLC-MS/MS | Agilent HPLC 1290 Infinity II + Agilent MS spectrometer 6470A Triple Quad |
| Column: | Phenomenex Kinetex C18, 1.7 μm, 2.1 x 50 mm |
| Column temperature: | 40°C |
| Flow: | 0.6 mL/min |
| Injection volume: | 2.5 µL |
| Mobile phase:  Elution: | A = LC-MS grade water with 0.2% formic acid and 5 mM ammonium formate  B = LC-MS grade methanol with 0.2% formic acid and 5 mM ammonium formate   |  |  |  | | --- | --- | --- | | Time (min) | % A | % B | | 0 | 70 | 30 | | 0.5 | 70 | 30 | | 3.0 | 0 | 100 | |
| Stop time: | 5 min |
| Post time: | 1 min |
| Divert value: | 0 min. to waste, 2 min to MS, 3.5 min. to waste |
| Source type: | ESI |
| Gas temperature: | 350°C |
| Gas flow: | 8 L/min |
| Nebulizer: | 40 psi |
| Sheath gas heater: | 400°C |
| Sheath gas flow: | 12 L/min |
| Capillary: | Positive mode 3500V  Negative mode 3000V |
| Vcharging: | 0 |
| Acquiring mode: | ESI positive and ESI negative, MRM (multi-reaction monitoring) |

|  |  |
| --- | --- |
| Analytical standards: | Difenoconazole  CAS No.: 119449-68-3  Batch No.: BCCD4900  Purity : 95.5% with 0.1% water (purity corrected for the water content: 95.4 %)  Expiry date: June, 2025 |
| Reagents: | Water, LC-MS grade  Cysteine HCl  Formic acid  Acetonitrile  Unbuffered QuEChERS Extraction Salt Packet  Ammonium formate  Methanol |
| Buffer,  reference standard: | Buffer, pH 4.00 ± 0.01 (25°C)  Batch No.: MKCR2269  Product No.: B5020  Certified value (pH); 4.01  Expiry date: January, 2024  Buffer, pH 7.00 ± 0.01 (25°C)  Batch No.: MKCR0856  Product No.: B4770  Certified value (pH); 7.00  Expiry date: December, 2023 |
| Matrix: | Honey (multiflower origin) purchased in a local market Esselunga, Broni (PV), Italy  Storage: frozen  Measured pH-value: 4.2 according to method CIPC MT 75.3 |

**Methods:**

The analytical method for the quantification of difenoconazole in the honey are presented below.

***2.A. Schematic diagram of the analytical method***

***Une image contenant texte, capture d’écran, Police, ligne

Description générée automatiquement***

***2.B. Quantification of difenoconazole in honey***

The analytical methods for the determination of difenoconazole were confirmed so as to comply with the Guidance Document SANTE/2020/12830 rev.2. The methods were confirmed in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

|  |  |
| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A 227: Recovery results from confirmatory method of difenoconazole using the analytical method

| Matrix | Analyte | Fortification level (mg/kg) (n = 5) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Honey | Difenoconazole  (product ion: 188.4 m/z) | At low level :  0.01 mg/kg (LOQ) | 99.3 | 4.1 | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 98.7 | 3.9 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

Table A 228: Characteristics for the analytical method used for confirmatory method of difenoconazole residues in honey

|  | difenoconazole |
| --- | --- |
| Specificity | No interfering signals were detected in the untreated matrix in amount higher than 30% of the LOQ level, that is in compliance with the guideline requirements described by SANTE/2020/12830 rev. 2.  ***Product ion (m/z = 188.4):***  % interference mean = 9.5 |
| Calibration (type, number of data points) | ***Product ion (m/z = 188.4):***  Equation : Y = 218.200199 \* x + 57.357271  Coefficient of correlation: r² = 99.97 |
| Calibration range | Accepted calibration range in concentration units 0.50 – 50. µg/L  Corresponding calibration range in mass ratio units for the sample (0.0020 – 0.200 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 7.93 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.5 µg/L  (0.002 mg/kg)  10.8 S/N at LOD level |

Conclusion

The confirmatory method for the quantification of difenoconazole residues in honey was successfully validated according to Guidance Document SANTE/2020/12830 rev. 2 by definition of the specificity, calibration (linearity), assessment of matrix effects, limits of determination and quantification for prothioconazole and prothioconazole-desthio.

Independent laboratory validation

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| --- | --- |
| Comments of zRMS: | The ILV of the analytical method developed and validated in GLP studies Code LBN-0092-2023 has been accepted.  The Prothioconazole, Prothioconazole-desthio and difenoconazole determination was conducted by LC-MS/MS in MRM mode, monitoring two MS/MS ion mass transitions. The validation parameters were in the required range. |

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| Reference: | KCP 5.3.2.8/03 |
| Report | Independent Laboratory Validation (ILV) of the analytical Method for the Determination of Difenoconazole, Prothioconazole, Prothioconazole-desthio and Triazole Derivatives Metabolites (TDMs) in Honey  Report No.: CH-0859-2023  Mattioli, B. 2023  ChemService S.r.l. Controlli e Ricerche, Novate Milanese (MI) - Italy |
| Guideline(s): | Yes  SANTE/2020/12830 rev. 2: Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes. |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

Materials and methods

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| **Aim of the method** | The aim of this study was to perform an Independent Laboratory Validation (ILV) of the analytical method developed and validated in GLP studies Code LBN-0092-2023 (Determination of Difenoconazole, Prothioconazole, Prothioconazole-desthio and difenoconazole in Honey) performed by LabAnalysis s.r.l.  The Test Facility ChemService Srl Controlli e Ricerche had re-validate the section of “Recovery and Repeatability” and of “Selectivity and Specificity” of the analytical method already adjusted and validated by LabAnalysis in GLP studies Code LBN-0092-2023.  The analytical method was based on an instrumental determination using a HPLC-MS/MS (high-performance liquid chromatography + triple-quadrupole mass spectrometry).  The limit of quantification (LOQ) was set to 0.01 mg/kg.  The analytical method was validated under GLP compliance according to SANTE/2020/12830 Rev.2. |

**Materials:**

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| --- | --- |
| HPLC-MS/MS | Agilent HPLC 1290 Infinity II + Agilent MS spectrometer 6470A Triple Quad |
| Column: | Phenomenex Kinetex C18, 1.7 μm, 2.1 x 50 mm |
| Column temperature: | 40°C |
| Eluent Flow: | 0.6 mL/min |
| Injection volume: | 2.5 µL |
| Eluent:  Solvent composition: | C = Methanol with 0.2% formic acid and 5 mM ammonium formate  D = Water with 0.2% formic acid and 5 mM ammonium formate   |  |  |  | | --- | --- | --- | | Time (min) | % C | % D | | 0 | 30 | 70 | | 0.5 | 30 | 70 | | 3 | 10 | 90 | | 5 | 30 | 70 | |
| Retention time: | Difenoconazole – about 3.3 minutes |
| Total analysis time: | 5 minutes + 1.0 minutes as post time |
| Source type: | ESI |
| Dry Gas temperature: | 300°C |
| Dry Gas flow: | 8 L/min |
| Nebulizer: | 40 psi |
| Sheath gas temp: | 400°C |
| Sheath gas flow: | 12 L/min |
| Capillary current: | 3000V |
| Vcharging: | 1500V |
| Dwell time: | 50 msec |

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| Analytical standards: | Difenoconazole  CAS No.: 119449-68-3  Batch No.: BCCD4900  Purity : 95.5%  Expiry date: June 01, 2025 |
|  |  |
| Reagents: | Water, LC-MS grade  Cysteine HCl  Formic acid  Acetonitrile, HPLC grade  Unbuffered QuEChERS Extraction Salt Packet  Ammonium formate  Methanol, LC-MS grade |
| Matrix: | Honey  Expiry date: September 13, 2024  Storage: frozen |

**Methods:**

The analytical method for the quantification of difenoconazole in the honey are presented below.

***2.A. Quantification of difenoconazole in honey***

The analytical methods for the determination of difenoconazole were confirmed so as to comply with the Guidance Document SANTE/2020/12830 rev.2. The methods were confirmed in terms of linearity, precision, accuracy and specificity and limits of detection and quantification.

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| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Precision: | It was determining by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision was the expressed as the relative standard deviation.  % RSD = 100 x S.D./mean  The acceptability of the precision was assessed using the modified Horwitz equation:  %RSDr = 0.67 x 2(1-0.5 log C) |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| Specificity: | The specificity ensures that the signal measured comes from the substance of interest (no interference from excipients and/or degradation products and/or impurities.). |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |
| LOD: | This was assessed according to the Guidance Document and corresponds to the lowest concentration in the sample than can be detected (but not necessarily quantified). |

Results and discussions

Table A 229: Recovery results from independent validation method of difenoconazole using the analytical method

| Matrix | Analyte | Fortification level (mg/kg) (n = 5) | Mean  recovery (%) | RSD (%) | Comments |
| --- | --- | --- | --- | --- | --- |
| Honey | Difenoconazole  (product ion: 251.1 m/z)  Primary dectecion | At low level :  0.01 mg/kg (LOQ) | 94.5 | 5.6 | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 92.4 | 3.8 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |
| Difenoconazole  (product ion: 188.4 m/z)  Confirmatory detection | At low level :  0.01 mg/kg (LOQ) | 92.9 | 10.5 | Recoveries range of 60 – 120 %  RSD ≤ 30 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 89.9 | 4.7 | Recoveries range of 70 – 120 %  RSD ≤ 20 % |

Table A 230: Characteristics for the analytical method used for independent validation method of difenoconazole residues in honey

|  | difenoconazole |
| --- | --- |
| Specificity | n.d. |
| Calibration (type, number of data points) | ***Product ion (m/z = 251.1) – Primary dectection:***  Equation : Y = 676.5 \* x + 4755.5  Coefficient of correlation: r² = 99.979  ***Product ion (m/z = 188.4) – Confirmatory dectection:***  Equation : Y = 71.4 \* x + 517.8  Coefficient of correlation: r² = 99.976 |
| Calibration range | Accepted calibration range in concentration units 0.50 – 50. µg/L  Corresponding calibration range in mass ratio units for the sample (0.0020 – 0.200 mg/kg) |
| Assessment of matrix effects is presented | Yes  Matrix effect = - 7.93 %  Not significant |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Limit of determination (LOD) | LOD = 0.5 µg/L  (0.002 mg/kg) |
| Stability of samples extracts | % residual analyte after storage = 102% |
| Stability of standard solutions | No degradation higher than 10% was observed during this storage period. |

Conclusion

The independent analytical method for the quantification of difenoconazole residues in honey was successfully validated according to Guidance Document SANTE/2020/12830 rev. 2 by definition of the accuracy, calibration (linearity), assessment of matrix effects, limits of determination and quantification and stability for difenoconazole.

* + - * 1. Analytical method 19

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| Comments of zRMS: | The validation of the analytical methods has been accepted.  Difenoconazole, prothioconazole, prothioconazole-desthio and the triazole-derivative metabolites (TDMs): triazole-alanine (TA), 1,2,4-triazole (1,2,4-T), triazole lactic acid (TLA), triazole acetic acid (TAA) in honey were determined by LC-MS/MS (MRM mode), monitoring two mass transitions.  Samples were analysed according to the analytical method AM1-LBN-0092-2023 and AM-LBN-0093-2023 (see relevant validations).  The limits of quantification (LOQs) were set to 0.01 mg/kg. The validation parameters were in the required range. |

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| Reference: | KCP 5.3.2.8/04 |
| Report | Analytical phase report – Magnitude of the residue of difenoconazole, prothioconazole, prothioconazole-desthio and triazole-derivative metabolites (TDMs) in honey after one application of IN233C1560 380 EC on Phacelia crop under semi-field conditions in four trials in Northern Europe and Southern Europe – 2023.  Multisite study 1111.4F.SAG23  Report No.: LBN-0108-2023  Rovetto, I. 2023  LabAnalysis s.r.l., Casanova Lonati (PV) - Italy |
| Guideline(s): | Yes  SANTE/2020/12830 rev. 2: Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes. |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

Materials and methods

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| **Principle of the method** | The analytical method to quantify Difenoconazole in honey was based on the QuEChERS method (EN 15662\_2018). The instrumental determination was carried out using a HPLC-MS/MS system (high-performance liquid chromatography + triple quadrupole mass spectrometry).  The analyses were carried out according to the following analytical methods validated under GLP compliance  according to SANTE/2020/12830 rev.2:  - Analytical method AM1-LBN-0092-2023 “Determination of Difenoconazole, Prothioconazole and  Prothioconazole-desthio in honey” validated under GLP compliance in the GLP study LBN-0092-2023  “Validation of an analytical method for the quantification of Difenoconazole, Prothioconazole and  Prothioconazole-desthio in honey”, Test Facility: LabAnalysis s.r.l., Study Director: Diego Longhi. |

**Materials:**

|  |  |
| --- | --- |
| HPLC-MS/MS | Agilent HPLC 1290 Infinity II + Agilent MS spectrometer 6470A Triple Quad |
| Column: | Phenomenex Kinetex C18, 1.7 μm, 2.1 x 50 mm |
| Column temperature: | 40°C |
| Flow: | 0.6 mL/min |
| Injection volume: | 2.5 µL |
| Mobile phase:  Elution: | A = LC-MS grade water with 0.2% formic acid and 5 mM ammonium formate  B = LC-MS grade methanol with 0.2% formic acid and 5 mM ammonium formate   |  |  |  | | --- | --- | --- | | Time (min) | % A | % B | | 0 | 70 | 30 | | 0.5 | 70 | 30 | | 3.0 | 0 | 100 | |
| Stop time: | 5 min |
| Post time: | 1 min |
| Divert value: | 0 min. to waste, 2 min to MS, 3.5 min. to waste |
| Source type: | ESI |
| Gas temperature: | 350°C |
| Gas flow: | 8 L/min |
| Nebulizer: | 40 psi |
| Sheath gas heater: | 400°C |
| Sheath gas flow: | 12 L/min |
| Capillary: | Positive mode 3500V  Negative mode 3000V |
| Vcharging: | 0 |
| Acquiring mode: | ESI positive and ESI negative, MRM (multi-reaction monitoring) |

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| Analytical standards: | Difenoconazole  CAS No.: 119449-68-3  Batch No.: BCCD4900  Purity : 95.5% with 0.1% water (purity corrected for the water content: 95.4 %)  Expiry date: June, 2025 |
| Reagents: | Water, LC-MS grade  Cysteine HCl  Formic acid  Acetonitrile  Unbuffered QuEChERS Extraction Salt Packet  Ammonium formate  Methanol |
| Buffer,  reference standard: | Buffer, pH 4.00 ± 0.01 (25°C)  Batch No.: MKCR2269  Product No.: B5020  Certified value (pH); 4.01  Expiry date: January, 2024  Buffer, pH 7.00 ± 0.01 (25°C)  Batch No.: MKCR0856  Product No.: B4770  Certified value (pH); 7.00  Expiry date: December, 2023 |
| Matrix: | Honey (multiflower origin) purchased in a local market Esselunga, Broni (PV), Italy  Storage: frozen  Measured pH-value: 4.2 according to method CIPC MT 75.3 |

**Methods:**

The analytical method for the quantification of difenoconazole in the honey are presented below.

***2.A. Schematic diagram of the analytical method***

***Une image contenant texte, capture d’écran, Police, ligne

Description générée automatiquement***

***2.B. Quantification of difenoconazole in honey***

The analytical methods for the determination of difenoconazole were confirmed so as to comply with the Guidance Document SANTE/2020/12830 rev.2. The methods were confirmed in terms of linearity, accuracy and limits quantification.

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| --- | --- |
| Linearity: | It was determining by calculating the regression line using a mathematical treatment of the results vs. analyte concentration and the range was reported. The coefficient of determination should be 99 %, at least. |
| Accuracy: | It was assessed by analyzing the above sample solutions used for the assessment of precision and comparing the measured value to the true value. |
| LOQ: | This was assessed according to the Guidance Document and corresponds to the lowest concentration of an analyte in the sample which can be determined with acceptable precision and accuracy. |

Results and discussions

Table A 231: Recovery results from analytical phase of difenoconazole using the analytical method for honey residue trials

| Matrix | Analyte | Fortification level (mg/kg) (n = 5) | Mean  recovery (%) | Comments |
| --- | --- | --- | --- | --- |
| Honey  CDS-23-1469 | Difenoconazole  (product ion: 251.1 m/z) | At low level :  0.01 mg/kg (LOQ) | 108 | Recoveries range of 60 – 120 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 117 | Recoveries range of 70 – 120 % |
| Honey  CDS-23-1565 | Difenoconazole  (product ion: 251.1 m/z) | At low level :  0.01 mg/kg (LOQ) | 93.2 | Recoveries range of 60 – 120 % |
| At high level:  0.1 mg/kg (10\*LOQ) | 100 | Recoveries range of 70 – 120 % |

Table A 232: Characteristics for the analytical method used for analytical phase of difenoconazole in honey residue trials

|  | difenoconazole |
| --- | --- |
| Calibration (type, number of data points) | ***Product ion (m/z = 251.1):***  Equation : Y = 1578.707297 \* x + 326.624111  Coefficient of correlation: r² = 99.988849 |
| Calibration range | Accepted calibration range in concentration units 0.50 – 50. µg/L  Corresponding calibration range in mass ratio units for the sample (0.0020 – 0.200 mg/kg) |
| Limit of quantification (LOQ) | LOQ = 0.01 mg/kg |
| Extraction | Samples were extracted and analysed within 30 days from the sampling.  All the extracts were analysed within 24 hours from their preparation, keeping them at a temperature of 5 ± 3°C until the analysis. |

Conclusion

The analytical phase according to the analytical validation method for the quantification of difenoconazole in honey residue study was successfully validated according to Guidance Document SANTE/2020/12830 rev. 2 by definition of the accuracy, calibration (linearity), limits of quantification and extraction for prothioconazole and prothioconazole-desthio.

* + 1. Methods for post-authorization control and monitoring purposes (KCP 5.2)

No new or additional studies have been submitted.