FINAL REGISTRATION REPORT

**Part B**

**Section 5**

**Analytical Methods**

Detailed summary of the risk assessment

Product code: GLOB2007bF

Product name: Observer Pro

Chemical active substances:

Zoxamide, 67.5 g/L
Propamocarb-HCl, 450 g/L

Central Zone

Zonal Rapporteur Member State: Poland

CORE ASSESSMENT

Applicant: Globachem NV

Submission date: November 2023

MS Finalisation date: 31/10/2024

# Version history

| When | What |
|---|---|
| November 2023 | Initial dossier submission by applicant for approval of new product |
| March 2024 | Dossier sent for evaluation |
| July 2024 | zRMS finalised evaluation |
| October 2024 | zRMS finalised evaluation after commenting period |

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zRMS comments:
This report has been completed by the Applicant.
The text highlighted in grey was provided by the zRMS.
The text highlighted in green was added/changed after the commenting period.

# 5 Analytical methods

## 5.1 Conclusion and summary of assessment

Sufficiently sensitive and selective analytical methods are ~~not~~ available for the active substance(s) and relevant impurities in the plant protection product.
~~Noticed data gaps are:~~

Sufficiently sensitive and selective analytical methods are available for all analytes included in the residue definitions.
Noticed data gaps are:
for propamocarb:

- a primary, confirmatory and ILV methods for the determination of propamocarb in drinking water
- a primary method for the determination of propamocarb in surface water
- a primary method and confirmation for the analysis of propamocarb in body tissues and body fluids

The indicated data gaps should be considered as gaps for the active substance and the Applicant should complete them post-registration after the renewal of approval for propamocarb.

| Commodity/crop | Supported/ Not supported |
|---|---|
| Seed, ware and starch potato | Supported |

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

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

Analytical methods for the determination of zoxamide and propamocarb-HCl in GLOB2007bF were not evaluated as part of the EU review of these active substances. Therefore all relevant data are provided here and are considered adequate.

### 5.2.1.1 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 zoxamide and propamocarb-HCl in plant protection product is provided as follows:

| | |
|---|---|
| Comments of zRMS: | The method of analysis of active substances content (zoxamide content (both stereoisomers content and total zoxamide content) and of propamocarb-HCl content) have been validated in GLP laboratory in compliance with Document SANCO/3030/99 - rev 5. The acceptance criteria are met for all the validation parameters. |

| | |
|---|---|
| Reference: | KCP 5.1.1 |
| Report | Validation of HPLC methods for the determination of zoxamide content (both stereoisomers content and total zoxamide content) and of propamocarb-HCl content in a formulation suspension concentrate (SC) containing 67.5 g/L zoxamide and 450 g/L propamocarb-HCl, De Ryckel, B., 2022, Centre Wallon De Recherches Agronomiques, Report No.: 25508 |
| Guideline(s): | SANCO 3030/99, rev. 5 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

**Materials and methods**

**Zoxamide**

The zoxamide content and the ratio of the stereoisomers of zoxamide is determined after dissolution of active substance in methanol. The separation is achieved using reverse phase liquid chromatography with ultra-violet detection and external standard.

**Calibration solutions**

Weigh (to the nearest 0.1 mg) about 20 and 30 mg of zoxamide analytical standard into two separate 25 mL volumetric flasks. Add methanol and place the flask in an ultrasonic bath until complete dissolution (minimum during 10 minutes). Fill to the mark at 20°C ± 1°C with methanol. Mix thoroughly. (**Stock calibration solutions 1 and 2)**

Transfer by pipette 5 mL of **stock calibration solutions**, at 20°C ± 1°C, into two separate 25 mL volumetric flasks and fill the volumetric flasks to the mark, at 20°C ± 1°C, with methanol. Mix well. (**calibration solutions 1B and 2B**).
[concentrations of about 160 µg/mL and 240 µg/mL]

**Preparation of sample**

Weigh (to the nearest 0.1 mg) about **325 mg** of test item into a 100 mL volumetric flask. Add methanol and disperse the test item during minimum 10 minutes. Fill to the mark, at 20°C ± 1°C, with methanol. Mix thoroughly.

**CHROMATOGRAPHIC PARAMETERS**

| | |
|---|---|
| Column: | Phenomenex Lux Cellulose-3, 3 µm, 150 x 4.6 mm i.d.<br>with pre-column AJO-8622 |
| Mobile phase: | methanol –water (80-20 v/v). |

| | |
|---|---|
| Flow rate: | 0.5 mL/min. |
| Column temperature: | 30°C. |
| Injection volume: | 10 µL. |
| Detector wavelength: | at 230 nm |
| Injection sequence: | calibration 1, sample weighing 1, sample weighing 2, calibration 2, sample weighing 3,…<br>Each solution is injected in duplicate. |

**Propamocarb-HCl**

Active substance content is determined after dispersion of the test item in a mixture methanol - water (80-20 v/v). The separation is achieved using reverse phase liquid chromatography with ultraviolet detection and external standard.

**Calibration solutions**

Weigh (to the nearest 0.1 mg) about 28 mg and 42 mg of propamocarb analytical standard into two separate 20 mL volumetric flasks. Add diluting solvent and place the flasks in an ultrasonic bath until complete dissolution (minimum 10 minutes). Fill to the mark at 20°C ± 1°C with diluting solvent. Mix thoroughly. (Calibration solutions Cal 2 and Cal 4) [concentrations of about 1400 µg/mL and 2100 µg/mL]

**Preparation of sample**

Weigh (to the nearest 0.1 mg) about 500 mg of test item into a 100 mL volumetric flask. Add diluting solvent and place the flask in an ultrasonic bath during minimum 10 minutes. Fill to the mark at 20°C ± 1°C with diluting solvent. Mix thoroughly and filter on a 0.45 µm PTFE filter.
This solution is prepared in triplicate.

**CHROMATOGRAPHIC PARAMETERS**

| | |
|---|---|
| Column: | Merck LiChrosorb Si 60, 10 µm, 250 x 4 mm i.d. |
| Mobile phase: | methanol - water - ammonia solution (80-19.3-0.7 v/v). |
| Flow rate: | 1 mL/min. |
| Column temperature: | 25°C. |
| Injection volume: | 10 µL. |
| Detector wavelength: | at 210 nm. |
| Injection sequence: | calibration solution 1, sample weighing 1, sample weighing 2, calibration solution 2, sample weighing 3,... |

Each solution is injected in duplicate.

**Validation - Results and discussions**

**Table 5.2-1: Methods suitable for the determination of active substances zoxamide and propamocarb-HCl in plant protection product GLOB2007bF**

| | Zoxamide | Propamocarb-HCl |
|---|---|---|
| **Author(s), year** | De Ryckel, B., 2023 | De Ryckel, B., 2023 |
| **Principle of method** | HPLC-UV | HPLC-UV |

| | **Zoxamide** | **Propamocarb-HCl** |
|---|---|---|
| **Linearity (linear between mg/L / % range of the declared content) (correlation coefficient, expressed as r)** | The response of the sum of the peaks of both stereoisomers of zoxamide is linear in the range 79.88 – 361.83 µg/mL zoxamide: $r^2$ = 1.0000 [r = 1.0000]. y= 37 046.2411x-71 581.2500 | The response of propamocarb is linear in the range 998.28 – 2534.46 µg/mL propamocarb (corresponding to the range 1191.25 – 3024.9 µg/ml propamocarb-HCl) : $r^2$ = 0.9999 **[r = 1.0000].** y= 1.8411x+5.2179 |
| **Precision – Repeatability Mean n = 6 (%RSD)** | Mean : 58.46 g/kg<br>Relative Standard Deviation : **0.11 %**<br>Horrat : **0.05** | Mean : 340.0 g/kg propamocarb (corresponding to 405.7 g/kg propamocarb-HCl<br>Relative Standard Deviation : **0.27 %**<br>Horrat : **0.17** |
| **Accuracy n = 6 (% Recovery)** | Level : 4.921% w/w (n = 2)<br>(80% of nominal concentration)<br>Mean : **102.8%**<br><br>Level : 6.340% w/w (n = 2)<br>(100% of nominal concentration)<br>Mean : **101.5%**<br><br>Level : 7.410% w/w (n = 2)<br>(120% of nominal concentration)<br>Mean : **101.3%**<br><br>Mean level : 6.224 % w/w (n = 3 x 2)<br>Mean : **101.9%**<br>Relative Standard Deviation : 0.99 % | Level : 27.34% w/w propamocarb (n = 2)<br>or 32.63% w/w propamocarb-HCl<br>(80% of nominal concentration)<br>Mean : **99.3%**<br><br>Level : 34.26% w/w propamocarb (n = 2)<br>or 40.88% w/w propamocarb-HCl<br>(100% of nominal concentration)<br>Mean : **99.2%**<br><br>Level : 41.31% w/w propamocarb (n = 2)<br>or 49.29% w/w propamocarb-HCl<br>(120% of nominal concentration)<br>Mean : **99.5%**<br><br>Mean level : 34.30 % w/w (n = 3 x 2)<br>or 40.93% w/w propamocarb-HCl<br>Mean : **99.3%**<br>Relative Standard Deviation : 0.33 % |
| **Interference/ Specificity** | **No interference** likely to affect the chromatographic peaks of both stereoisomers of zoxamide.<br>The peaks of both stereoisomers of zoxamide are free from co-eluents.<br><br>No **significant** deviation of the retention times of both stereoisomers of zoxamide from sample solution by comparison with the calibration solution. | **No interference** likely to affect the chromatographic peak of propamocarb.<br>The peak of propamocarb is free from co-eluents.<br><br>No **significant** deviation of the retention time of propamocarb from sample solution by comparison with the calibration solution. |
| **Comment** | | |

## Conclusion

The method of analysis of active substances content has been validated in compliance with Document SANCO/3030/99 – rev 5. The acceptance criteria are met for all the validation parameters.

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

No relevant impurities are present in GLOB2007bF.

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

Not required.

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

A CIPAC method (399) for determination of Propamocarb-HCl exists for the determination of Propamocarb hydrochloride in an SL formulation.
There are no CIPAC methods available for the determination of zoxamide.
In conclusion: there are no CIPAC methods available for the determination of Propamocarb-HCl and zoxamide in the SC formulation GLOB2007bF.

## 5.2.2 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 zoxamide, its metabolites RH-141455 and RH-141452 and propamocarb-HCl for the generation of pre-authorization data is given in the following table. For the detailed evaluation of new/ additional studies it is referred to Appendix 2.

**Table 5.2-2: Validated methods for the generation of pre-authorization data**

| Matrix type | Method type | Method LOQ | Principle of method (i.e. GC-MS or HPLC-UV) | Author(s), year / missing / EU agreed |
|---|---|---|---|---|
| **Component of residue definition: zoxamide** | | | | |
| Food/feed of plant origin (Residues) | Primary | 0.01 mg/kg | QuEChERS multi-residue method, LC-MS/MS | EFSA Journal 2017;15(9):4980 |
| Food/feed of animal origin (Residues) | Pending (data gap) | | | EFSA Journal 2017;15(9):4980 |
| Soil (Environmental fate, Efficacy, Ecotoxicology) | Primary | 0.05 mg/kg | LC-MS/MS | EFSA Journal 2017;15(9):4980 |
| Drinking and | Primary | 0.1 µg/L | LC-MS/MS | EFSA Journal 2017;15(9):4980 |

| Matrix type | Method type | Method LOQ | Principle of method (i.e. GC-MS or HPLC-UV) | Author(s), year / missing / EU agreed |
|---|---|---|---|---|
| **Component of residue definition: zoxamide** | | | | |
| surface water (Environmental fate, Efficacy, Ecotoxicology) | | | | |
| Air (Exposure) | Primary | 90 µg/m$^3$ | LC-MS/MS | EFSA Journal 2017;15(9):4980 |
| Feed, body fluids,... (Toxicology) | Primary | 0.01 mg/kg | LC-MS/MS | Gustloff, C., 2023 |
| **Component of residue definition: RH-141455 and RH-141452** | | | | |
| Food/Feed of plant origin (Residues) | Primary | 0.01 mg/kg (potato tubers)<br>0.05 mg/kg (potato chips and flakes) | LC-MS/MS | EFSA Journal 2017;15(9):4980 |

zRMS comments:
The Applicant did not complete the data regarding propamocarb. The table below contains data on this active substance.

| Matrix type | Method type | Method LOQ | Principle of method (i.e. GC-MS or HPLC-UV) | Author(s), year / missing / EU agreed |
|---|---|---|---|---|
| **Component of residue definition: Propamocarb-HCl** | | | | |
| Food/feed of plant origin (Residues) | Primary | 0.01 mg/kg (Wheat and Barley grain, Rape seed, Tomato, Orange fruit)<br>0.05 mg/kg (Wheat, Barley forage and straw) | HPLC-MS/MS | EFSA Scientific Report (2006) 78, 1-80 |
| Food/feed animal origin (Residues) | - | Not required | - | EFSA Scientific Report (2006) 78, 1-80 |
| Soil (Environmental fate, Efficacy, Ecotoxicology) | Primary | 0.02 mg/kg | HPLC-MS/MS | EFSA Scientific Report (2006) 78, 1-80 |
| Surface water and drinking water (Environmental fate, Efficacy, | Primary | 0.05 mg/kg | HPLC-MS/MS | EFSA Scientific Report (2006) 78, 1-80 |

| Matrix type | Method type | Method LOQ | Principle of method (i.e. GC-MS or HPLC-UV) | Author(s), year / missing / EU agreed |
|---|---|---|---|---|
| **Component of residue definition: Propamocarb-HCl** | | | | |
| Ecotoxicology) | | | | |
| Feed, body fluids,... (Toxicology) | - | Required (data gap) | - | EFSA Scientific Report (2006) 78, 1-80<br>Commission Regulation (EU) No 283/2013 |
| Air (Exposure) | Primary | 9 µg/m$^3$ | LC-MS/MS | EFSA Scientific Report (2006) 78, 1-80 |
| | Primary | 0.4 µg/m$^3$ | GC-MS/MS | EFSA Scientific Report (2006) 78, 1-80 |

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

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

Analytical methods for the determination of the active substance 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.

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

#### 5.3.2.1 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.

**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 | Zoxamide (fruit crops and pulses and oilseed)<br><br>sum of metabolites RH-141455 and RH-141452 (root crops) | ~~0.01 mg/kg~~<br>0.02 mg/kg | EFSA Journal 2017;15(9):4980<br>Reg. (EU) 2017/171 |
| Plant, high acid content | | ~~0.01 mg/kg~~<br>0.02 mg/kg | EFSA Journal 2017;15(9):4980<br>Reg. (EU) 2017/171 |
| Plant, high protein/high starch content (dry | | ~~0.01mg/kg~~<br>0.02 mg/kg | EFSA Journal 2017;15(9):4980 |

| Matrix | Residue definition | MRL / limit | Reference for MRL/level Remarks |
|---|---|---|---|
| commodities) | only zoxamide for all products - MRL regulation | | Reg. (EU) 2017/171 |
| Plant, high oil content | | ~~0.01 mg/kg~~<br>0.02 mg/kg | EFSA Journal 2017;15(9):4980<br>Reg. (EU) 2017/171 |
| Plant, difficult matrices (hops, spices, tea) | | ~~0.01 mg/kg~~<br>0.05 mg/kg | EFSA Journal 2017;15(9):4980<br>Reg. (EU) 2017/171 |
| Muscle | Open<br>zoxamide for all products - MRL regulation | 0.01 mg/kg | EFSA Journal 2017;15(9):4980<br>Reg. (EU) 2017/171 |
| Milk | | | |
| Eggs | | | |
| Fat | | | |
| Liver, kidney | | | |
| Soil (Ecotoxicology) | At least zoxamide but open regarding metabolites RH-163353 and RH-141455 | 0.05 mg/kg (zoxamide) | General limit for soil SANCO/825/00 rev. 8.1<br>EFSA Journal 2017;15(9):4980 |
| Drinking water (Human toxicology) | At least zoxamide but open regarding RH -141455 | 0.1 µg/L (zoxamide) | general limit for drinking water<br>SANCO/825/00 rev. 8.1 |
| Surface water (Ecotoxicology) | At least zoxamide but open regarding RH-127450, RH-24549, RH-163353 & RH-141455 | 3.48 µg/L (zoxamide) | EFSA Journal 2017;15(9):4980<br>NOEC (*Oncorhynchus mykiss*) |
| Air | zoxamide | 90 µg/m$^3$ | EFSA Journal 2017;15(9):4980 |
| Tissue (meat or liver) | zoxamide | ~~not required~~<br>data gap | not classified as T |
| Body fluids | | ~~not required~~<br>data gap | not classified as T |

### 5.3.2.2 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 zoxamide in plant matrices is given in the following tables. For the detailed evaluation of new/ additional studies it is referred to Appendix 2.

**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: zoxamide | | | | |
|---|---|---|---|---|
| **Matrix type** | **Method type** | **Method LOQ** | **Principle of method (i.e. GC-MS or HPLC-UV)** | **Author(s), year / missing / EU agreed** |
| Potato (tuber, chips and flakes), grapes (berries, juice, wine and raisins), lettuce, dry bean and oilseed rape seed | Primary | 0.01 mg/kg | QuEChERS multi-residue method, LC-MS/MS | EFSA Journal 2017;15(9):4980 |
| | ILV (potato tuber, grape vine and lettuce) | 0.01 mg/kg | | EFSA Journal 2017;15(9):4980 |
| **Component of residue definition: RH-141452 and RH-141455** | | | | |
| High starch content (dry) (potato) | Primary | Potato tubers: 0.01 mg/kg Potato chips and flakes: 0.05 mg/kg | LC-MS/MS | EFSA Journal 2017;15(9):4980 |
| | ILV | data gap | | EFSA Journal 2017;15(9):4980 |

For any special comments or remarkable points concerning the analytical methods for the determination of residues in plant matrices, please refer to Appendix 2.

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

| | **Method for products of plant origin** |
|---|---|
| Required, available from: | EFSA Journal 2017;15(9):4980<br>Extraction efficiency was addressed in high water content commodities (pea whole plant) and dry commodities (dry peas) (Latvia, 2017). |
| Not required, because: | - |

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

An analytical method is not required due to fact that no MRL is proposed.

zRMS comments:
zRMS does not agree with the Applicant's explanation. MRLs for zoxamide in products of animal origin were proposed by the Reg. (EU) No 2017/171 (0.01 mg/kg for all animal products, except honey for which the MRL value is 0.05 mg/kg). They are still valid.
According to the EFSA Journal 2017;15(9):4980: "An analytical method for food of animal origin is not proposed due to the fact that the residue definition for animals is currently open."

Taking into account that no residues are expected in potatoes or products of animal origin after use in accordance with the proposed GAP, the above mentioned lack of data is not considered critical for this dossier.

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

An overview on the acceptable methods and possible data gaps for analysis of zoxamide in soil is given in the following tables. ~~For the detailed evaluation of new/ additional studies it is referred to Appendix 2.~~

**Table 5.3-4: Validated methods for soil (if appropriate)**

| Method type | Method LOQ | Principle of method (*i.e.* GC-MS or HPLC-UV) | Author(s), year / missing |
|---|---|---|---|
| **Component of residue definition: zoxamide** | | | |
| Primary | 0.05 mg/kg | LC-MS/MS | EFSA Journal 2017;15(9):4980 |

~~For any special comments or remarkable points concerning the analytical methods for soil please refer to Appendix 2.~~

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

An overview on the acceptable methods and possible data gaps for analysis of zoxamide in surface and drinking water is given in the following tables. ~~For the detailed valuation of new/ additional studies it is referred to Appendix 2.~~

**Table 5.3-5: Validated methods for water (if appropriate)**

| Matrix type | Method type | Method LOQ | Principle of method (i.e. GC-MS or HPLC-UV) | Author(s), year / missing |
|---|---|---|---|---|
| **Component of residue definition: zoxamide** | | | | |
| Drinking water | Primary | 0.1 µg/L | LC-MS/MS | EFSA Journal 2017;15(9):4980 |
| | ILV | 0.1 µg/L | LC-MS/MS | EFSA Journal 2017;15(9):4980 study R B4049 |
| Surface water | Primary | 0.1 µg/L | LC-MS/MS | EFSA Journal 2017;15(9):4980 |

For any special comments or remarkable points concerning the analytical methods for water please refer to Appendix 2.

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

An overview on the acceptable methods and possible data gaps for analysis of zoxamide in air is given in

the following tables. ~~For the detailed evaluation of new/ additional studies please refer to Appendix 2~~.

**Table 5.3-6: Validated methods for air (if appropriate)**

| Component of residue definition: zoxamide | | | |
|---|---|---|---|
| **Method type** | **Method LOQ** | **Principle of method (i.e. GC-MS or HPLC-UV)** | **Author(s), year / missing** |
| Primary | 90 µg/m$^3$ | LC-MS/MS | EFSA Journal 2017;15(9):4980 |

~~For any special comments or remarkable points concerning the analytical methods for air it is referred to Appendix 2.~~

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

In the EFSA review on zoxamide (EFSA Journal 2017; 15(9):4980), a data gap was identified for an analytical method to monitor zoxamide in body fluids and tissues, Therefore, the applicant provides this method in the current dossier. An overview of this method is given in the following table. For the detailed evaluation of this new study, it is referred to Appendix 2.

**Table 5.3-7: Methods for body fluids and tissues (if appropriate)**

| Component of residue definition: zoxamide and RH-141452 | | | |
|---|---|---|---|
| **Method type** | **Method LOQ** | **Principle of method (i.e. GC-MS or HPLC-UV)** | **Author(s), year / missing** |
| Primary | 0.01 mg/kg for bovine (liver) and porcine (urine) | LC-MS/MS | Gustloff, C., 2023 |

For any special comments or remarkable points concerning the analytical methods for body fluids and tissues please refer to Appendix 2.

### 5.3.2.8 Other studies/ information

In several ecotoxicological studies summarized in section B9 of the dRR, analytical methods were used for the detection of zoxamide and propamocarb-HCl in the different test mediums. The analytical part of these studies is summarized in Appendix 2.

## 5.3.3 Description of analytical methods for the determination of residues of

**propamocarb-HCl (KCP 5.2)**

### 5.3.3.1 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.

**Table 5.3-8: 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 | Sum of propamocarb and its salts, expressed as propamocarb | 0.01 mg/kg | EFSA, 2006<br>Reg. (EU) ~~2020/856~~ 2024/1439 |
| Plant, high acid content | | 0.01 mg/kg | EFSA, 2006<br>Reg. (EU) 2024/1439 |
| Plant, high protein/high starch content (dry commodities) | | 0.01 mg/kg | EFSA, 2006<br>Reg. (EU) 2024/1439 |
| Plant, high oil content | | 0.01 mg/kg | EFSA, 2006<br>Reg. (EU) ~~2020/856~~ 2024/1439 |
| Muscle | N-oxide propamocarb in ruminant and pig matrices and N-desmethyl propamocarb in poultry matrices | ~~not required~~<br>0.01 mg/kg | EFSA, 2006<br>Reg. (EU) 2024/1439 |
| Milk | | ~~not required~~<br>0.01 mg/kg | EFSA, 2006<br>Reg. (EU) 2024/1439 |
| Eggs | | ~~not required~~<br>0.05 mg/kg | EFSA, 2006<br>Reg. (EU) 2024/1439 |
| Fat | | ~~not required~~<br>0.01 mg/kg | EFSA, 2006<br>Reg. (EU) 2024/1439 |
| Liver, kidney | | ~~not required~~<br>0.01 mg/kg | EFSA, 2006<br>Reg. (EU) 2024/1439 |
| Soil (Ecotoxicology) | Sum of propamocarb and its salts, expressed as propamocarb | 0.05 mg/kg | General limit for soil<br>SANCO/825/00 rev. 8.1 |
| Drinking water (Human toxicology) | Sum of propamocarb and its salts, expressed as propamocarb | 0.1 µg/L | general limit for drinking water<br>SANCO/825/00 rev. 8.1 |
| Surface water (Ecotoxicology) | Sum of propamocarb and its salts, expressed as propamocarb | 6300 µg/L | EFSA, 2006<br>NOEC (*Lepomis macrochirus*) |
| Air | Sum of propamocarb and its salts, expressed as propamocarb | 87 µg/m$^3$ | EFSA, 2006 |

| Matrix | Residue definition | MRL / limit | Reference for MRL/level Remarks |
|---|---|---|---|
| Tissue (meat or liver) | - | ~~not required~~<br>0.01 mg/kg | Not classified as T / T+<br>Commission Regulation (EU) No 283/2013<br>SANTE/2020/12380 rev. 2. |
| Body fluids | | ~~not required~~<br>0.01 mg/L | Not classified as T / T+<br>Commission Regulation (EU) No 283/2013<br>SANTE/2020/12380 rev. 2. |

### 5.3.3.2 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 propamocarb-HCl in plant matrices is given in the following tables.

**Table 5.3-9: 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: Sum of propamocarb and its salts, expressed as propamocarb | | | | |
|---|---|---|---|---|
| **Matrix type** | **Method type** | **Method LOQ** | **Principle of method (i.e. GC-MS or HPLC-UV)** | **Author(s), year / missing / EU agreed** |
| High water content | Primary | 0.01 mg/kg | HPLC-MS/MS | EFSA, 2006 |
| High acid content | | | | |
| High oil content | | | | |
| High protein/high starch content (dry) | | | | |

**Table 5.3-10: Statement on extraction efficiency**

| | Method for products of plant origin |
|---|---|
| Required, available from: | RAR of propamocarb-HCl (2017), expert statement (KCA 4.1.2/15; Theurig, M.; 2006; M-267054-01-1 Non-GLP, unprotected) |

### 5.3.3.3 Description of analytical methods for the determination of residues in animal

**matrices (KCP 5.2)**

An analytical method is not required due to fact that no MRL is proposed.

zRMS comments:
zRMS does not agree with the Applicant's explanation. MRLs for propamocarb in products of animal origin were proposed by the Reg. (EU) No 289/2014. They are still valid.
According to the EFSA Journal 2013;11(4):3214: During the peer review under Directive 91/414/EEC, an analytical method using HPLC-MS/MS and its ILV was reported for the determination of propamocarb in food of animal origin with an LOQ of 0.01mg/kg in milk, meat, liver, kidney and eggs (Ireland, 2004; FAO, 2006b). In addition, after Annex I inclusion, the RMS also reported an HPLC-MS/MS method for the determination of propamocarb with an LOQ of 0.01 mg/kg in meat, fat, liver, kidney, milk and eggs (Ireland, 2012). Nevertheless, as the residue for enforcement is defined as N-oxide propamocarb in ruminant and pig matrices and N-desmethyl propamocarb in poultry matrices, a fully validated analytical method, with its ILV and a confirmatory method for the determination of each analyte are required.
Taking into account that no residues are expected in potatoes or products of animal origin after use in accordance with the proposed GAP, the above mentioned lack of data is not considered critical for this dossier.

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

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

**Table 5.3-11: Validated methods for soil (if appropriate)**

| **Component of residue definition: Sum of propamocarb and its salts, expressed as propamocarb** | | | |
|---|---|---|---|
| **Method type** | **Method LOQ** | **Principle of method (*i.e.* GC-MS or HPLC-UV)** | **Author(s), year / missing** |
| Primary | 0.02 mg/kg | HPLC-MS/MS | EFSA, 2006 |
| Confirmatory | 0.02 mg/kg | HPLC-MS/MS | EFSA, 2006 |

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

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

**Table 5.3-12: Validated methods for water (if appropriate)**

| **Component of residue definition: Sum of propamocarb and its salts, expressed as propamocarb** | | | | |
|---|---|---|---|---|
| **Matrix type** | **Method type** | **Method LOQ** | **Principle of method (i.e. GC-MS or HPLC-UV)** | **Author(s), year / missing** |
| Drinking water | Primary | 0.05 mg/kg | HPLC-MS/MS | EFSA, 2006 |
| Surface water | | | | |

zRMS comments:
The methods used in the DAR and in the EFSA Scientific Report (2006) 78, 1-80 of propamocarb, include dichloromethane, both for surface and drinking water, which does not meet SANTE 2020/12830 rev. 2 requirements. Therefore, a primary, confirmatory and ILV methods in drinking water is missing - data gap. In addition, a primary method is missing for the determination of propamocarb in surface water - data gap. In the opinion of the zRMS, the Applicant should complete them post-registration after the renewal of approval for propamocarb.

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

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

**Table 5.3-13: Validated methods for air (if appropriate)**

| **Component of residue definition: Sum of propamocarb and its salts, expressed as propamocarb** | | | |
|---|---|---|---|
| **Method type** | **Method LOQ** | **Principle of method (i.e. GC-MS or HPLC-UV)** | **Author(s), year / missing** |
| Primary | 9 µg/m$^3$ | LC-MS/MS | EFSA, 2006 |
| Primary | 0.4 µg/m$^3$ | GC-MS/MS | EFSA, 2006 |

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

No method is required because GLOB2007bF and propamocarb hydrochloride are not classified as *toxic* or *very toxic*.

zRMS comments:
According to Regulation No. 283/2013 a primary method and confirmation is required for the analysis of propamocarb in body tissues and body fluids - data gap. Data should be completed after renewal of propamocarb.

### 5.3.3.8 Other studies/ information

In several ecotoxicological studies summarized in section B9 of the dRR, analytical methods were used for the detection of zoxamide and propamocarb-HCl in the different test mediums. The analytical part of these studies is summarized in Appendix 2.

# Appendix 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<br>Company Report No.<br>Source (where different from company)<br>GLP or GEP status<br>Published or not | Vertebrate study Y/N | Owner |
|---|---|---|---|---|---|
| KCP 5.1.1 | De Ryckel, B. | 2022 | Validation of HPLC methods for the determination of zoxamide content (both stereoisomers content and total zoxamide content) and of propamocarb-HCl content in a formulation suspension concentrate (SC) containing 67.5g/L zoxamide and 450g/L propamocarb-HCl, Centre Wallon De Recherches Agronomiques, Report No.: 25508, GLP, Unpublished | N | Globachem NV |
| KCP 10.2.1 (filed in Part B Section 9) | xxxxxxxx | 2023 | GLOB2007bF: Acute Toxicity to Rainbow Trout (*Oncorhynchus mykiss*) in a 96-hour Semi-Static Test, xxxxxxxxxx, Report No.: 169561230, GLP, Unpublished | Y | Globachem NV |
| KCP 10.2.1 (filed in Part B Section 9) | Thorpe, K. | 2023 | GLOB2007bF: *Daphnia magna* Acute Immobilisation Test, Fera Science Ltd, Report No.: FR/002723, GLP, Unpublished | N | Globachem NV |
| KCP 10.2.1 (filed in Part B Section 9) | Wright, E. | 2023 | GLOB2007bF: *Pseudokirchneriella subcapitata* Growth Inhibition Test, Fera Science Ltd, Report No.: FR/002722, GLP, Unpublished | N | Globachem NV |

| Data point | Author(s) | Year | Title<br>Company Report No.<br>Source (where different from company)<br>GLP or GEP status<br>Published or not | Vertebrate study Y/N | Owner |
|---|---|---|---|---|---|
| KCP 10.3.1.1 (filed in Part B Section 9) | Chwiesko, D. | 2023 | GLOB2007bF: Acute Contact and Oral Toxicity to Bumblebees (*Bombus terrestris* L.) in the Laboratory, Ibacon Gmbh, Report No.: 169561105, GLP, Unpublished | N | Globachem NV |
| KCP 10.3.1.2 (filed in Part B Section 9) | Schabio, S. | 2023 | GLOB2007bF: Chronic Oral Toxicity Test on the Honey Bee (*Apis mellifera* L.) in the Laboratory, Ibacon Gmbh, Report No.: 169561136, GLP, Unpublished | N | Globachem NV |
| KCP 10.3.1.3 (filed in Part B Section 9) | Colli, M. | 2022 | Honey Bee Larvae Toxicity Test (*Apis mellifera*), Biotecnologie Bt S.R.L., Report No.: BT127/22, GLP, Unpublished | N | Globachem NV |
| KCP 10.6.2 (filed in Part B Section 9) | Davies, C. | 2023 | GLOB2007bF: OECD Terrestrial Plant Test - Vegetative Vigour Test, Stockbridge Technology Centre Ltd., Report No.: STC/22/E1555, GLP, Unpublished | N | Globachem NV |
| KCP 10.6.2 (filed in Part B Section 9) | Stead, A. | 2023 | GLOB2007bF: OECD Terrestrial Plant Test - Seedling Emergence and Seedling Growth Test, Stockbridge Technology Centre Ltd., Report No.: STC/22/E1556, GLP, Unpublished | N | Globachem NV |
| KCA 4.1.2 | DeVellis, S. | 2023 | Zoxamide Metabolite (RH-163353) - Analytical Method Validation for the Determination of a Test Substance in Aqueous Solutions, Smithers Ers Ltd, Report No.: 14365.6100, GLP, Unpublished | N | Globachem NV |

| Data point | Author(s) | Year | Title<br>Company Report No.<br>Source (where different from company)<br>GLP or GEP status<br>Published or not | Vertebrate study Y/N | Owner |
|---|---|---|---|---|---|
| KCA 4.1.2 | Liu, Y. | 2023 | RH-139432 in Salt Water Enforcement Analytical Method, Stillmeadow Inc, Report No.: 26104-22, GLP, Unpublished | N | Globachem NV |
| KCA 4.1.2 | Liu, Y. | 2023 | RH-139432 in Algae Media Enforcement Analytical Method, Stillmeadow Inc, Report No.: 26103-22, GLP, Unpublished | N | Globachem NV |
| KCA 4.1.2 | Liu, Y. | 2023 | RH-127450 in Salt Water Enforcement Analytical Method, Stillmeadow Inc, Report No.: 26101-22, GLP, Unpublished | N | Globachem NV |
| KCA 4.1.2 | Liu, Y. | 2023 | RH-127450 in Algae Media Enforcement Analytical Method, Stillmeadow Inc, Report No.: 26102-22, GLP, Unpublished | N | Globachem NV |
| KCA 4.1.2 | Liu, Y. | 2023 | RH-141455 in Salt Water Enforcement Analytical Method, Stillmeadow Inc, Report No.: 26105-22, GLP, Unpublished | N | Globachem NV |
| KCA 4.1.2 | Liu, Y. | 2023 | RH-24549 in Salt Water Enforcement Analytical Method, Stillmeadow Inc, Report No.: 26107-22, GLP, Unpublished | N | Globachem NV |
| KCA 4.2 | Gustloff, C. | 2023 | Validation of an Analytical Method for Determination of Zoxamide in Body Fluids and Animal Tissues, Eurofins Agroscience Services Chem Gmbh, Report No.: S23-100691, GLP, Unpublished | N | Globachem NV |
| KCA 8.2.6.1 (filed in Part B Section 9) | Jarratt, N. | 2023 | Zoxamide Technical: *Pseudokirchneriella subcapitata* Growth Inhibition Test, Fera Science Ltd, Report No.: FR/002786, GLP, Unpublished | N | Globachem NV |
| KCA 8.3.1.3 (filed in Part B Section 9) | Aguilar-Alberola, J. | 2023 | Zoxamide technical: Honey Bee (*Apis mellifera* L.) Larval Toxicity Test following Repeated Exposure under laboratory conditions, Eurofins Trialcamp S.L.U., Report No.: S23-106642, GLP, Unpublished | N | Globachem NV |

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

*Studies in the table below were generated to data match the AIR protected studies from the main notifier. The data matching package has been evaluated by the RMS Latvia and a copy was already sent to all MS.

| Data point | Author(s) | Year | Title<br>Company Report No.<br>Source (where different from company)<br>GLP or GEP status<br>Published or not | Vertebrate study Y/N | Owner |
|---|---|---|---|---|---|
| KCA 4.1.2 | Gustloff, C. | 2022 | Validation of Analytical Methods to Determine Residues of Zoxamide in Plant Matrices, Eurofins Agroscience Services Chem Gmbh, Report No.: S21-07039, GLP, Unpublished | N | Globachem NV |
| KCA 4.1.2 | Gustloff, C. | 2022 | Validation of an Analytical Method to Determine Residues of Zoxamide Metabolites (RH-1452 and RH-1455) in Grape and Potato Matrices, Eurofins Agroscience Services Chem Gmbh, Report No.: S21-07040, GLP, Unpublished | N | Globachem NV |
| KCA 4.2 | Homazava, N. | 2022 | Validation of LC-MS/MS Analytical Method for Zoxamide in Soil, Innovative Environmental Services, Report No.: 20210506, GLP, Unpublished | N | Globachem NV |
| KCA 4.2 | Homazava, N. | 2022 | Validation of LC-MS/MS Analytical Method for Zoxamide in Water Matrices, Innovative Environmental Services, Report No.: 20210507, GLP, Unpublished | N | Globachem NV |
| KCA 4.2 | Homazava, N. | 2022 | Validation of LC-MS/MS Analytical Method for Zoxamide in Air, Innovative Environmental Services, Report No.: 20210508, GLP, Unpublished | N | Globachem NV |
| KCP 4.2 | Ducat, N. | 2022 | Determination of zoxamide residues in drinking water. Independent Laboratory Validation (ILV) of the analytical method described in the final report IES study 20210507 of Innovative Environmental Services (IES) Ltd, Switzerland for Globachem., Centre Wallon De Recherches Agronomiques, Report No.: 25674, GLP, Unpublished | N | Globachem NV |
| KCP 5.1.1 | Świstak, M. | 2021 | Validation of analytical method for the determination of active substance – zoxamide of the test item Zoxamide 450 SC in 50% sucrose solution, Sorbolab Research Laboratory Llc, Report No.: 0064/0014/FA, GLP, Unpublished | N | Globachem NV |
| KCP 5.1.1 | Świstak, M. | 2021 | Validation of analytical method for the determination of active substance – zoxamide of the test item Zoxamide 450 SC in aqueous solutions, Sorbolab Research Laboratory Llc, Report No.: 0064/0011/FA, GLP, Unpublished | N | Globachem NV |

| Data point | Author(s) | Year | Title<br>Company Report No.<br>Source (where different from company)<br>GLP or GEP status<br>Published or not | Vertebrate study Y/N | Owner |
|---|---|---|---|---|---|
| KCP 10.2.1 | Wright, E. | 2023 | GLOB2013F: Pseudokirchneriella subcapitata Growth Inhibition Test, Fera Science Ltd, Report No.: FR/002720, GLP, Unpublished | N | Globachem NV |

# Appendix 2 Detailed evaluation of submitted analytical methods

## A 2.1 Analytical methods for zoxamide

### A 2.1.1 Methods used for the generation of pre-authorization data (KCP 5.1)

#### A 2.1.1.1 Description of analytical methods for the determination of residues in aqueous solutions (KCP 5.1)

##### A 2.1.1.1.1 Fish

###### A 2.1.1.1.1.1 Method validation

| | |
|---|---|
| Comments of zRMS: | The purpose of the analytical part was to perform the analysis of the concentrations of zoxamide and propamocarb-HCL of the test item GLOB2007bF in the test samples.<br>Two fortification concentrations with 5 replicates per level were assessed. Both levels have a mean recovery in the range of 70 – 120% and the RSD is ≤ 20 % per level for both active ingredients.<br>The method was successfully validated according to SANTE/2020/12830 rev. 2 at an LOQ of 0.30 mg test item/L for zoxamide (corresponding to 17.5 µg a.i./L) and propamocarb-HCl (corresponding to 121.71 µg propamocarb-HCL/L equivalent to 102 µg propamocarb/L). |

| | |
|---|---|
| Reference: | KCP 10.2.1 |
| Report | GLOB2007bF: Acute Toxicity to Rainbow Trout (*Oncorhynchus mykiss*) in a 96-hour Semi-Static Test, xxxxxxxx, 2022, xxxxxxxx, Report No.: 169561230 |
| Guideline(s): | SANTE/2020/12830, rev. 2 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

**Materials and methods**

The quantification of the active ingredients Zoxamide and Propamocarb-HCl of the test item GLOB2007bF in the test samples was performed using liquid chromatography with MS/MS detection.

**Results and discussions**

Zoxamide:
In the freshly prepared test media at the start of the test and at the renewal of the test media, 92 % of the

nominal test concentrations were found (average of all test concentrations). In the aged test media after 24 hours test duration, 65 % of the nominal value was determined (average of all test concentrations).
During the test, the test organism were exposed to a mean of 79 % of nominal.
The determined recovery values correspond to time-weighted average concentrations of 0.583, 1.04, 2.25, 4.98 and 9.45 mg test item/L (0.0341, 0.0608, 0.131, 0.291, 0.552 mg Zoxamide/L) for the nominal test concentrations of 0.75, 1.5, 3, 6 and 12 mg test item/L, respectively.

Propamocarb-HCl
In the freshly prepared test media at the start of the test and at the renewal of the test media, 108 % of the nominal test concentrations were found (average of all test concentrations). In the aged test media after 24 hours test duration, 108 % of the nominal value was determined (average of all test concentrations).
During the test, the test organism were exposed to a mean of 108 % of nominal.

**Validity Criteria of the Analytical Part**

Matrix Effect:

Zoxamide:
No significant matrix effect (< 20 %) was determined for the analyte Zoxamide in matrix compared to pure solvent. Independently of the determined matrix effect, matrix-matched calibration standards were used for quantification.
Details are presented in Table 17.

Propamocarb:
No significant matrix effect (< 20 %) was determined for the analyte Zoxamide in matrix compared to pure solvent. Independently of the determined matrix effect, matrix-matched calibration standards were used for quantification.
Details are presented in Table 18.

Calibration:

Calibration Range:
Zoxamide:
0.075 – 1.50 µg a.i./L (7 standard solutions)
The calibration range corresponds to minus 79 % of the lowest analysed concentration (LOQ) and plus 54 % of the highest analysed concentration (nominal 15 mg/L diluted by factor 900).

Propamocarb:
0.500 – 10.0 µg a.i./L (7 standard solutions)
The calibration range corresponds to minus 75 % of the lowest analysed concentration (LOQ) and plus 77 % of the highest analysed concentration (nominal 15 mg/L diluted by factor 900).

Linearity of Response:
Correlation of peak area of different standard solutions with their corresponding concentrations, using a linear regression

Correlation Coefficients:
Zoxamide:
r = 0.9996

Propamocarb:
r = 0.9999

Calibration Curves:
Zoxamide:
y = 132917 * x – 2549 (see Figure 1)

| | |
|---|---|
| | Propamocarb:<br>y = 95157 * x + 6690 (see Figure 3)<br><br>Regression residual:<br>The regression residual plot of the calibration curves (see Figure 2 and Figure 4) demonstrates random distribution of the regression residuals without a visible trend.<br>The used calibration curves consist of at least five calibration standards, the calibration range was at least -70% and +20% of the relevant analyte concentrations, the correlation coefficients were >0.99 and the regression residual plots show no visible trend. Thus, the used calibration curves fulfill the validity criteria specified by SANTE/2020/12830 rev.2. |
| Recovery and Repeatability: | Mean recovery rates in fortified samples:<br>Zoxamide:<br>0.30 mg test item/L (=LOQ): 83% (n = 5, RSD 10%)<br>15 mg test item/L: 78% (n = 5, RSD 4%)<br><br>Propamocarb-HCL:<br>0.30 mg test item/L (=LOQ): 118% (n = 5, RSD 3%)<br>15 mg test item/L: 115% (n = 5, RSD 3%)<br><br>Two fortification concentrations with 5 replicates per level were assessed. Both levels have a mean recovery in the range of 70 – 120% and the RSD is ≤ 20 % per level for both active ingredients. Thus, the requirements for recovery and repeatability according to SANTE/2020/12830 rev.2. are fulfilled. |
| Selectivity and Specificity: | No significant (< 30%) interference of total peak area for the target analyte was found with respect to the LOQ level.<br>The representative standard, blank control and fortification chromatograms (see Figure 5 to Figure 7 for Zoxamide and Figure 13 to Figure 15 for Propamocarb) show no significant interfering signals at the retention time of the analyte.<br>The product ion spectrum for Zoxamide shown in Figure 12 demonstrates the assigning of the chosen mass transitions of m/z 338 -> 189 (quantifier) and m/z 338 -> 187 (qualifier) to the target analyte.<br>The product ion spectrum for Propamocarb shown in Figure 20 demonstrates the assigning of the chosen mass transitions of m/z 189 -> 102 (quantifier) and m/z 189 -> 144 (qualifier) to the target analyte.<br>In summary, selectivity and specificity criteria set forth by SANTE/2020/12830 rev. 2 were fulfilled. |
| Extract and Standard Stability: | Storage stability of Zoxamide or Propamocarb in final extracts and standard solutions was not investigated since all prepared samples were not stored between end of sample preparation and beginning of analysis. |

**Conclusion**

The validity criteria for the analytical method have been met according to SANTE/2020/12830 rev.2.

## A 2.1.1.1.2 Aquatic invertebrates

### A 2.1.1.1.2.1 Method validation

| | |
|---|---|
| Comments of zRMS: | The purpose of the analytical part was to perform the analysis of the concentrations of zoxamide and propamocarb-HCL in test medium (M4).<br>Two fortification concentrations with 5 replicates per level were assessed. Both levels have a mean recovery in the range of 70 – 120% and the RSD is ≤ 20 % per level for both active ingredients.<br>The method was successfully validated according to SANTE/2020/12830 rev. 2 at an LOQ of 0.11 mg /L for zoxamide and 0.76 mg/L for propamocarb-HCl. |

| | |
|---|---|
| Reference: | KCP 10.2.1 |
| Report | GLOB2007bF: *Daphnia magna* Acute Immobilisation Test, Thorpe, K., 2023, Fera Science Ltd, Report No.: FR/002723 |
| Guideline(s): | SANTE/2020/12830, rev. 2 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

**Materials and methods**

Method reference: FR/002723A v1
This method is intended for the determination of zoxamide and propamocarb (as hydrochloride) in test medium (M4) used to conduct *Daphnia* sp. acute immobilisation tests according to OECD guideline test no. 202. In this analytical method, samples are diluted (if required), filtered through PVDF and analysed by liquid chromatography – mass spectrometry/mass spectrometry (LC-MS/MS).

**Results and discussions**

**Method FR/002723A v1 Assessment – Zoxamide**

The analytical method was validated at 1.9 mg test item (TI)/L (low-level validation, LLV), and at 82 mg TI/L (high-level validation, HLV), equivalent to 0.11 mg zoxamide/L, and 4.7 mg zoxamide/L, respectively. The data from LIMS runs 65395 and 65409 show that the analytical method satisfies the validation guidelines in SANTE/2020/12830, Rev.2 as follows:

**Matrix-Effects**

An assessment of matrix effects on the detector response was performed by comparing the slope (gradient) of the calibration function prepared with standards in solvent with that for the calibration with standards in matrix.

The matrix effects were calculated by comparison of the slopes of the 2 calibration curves using the equation:

*Matrix effects (%) = 100 x slope (matrix) / slope (solvent) - 100*

For zoxamide, the matrix effect was determined as 2.3%. As the matrix effect did not exceed ±20% in enhancement or suppression, it was not considered to be significant. Nevertheless, calibration curves were still constructed using matrix matched standards.

**Calibration**

The calibration consisted of five levels with duplicate injections, bracketing the samples. The calibration range covered 0.13 to 2.1 ng zoxamide/mL, equivalent to 0.020 – 0.32 mg zoxamide/L in a sample (when diluted by lowest factor of 150). The LLV (= limit of quantification, LOQ) was performed at 0.11 mg zoxamide/L (1.9 mg TI/L); 30% of this is 0.033 mg zoxamide/L, which is equivalent to 0.22 ng/mL when diluted according to method FR/002722A v2. The lowest calibration level, 0.13 ng/mL is, therefore, <30% of the LOQ. The highest concentration samples were diluted sufficiently to ensure that the highest calibration level was at least 20% above the concentration being measured. Therefore, the calibration range met or exceeded the requirements of SANTE/2020/12830, Rev.2 (≤ 30% of LOQ to ≥ 20% above the highest measured solution concentration). Inspection of the plots of the residuals from the unweighted linear regressions showed that they were reasonably evenly distributed, therefore, the fitted line was considered acceptable. The correlation co-efficients (LIMS runs 65395), r, was 0.996941 ($r^2$ = 0.993891). The calibration graphs meet the principles of SANTE/2020/12830, Rev.2.

**Limit of detection**

The lowest calibration level is 0.13 ng zoxamide/mL, equivalent to 0.020 mg zoxamide/L in undiluted sample.

**Limit of Quantification**

This is equal to the lowest validation level which satisfies the SANTE/2020/12830, Rev.2 criteria, i.e. 0.11 mg zoxamide/L (1.9 mg TI/L).

**Recovery and repeatability**

Two sets of fortified control samples were analysed, one set below the lowest expected sample concentration and one set above the highest expected sample concentration. Calculated per cent recoveries and per cent relative standard deviations (RSDs) in the fortified controls are shown below:

| Fortification level (mg TI/L) | Fortification level (mg zoxamide/L) | Recovery (%) | Mean recovery (%) | RSD (%) |
|---|---|---|---|---|
| 1.9 | 0.11 | 108.3094<br>110.4211<br>115.2457<br>101.6709<br>105.3220 | 108 | 4.7 |
| 88 | 4.8 | 105.0460<br>116.3354<br>108.7344<br>104.6442<br>113.4282 | 110 | 4.7 |

For the method to be deemed fit for purpose, SANTE/2020/12830, Rev.2. states that mean recoveries must be in the range 70-120% and the RSDs must be ≤ 20%. The data meet these requirements and are therefore acceptable.

**Selectivity and specificity**

There was no response ≥ 30% of the LOQ in the unfortified control media at the retention time of zoxamide, therefore, the method meets the requirements of SANTE/2020/12830/Rev.2 in this regard.

**Extract Stability**

Fortified control solutions were analysed on the day of preparation, and there was no evidence of degradation before or during analysis. During analysis of actual study samples, a Procedural Recovery sample was prepared alongside the samples and analysed on the same day. Any degradation of sample residues would be seen as significant changes in the recovery values calculated from the Procedural Recovery sample. The data from the validation batch provide no evidence of stability issues in sample matrix under the circumstances used for its preparation and analysis.

**Standard Stability**

Stability of the analyte in the stored stock solution was determined in Fera study number FR/002722, where the stock zoxamide standard has been shown to be stable in acetonitrile for at least 26 days when stored in a freezer.

**Method FR/002723A v1 Assessment – Propamocarb hydrochloride**

The analytical method was validated at 1.9 mg test item (TI)/L (low-level validation, LLV), and at 82 mg TI/L (high-level validation, HLV), equivalent to 0.75 mg propamocarb hydrochloride/L and 33 mg propamocarb hydrochloride/L, respectively. The data from LIMS runs 65395 and 65409 show that the analytical method satisfies the validation guidelines in SANTE/2020/12830 rev.2 as follows:

**Matrix-Effects**

An assessment of matrix effects on the detector response was performed by comparing the slope (gradient) of the calibration function prepared with standards in solvent with that for the calibration with standards in matrix.

The matrix effects were calculated by comparison of the slopes of the 2 calibration curves using the equation:

*Matrix effects (%) = 100 x slope (matrix) / slope (solvent) - 100*

For propamocarb hydrochloride, the matrix effect was determined as 169%. As the matrix effect exceeded ±20% in enhancement or suppression, it was considered to be significant. However, calibration curves were constructed using matrix matched standards.

**Calibration**

The calibration consisted of five levels with duplicate injections, bracketing the samples. The calibration range covered 0.93 to 15 ng propamocarb hydrochloride/mL, equivalent to 0.14 – 2.3 mg propamocarb hydrochloride/L in a sample (when diluted by lowest factor of 150). The LLV (= limit of quantification, LOQ) was performed at 0.75 mg propamocarb hydrochloride/L (1.9 mg TI/L); 30% of this is 0.23 mg propamocarb hydrochloride/L, which is equivalent to 1.5 ng/mL when diluted according to method

FR/002722A v2. The lowest calibration level, 0.93 ng/mL is, therefore, <30% of the LOQ. The highest concentration samples were diluted sufficiently to ensure that the highest calibration level was at least 20% above the concentration being measured. Therefore, the calibration range met or exceeded the requirements of SANTE/2020/12830, Rev.2. (≤ 30% of LOQ to ≥ 20% above the highest measured solution concentration). Inspection of the plots of the residuals from the unweighted linear regressions showed that they were reasonably evenly distributed, therefore, the fitted line was considered acceptable. The correlation co-efficients (LIMS runs 65395), r, was 0.999637 ($r^2$ = 0.999273). The calibration graphs meet the principles of SANTE/2020/12830, Rev.2.

**Limit of detection**

The lowest calibration level is 0.93 ng propamocarb hydrochloride/mL, equivalent to 0.14 mg propamocarb hydrochloride/L.

**Limit of quantification**

This is equal to the lowest validation level which satisfies the SANTE/2020/12830, Rev.2 criteria, i.e. 0.75 mg propamocarb hydrochloride/L (1.9 mg TI/L).

**Recovery and repeatability**

Two sets of fortified control samples were analysed, one set below the lowest expected sample concentration and one set above the highest expected sample concentration. Calculated per cent recoveries and per cent relative standard deviations (RSDs) in the fortified controls are shown below:

| Fortification level (mg TI/L) | Fortification level (mg propamocarb hydrochloride/L) | Recovery (%) | Mean recovery (%) | RSD (%) |
|---|---|---|---|---|
| 1.9 | 0.75 | 109.5053<br>113.0820<br>110.2835<br>107.8670<br>104.7734 | 109 | 2.8 |
| 82 | 33 | 108.4907<br>107.3667<br>106.2124<br>106.2128<br>106.2773 | 107 | 0.95 |

For the method to be deemed fit for purpose, SANTE/2020/12830, Rev.2 states that mean recoveries must be in the range 70-120% and the RSDs must be ≤ 20%. The data meet these requirements and are therefore acceptable.

**Selectivity and specificity**

There was no response ≥ 30% of the LOQ in the unfortified control media at the retention time of propamocarb hydrochloride, therefore, the method meets the requirements of SANTE/2020/12830, Rev.2 in this regard.

**Extract Stability**

Fortified control solutions were analysed on the day of preparation, and there was no evidence of degradation before or during analysis. During analysis of actual study samples, a Procedural Recovery sample was prepared alongside the samples and analysed on the same day. Any degradation of sample residues would be noticed as significant changes in the recovery values calculated from the Procedural Recovery. The data from the validation batches provide no evidence of stability issues in sample matrix under the circumstances used for its preparation and analysis.

**Standard Stability**

Stability of the analyte in the stored stock solution was determined in Fera study number FR/002722, where the stock propamocarb hydrochloride standard has been shown to be stable in acetonitrile for at least 26 days when stored in a freezer.

**Conclusion**

Method FR/002723A v1 was assessed as being fit-for-purpose for this study.

## A 2.1.1.1.2.2 Method validation RH-163353

| | |
|---|---|
| Comments of zRMS: | The method was successfully validated according to SANTE/2020/12830 rev. 2. |

| | |
|---|---|
| Reference: | KCA 4.1.2 |
| Report | Zoxamide Metabolite (RH-163353) - Analytical Method Validation for the Determination of a Test Substance in Aqueous Solutions, DeVellis, S., 2023, Smithers Ers Ltd, Report No.: 14365.6100 |
| Guideline(s): | SANTE/2020/12830, rev. 2 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

**Materials and methods**

The purpose of this study was to validate an analytical method used to determine the content of RH-163353 in aqueous solutions. The method was validated (24 April to 26 May 2023) to quantify the concentrations of RH-163353 present in recovery samples prepared in Algal Assay Procedure (AAP) medium and dilute, natural, filtered seawater (FSW). The analytical method was validated with regards to selectivity and specificity, calibration, recovery and repeatability, limit of quantitation (LOQ), limit of detection (LOD), matrix effects, confirmation, and extract and stock stability in accordance with SANTE/2020/12830 rev. 2 (14 February 2023).

The method was validated in Algal Assay Procedure (AAP) medium and natural, dilute, filtered seawater (FSW) by fortification with RH-163353 at concentrations of 1.00 (LOQ) and 100,000 (High) µg/L. For each matrix, five replicate samples were produced for each concentration level. Additionally, two samples were left unfortified to serve as controls and one reagent blank was also prepared and processed with the fortified samples. Recovery samples were diluted with 20/80 acetonitrile/purified reagent water (v/v) for a final composition of 18/10/72 acetonitrile/test matrix/purified reagent water (v/v/v). The High-level recovery samples were further diluted into the calibration range with 18/10/72 acetonitrile/test matrix/purified reagent water (v/v/v). All samples were analyzed using liquid chromatography with tandem mass spectrometry detection (LC-MS/MS).

**Results and discussions**

The method validation with RH-163353 in AAP medium met the performance criteria as presented in the following table:

| Criterion | Acceptable Limits | Study Performance | |
|---|---|---|---|
| | | Primary | Confirmatory |
| Specificity | Peaks attributable to the test substance should be sufficiently resolved from any peaks found in the samples of control matrix to enable quantification. | No extraneous peaks occurred which could interfere with quantification of the peak attributable to the test substance. | No extraneous peaks occurred which could interfere with quantification of the peak attributable to the test substance. |
| Linearity: Coefficient of Determination | The data should have a coefficient of determination ($r^2$) of not less than 0.990. | $r^2 = 0.994$ | $r^2 = 0.991$ |
| Linearity: Matrix Effects | Possible effects of sample components will be evaluated. The effects of matrix enhancement or suppression will be evaluated through the assessment of solvent-based and matrix-matched calibration standards. | The matrix effects assessment indicated that there was no significant matrix effect observed (matrix effect <20%). This result is an indication that no significant matrix effect was observed. Even though no matrix effect was observed, there is a potential for matrix issues to become a concern during testing as the test vessels experience aging over the course of the exposure. As a result and as a conservative measure, matrix-matched calibration standards will be utilized for future testing with this method. | |
| Accuracy: Mean Recoveries | Mean recoveries of 70.0 to 120% for each fortification level will be considered acceptable. | LOQ, 1.00 µg/L: 98.2% | LOQ, 1.00 µg/L: 104% |
| | | High, 100,000 µg/L: 98.3% | High, 100,000 µg/L: 98.0% |
| Accuracy: Test Concentrations | The study will be performed at two fortification levels which are set by anticipated testing levels, the lowest of which is the LOQ for this analysis and the high being the highest predicted level to be used during testing. | This portion of the study was performed at levels of 1.00 and 100,000 µg/L; 1.00 µg/L was set as the LOQ. | |
| Precision: Relative Standard Deviation (RSD) | Relative Standard Deviation (RSD) ≤20% for each fortification level will be considered acceptable. | LOQ, 1.00 µg/L: 3.41% | LOQ, 1.00 µg/L: 5.30% |
| | | High, 100,000 µg/L: 2.25% | High, 100,000 µg/L: 6.34% |
| Precision: Repeatability of Recovery | Five determinations will be made at each fortification level. | Five replicates were prepared and analyzed for each of the two fortification levels. | |
| Final extract stability | Analyte stability in final extract will be sufficiently proven if recoveries of fortified samples are within 70.0 to 120% for each fortification level. | LOQ, 1.00 µg/L: 101 ± 1.50 %<br>High, 100,000 µg/L: 113 ± 1.42 %<br><br>This is an indication that the analyte was stable after 8 days of storage under refrigerated conditions (2 to 8 °C) | |
| Stock stability | Analyte stability in stock solution will be sufficiently proven if the means from at least 5 replicate measurements of a refrigerated aged and a freshly prepared stock solution do not differ by more than 10% | Primary Stock Solutions: 1000 mg/L: 2.77% Difference<br>Secondary Stock Solutions: 10.0 mg/L: 5.18% Difference<br><br>This is an indication that the analyte was stable after 37 days of storage under refrigerated conditions (2 to 8 °C). | |
| Limit Of Quantitation (LOQ) | Blank values (reagent blanks and untreated control samples) should not exceed 30% of the LOQ. | All blank sample values were <30% of the LOQ (1.00 µg/L). | All blank sample values were <30% of the LOQ 1.00 µg/L). |
| Limit Of Detection (LOD) | The LOD will be set at the lowest concentration that can be detected in test solution samples. This value is calculated based on the concentration of the low | 0.300 µg/L | 0.300 µg/L |

| Criterion | Acceptable Limits | Study Performance | |
|---|---|---|---|
| | | Primary | Confirmatory |
| | calibration standard and the dilution factor of the control samples. | | |
| Confirmation of Analyte Identification | A chromatographic confirmatory method will be used to determine test solution concentrations during validation. | Primary ion: 330.3/258.2<br><br>Meets all method and guideline specifications outlined in this table. | Confirmatory ion: 330.3/159.0<br><br>Meets all method and guideline specifications outlined in this table. |

The method validation with RH-163353 in FSW met the performance criteria as presented in the following table:

| Criterion | Acceptable Limits | Study Performance | |
|---|---|---|---|
| | | Primary | Confirmatory |
| Specificity | Peaks attributable to the test substance should be sufficiently resolved from any peaks found in the samples of control matrix to enable quantification. | No extraneous peaks occurred which could interfere with quantification of the peak attributable to the test substance. | No extraneous peaks occurred which could interfere with quantification of the peak attributable to the test substance. |
| Linearity: Coefficient of Determination | The data should have a coefficient of determination ($r^2$) of not less than 0.990. | $r^2 = 0.995$ | $r^2 = 0.993$ |
| Linearity: Matrix Effects | Possible effects of sample components will be evaluated. The effects of matrix enhancement or suppression will be evaluated through the assessment of solvent-based and matrix-matched calibration standards. | The matrix effects assessment indicated that there was no significant matrix effect observed (matrix effect <20%). This result is an indication that no significant matrix effect was observed. Even though no matrix effect was observed, there is a potential for matrix issues to become a concern during testing as the test vessels experience aging over the course of the exposure. As a result and as a conservative measure, matrix-matched calibration standards will be utilized for future testing with this method. | |
| Accuracy: Mean Recoveries | Mean recoveries of 70.0 to 120% for each fortification level will be considered acceptable. | LOQ, 1.00 µg/L: 101% | LOQ, 1.00 µg/L: 97.0% |
| | | High, 100,000 µg/L: 86.3% | High, 100,000 µg/L: 83.7% |
| Accuracy: Test Concentrations | The study will be performed at two fortification levels which are set by anticipated testing levels, the lowest of which is the LOQ for this analysis and the high being the highest predicted level to be used during testing. | This portion of the study was performed at levels of 1.00 and 100,000 µg/L; 1.00 µg/L was set as the LOQ. | |
| Precision: Relative Standard Deviation (RSD) | Relative Standard Deviation (RSD) ≤20% for each fortification level will be considered acceptable. | LOQ, 1.00 µg/L: 5.76% | LOQ, 1.00 µg/L: 4.18% |
| | | High, 100,000 µg/L: 4.71% | High, 100,000 µg/L: 2.28% |
| Precision: Repeatability of Recovery | Five determinations will be made at each fortification level. | Five replicates were prepared and analyzed for each of the two fortification levels. | |
| Final extract stability | Analyte stability in final extract will be sufficiently proven if recoveries of fortified samples are within 70.0 to 120% for each fortification level. | LOQ, 1.00 µg/L: 104± 9.58 %<br>High, 100,000 µg/L: 98.9 ± 5.59 %<br><br>This is an indication that the analyte was stable after 8 days of storage under refrigerated conditions (2 to 8 °C) | |
| Limit Of Quantitation (LOQ) | Blank values (reagent blanks and untreated control samples) should not exceed 30% of the LOQ. | All blank sample values were <30% of the LOD (1.00 µg/L). | All blank sample values were <30% of the LOQ 1.00 µg/L). |
| Limit Of Detection (LOD) | The LOD will be set at the lowest concentration that can be detected in test solution samples. This value is calculated based on the concentration of the low calibration standard and the dilution factor of the control samples. | 0.300 µg/L | 0.300 µg/L |
| Confirmation of Analyte Identification | A chromatographic confirmatory method will be used to determine test solution concentrations during validation. | Primary ion: 330.3/258.2<br><br>Meets all method and guideline specifications outlined in this table. | Confirmatory ion: 330.3/159.0<br><br>Meets all method and guideline specifications outlined in this table. |

## Conclusion

The validity criteria for the analytical method have been met according to SANTE/2020/12830 rev.2.

## A 2.1.1.1.2.3 Method validation RH-127450

| | |
|---|---|
| Comments of zRMS: | The method was successfully validated according to SANTE/2020/12830 rev. 1. |

| | |
|---|---|
| Reference: | KCA 4.1.2 |
| Report | RH-127450 in Salt Water Enforcement Analytical Method, Liu, Y., 2023, Stillmeadow Inc., Report No.: 26101-22 |
| Guideline(s): | OCSPP 830.1800<br>SANTE/2020/12830, rev. 1 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

**Materials and methods**

The purpose of this study was to validate an enforcement analytical method suitable for active ingredient analysis of the test substance, RH-127450, within a salt water matrix. Confirmation of the identity of the test substance active ingredient was evaluated against a certified analytical grade standard using ultraviolet spectroscopy (UV/Vis). The concentration of the active ingredient, 3,5-dichloro-4-methyl-N-(3-methyl-2-oxopentan-3-yl) benzamide, was determined by high-performance liquid chromatography (HPLC). The method for active ingredient determination was validated by assessment of matrix effects, linearity, Limit of Detection (LOD), Limit of Quantification (LOQ), recovery and repeatability, selectivity and specificity and stability.

**Results and discussions**

| **Method Validation** | |
|---|---|
| **Specificity and Selectivity** | Confirmation of identity by UV/Visible Spectra retention time and absorbance |
| **Average Accuracy of Standards** | 99.93% |
| **Linearity (Correlation Coefficient)** | 1.0000* |
| **Precision (RSD from multiple injections of Check Standard)** | 0.45% (Check Standard C)<br>2.80% (Check Standard G) |
| **Percent Recovery of Spiked Samples** | 102% |
| **Limit of Detection** | 0.13 mg/L |
| **Limit of Quantification** | 0.334 mg/L |
| **Matrix Effect (<20%)** | 1.9849% |
| **Blank Matrix Samples (at least 2)** | Blanks values were 0. |

* Correlation coefficient is the same for both the High Curve and the Low Curve for the calibration curve

**Conclusion**

The validity criteria for the analytical method have been met according to SANTE/2020/12830 rev.1.

## A 2.1.1.1.2.4 Method validation RH-24549

| | |
|---|---|
| Comments of zRMS: | The method was successfully validated according to SANTE/2020/12830 rev. 1. |

| | |
|---|---|
| Reference: | KCA 4.1.2 |
| Report | RH-24549 in Salt Water Enforcement Analytical Method, Liu, Y., 2023, Stillmeadow Inc., Report No.: 26107-22 |
| Guideline(s): | OCSPP 830.1800<br>SANTE/2020/12830, rev. 1 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

**Materials and methods**

The purpose of this study was to validate an enforcement analytical method suitable for active ingredient analysis of the test substance, RH-24549, within a salt water matrix. Confirmation of the identity of the test substance active ingredient was evaluated against a certified analytical grade standard using ultraviolet spectroscopy (UV/Vis). The concentration of the active ingredient, 3,5-Dichloro-4-methylbenzoic acid (RH-24549), was determined by high-performance liquid chromatography (HPLC). The method for active ingredient determination was validated by assessment of matrix effects, linearity, Limit of Detection (LOD), Limit of Quantification (LOQ), recovery and repeatability, selectivity and specificity, and stability.

**Results and discussions**

| **Method Validation** | |
|---|---|
| **Specificity and Selectivity** | Confirmation of identity by UV/Visible Spectra retention time and absorbance |
| **Average Accuracy of Standards** | 99.56% |
| **Linearity (Correlation Coefficient)** | 0.9999 |
| **Precision (RSD from multiple injections of Check Standard)** | 1.62% (Check Standard C)<br>2.27% (Check Standard F) |
| **Percent Recovery of Spiked Samples** | 112% |
| **Limit of Detection** | 0.32 mg/L |
| **Limit of Quantification** | 1.159 mg/L |
| **Matrix Effect (<20%)** | 3.6819% |
| **Blank Matrix Samples (at least 2)** | Blanks values were 0. |

RSD - Relative Standard Deviation

**Conclusion**

The validity criteria for the analytical method have been met according to SANTE/2020/12830 rev.1.

## A 2.1.1.1.2.5 Method validation RH-141455

| | |
|---|---|
| Comments of zRMS: | The method was successfully validated according to SANTE/2020/12830 rev. 1. |

| | |
|---|---|
| Reference: | KCA 4.1.2 |

| | |
|---|---|
| Report | RH-141455 in Salt Water Enforcement Analytical Method, Liu, Y., 2023, Stillmeadow Inc., Report No.: 26105-22 |
| Guideline(s): | OCSPP 830.1800<br>SANTE/2020/12830, rev. 1 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

**Materials and methods**

The purpose of this study was to validate an enforcement analytical method suitable for active ingredient analysis of the test substance, RH-141455, within a salt water matrix. Confirmation of the identity of the test substance active ingredient was evaluated against a certified analytical grade standard using ultraviolet spectroscopy (UV/Vis). The concentration of the active ingredient, 2,6-Dichloroterephthalic acid (RH-141455), was determined by high-performance liquid chromatography (HPLC). The method for active ingredient determination was validated by assessment of matrix effects, linearity, Limit of Detection (LOD), Limit of Quantification (LOQ), recovery and repeatability, selectivity and specificity, and stability.

**Results and discussions**

| **Method Validation** | |
|---|---|
| **Specificity and Selectivity** | Confirmation of identity by UV/Visible Spectra retention time and absorbance |
| **Average Accuracy of Standards** | 101.43% |
| **Linearity (Correlation Coefficient)** | 0.9998 (High Calibration Curve)<br>0.1000 (Low Calibration Curve) |
| **Precision (RSD from multiple injections of Check Standard)** | 0.96% (Check Standard C)<br>2.47% (Check Standard G) |
| **Percent Recovery of Spiked Samples** | 108% (Control)<br>70% (Low Spike)<br>104% (High Spike) |
| **Limit of Detection** | 0.62 mg/L |
| **Limit of Quantification** | 31.592 mg/L |
| **Matrix Effect (<20%)** | -1.5733% |
| **Blank Matrix Samples (at least 2)** | Blanks values were 0. |

RSD - Relative Standard Deviation

**Conclusion**

The validity criteria for the analytical method have been met according to SANTE/2020/12830 rev.1.

## A 2.1.1.1.2.6 Method validation RH-139432

| | |
|---|---|
| Comments of zRMS: | The method was successfully validated according to SANTE/2020/12830 rev. 1. |

| | |
|---|---|
| Reference: | KCA 4.1.2 |

| | |
|---|---|
| Report | RH-139432 in Salt Water Enforcement Analytical Method, Liu, Y., 2023, Stillmeadow Inc., Report No.: 26104-22 |
| Guideline(s): | OCSPP 830.1800<br>SANTE/2020/12830, rev. 1 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

**Materials and methods**

The purpose of this study was to validate an enforcement analytical method suitable for active ingredient analysis of the test substance, RH-139432, within a salt water matrix. Confirmation of the identity of the test substance active ingredient was evaluated against a certified analytical grade standard using ultraviolet spectroscopy (UV/Vis). The concentration of the active ingredient, 3,5-dichloro-4-methylbenzamide, was determined by high-performance liquid chromatography (HPLC). The method for active ingredient determination was validated by assessment of matrix effects, linearity, Limit of Detection (LOD), Limit of Quantification (LOQ), recovery and repeatability, selectivity and specificity and stability.

**Results and discussions**

| **Method Validation** | |
|---|---|
| **Specificity and Selectivity** | Confirmation of identity by UV/Visible Spectra retention time and absorbance |
| **Average Accuracy of Standards** | 100.96% |
| **Linearity (Correlation Coefficient)** | 1.0000* |
| **Precision (RSD from multiple injections of Check Standard C)** | 0.59% for active ingredient analysis<br>0.830% for storage stability analysis |
| **Percent Recovery of Spiked Samples** | 99% |
| **Limit of Detection** | 0.09 mg/L |
| **Limit of Quantification** | 0.348 mg/L |
| **Matrix Effect (<20%)** | 1.5434% |
| **Blank Matrix Samples (at least 2)** | Blanks values were 0. |

RSD - Relative Standard Deviation
* Correlation coefficient is the same for both the High Curve and the Low Curve for the calibration curve

**Conclusion**

The validity criteria for the analytical method have been met according to SANTE/2020/12830 rev.1.

## A 2.1.1.1.3 Algae

## A 2.1.1.1.3.1 Method validation

| | |
|---|---|
| Comments of zRMS: | The method was successfully validated according to SANTE/2020/12830 rev. 2. Method FR/002722A v2 was assessed as being fit-for-purpose for this study. |

| | |
|---|---|
| Reference: | KCP 10.2.1 |
| Report | GLOB2007bF: *Pseudokirchneriella subcapitata* Growth Inhibition Test, Wright, E., 2023, Fera Science Ltd, Report No.: FR/002722 |
| Guideline(s): | SANTE/2020/12830, rev. 2 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

**Materials and methods**

Method reference: FR/002722A v2
This method is intended for the determination of zoxamide and propamocarb (as hydrochloride) in test medium used to conduct freshwater alga growth inhibition tests according to OECD 201. In this analytical method, samples are diluted (if required), filtered through PVDF and analysed by LC MS/MS.

**Results and discussions**

**Method FR/002722A v2 Assessment – Zoxamide**

The analytical method was validated at 0.047 mg test item (TI)/L (low-level validation, LLV), at 2.1 mg TI/L, and at 8 mg TI/L (high-level validation, HLV), equivalent to 0.0027 mg zoxamide/L, 0.12 mg zoxamide/L and 0.47 mg zoxamide/L, respectively. The data from LIMS runs 65369, 65419 and 65420 show that the analytical method satisfies the validation guidelines in SANTE/2020/12830, Rev.2 as follows:

**Matrix-Effects**

An assessment of matrix effects on the detector response was performed by comparing the slope (gradient) of the calibration function prepared with standards in solvent with that for the calibration with standards in matrix.

The matrix effects were calculated by comparison of the slopes of the 2 calibration curves using the equation:

*Matrix effects (%) = 100 x slope (matrix) / slope (solvent) - 100*

For zoxamide, the matrix effect was determined as 2.0%. As the matrix effect did not exceed ±20% in enhancement or suppression, it was not considered to be significant. Nevertheless, calibration curves were still constructed using matrix matched standards.

**Calibration**

The calibration consisted of five levels with duplicate injections, bracketing the samples. The calibration range covered 0.13 to 2.1 ng zoxamide/mL, equivalent to 0.00065 – 0.011 mg zoxamide/L in a sample (when diluted by lowest factor of 5). The LLV (= limit of quantification, LOQ) was performed at 0.0027 mg zoxamide/L (0.047 mg TI/L); 30% of this is 0.00081 mg zoxamide/L, which is equivalent to

0.16 ng/mL when diluted according to method FR/002722A v2. The lowest calibration level, 0.13 ng/mL is, therefore, <30% of the LOQ. The highest concentration samples were diluted sufficiently to ensure that the highest calibration level was at least 20% above the concentration being measured. Therefore, the calibration range met or exceeded the requirements of SANTE/2020/12830, Rev.2 (≤ 30% of LOQ to ≥ 20% above the highest measured solution concentration). Inspection of the plots of the residuals from the unweighted linear regressions showed that they were reasonably evenly distributed, therefore, the fitted line was considered acceptable The correlation co-efficients (LIMS runs 65369, 65419, 65420), r, ranged from 0.997966 to 0.999906 ($r^2$ = 0.995936 to 0.999813). The calibration graphs meet the principles of SANTE/2020/12830, Rev.2.

**Limit of detection**

The lowest calibration level is 0.13 ng zoxamide/mL, equivalent to 0.00065 mg zoxamide/L in undiluted sample.

**Limit of Quantification**

This is equal to the lowest validation level which satisfies the SANTE/2020/12830, Rev.2 criteria, i.e. 0.0027 mg zoxamide/L (0.047 mg TI/L).

**Recovery and repeatability**

Three sets of fortified control samples were analysed, one set below the lowest expected sample concentration, one set above the highest expected sample concentration and one set at an intermediate concentration. Calculated per cent recoveries and per cent relative standard deviations (RSDs) in the fortified controls are shown below:

| Fortification level (mg TI/L) | Fortification level (mg zoxamide/L) | Recovery (%) | Mean recovery (%) | RSD (%) |
|---|---|---|---|---|
| 0.047 | 0.0027 | 101.5957<br>107.4911<br>108.4933<br>109.6841<br>87.6376 | 103 | 8.9 |
| 2.1 | 0.12 | 92.3280<br>90.6252<br>94.2419<br>93.0554<br>95.8939<br>104.4809<br>108.3463<br>111.5370<br>108.1398<br>105.2837 | 100 | 7.9 |
| 8 | 0.47 | 89.5278<br>99.7250<br>93.6007<br>95.1146<br>86.3811 | 92.9 | 5.5 |

For the method to be deemed fit for purpose, SANTE/2020/12830, Rev.2. states that mean recoveries must be in the range 70-120% and the RSDs must be ≤ 20%. The data meet these requirements and are therefore acceptable.

**Selectivity and specificity**

There was no response ≥ 30% of the LOQ in the unfortified control media at the retention time of zoxamide, therefore, the method meets the requirements of SANTE/2020/12830/Rev.2 in this regard.

**Extract Stability**

Fortified control solutions were analysed on the day of preparation, and there was no evidence of degradation before or during analysis. During analysis of actual study samples, a Procedural Recovery sample was prepared alongside the samples and analysed on the same day. Any degradation of sample residues would be seen as significant changes in the recovery values calculated from the Procedural Recovery sample. The data from the validation batch provide no evidence of stability issues in sample matrix under the circumstances used for its preparation and analysis.

**Standard Stability**

Stability of the analyte in the stored stock solution was assessed (LIMS run 65369) by comparing the response of a calibration standard prepared from a freshly prepared stock with a calibration standard freshly prepared from a stored stock solution. The mean peak area of the older standard was 9% lower than the mean peak area of the fresh standard. As the responses were within 10% of each other, the stock zoxamide standard has been shown to be stable in acetonitrile for at least 26 days when stored in a freezer.

**Method FR/002722A v2 Assessment – Propamocarb hydrochloride**

The analytical method was validated at 0.047 mg test item (TI)/L (low-level validation, LLV), at 2.1 mg TI/L, and at 8 mg TI/L (high-level validation, HLV), equivalent to 0.019 mg propamocarb hydrochloride/L, 0.85 mg propamocarb hydrochloride/L and 3.2 mg propamocarb hydrochloride/L, respectively. The data from LIMS runs 65369, 65379 and 65402 show that the analytical method satisfies the validation guidelines in SANTE/2020/12830 rev.2 as follows:

**Matrix-Effects**

An assessment of matrix effects on the detector response was performed by comparing the slope (gradient) of the calibration function prepared with standards in solvent with that for the calibration with standards in matrix.

The matrix effects were calculated by comparison of the slopes of the 2 calibration curves using the equation:

*Matrix effects (%) = 100 x slope (matrix) / slope (solvent) - 100*

For propamocarb hydrochloride, the matrix effect was determined as 36%. As the matrix effect exceeded ±20% in enhancement or suppression, it was considered to be significant. However, calibration curves were constructed using matrix matched standards.

### Calibration

The calibration consisted of five levels with duplicate injections, bracketing the samples. The calibration range covered 0.93 to 15 ng propamocarb hydrochloride/mL, equivalent to 0.0047 – 0.075 mg propamocarb hydrochloride/L in a sample (when diluted by lowest factor of 5). The LLV (= limit of quantification, LOQ) was performed at 0.019 mg propamocarb hydrochloride/L (0.047 mg TI/L); 30% of this is 0.0057 mg propamocarb hydrochloride/L, which is equivalent to 1.1 ng/mL when diluted according to method FR/002722A v2. The lowest calibration level, 0.93 ng/mL is, therefore, <30% of the LOQ. The highest concentration samples were diluted sufficiently to ensure that the highest calibration level was at least 20% above the concentration being measured. Therefore, the calibration range met or exceeded the requirements of SANTE/2020/12830, Rev.2. (≤ 30% of LOQ to ≥ 20% above the highest measured solution concentration). Inspection of the plots of the residuals from the unweighted linear regressions showed that they were reasonably evenly distributed, therefore, the fitted line was considered acceptable. The correlation co-efficients (LIMS runs 65369, 65379 and 65402), r, ranged from 0.999467 to 0.999847 ($r^2$ = 0.998934 to 0.999693). The calibration graphs meet the principles of SANTE/2020/12830, Rev.2.

### Limit of detection

The lowest calibration level is 0.93 ng propamocarb hydrochloride/mL, equivalent to 0.0047 mg propamocarb hydrochloride/L.

### Limit of quantification

This is equal to the lowest validation level which satisfies the SANTE/2020/12830, Rev.2 criteria, i.e. 0.019 mg propamocarb hydrochloride/L (0.047 mg TI/L).

### Recovery and repeatability

Three sets of fortified control samples were analysed, one set below the lowest expected sample concentration, one set above the highest expected sample concentration and one set at an intermediate concentration. Calculated per cent recoveries and per cent relative standard deviations (RSDs) in the fortified controls are shown below:

| Fortification level (mg TI/L) | Fortification level (mg propamocarb hydrochloride/L) | Recovery (%) | Mean recovery (%) | RSD (%) |
|---|---|---|---|---|
| 0.047 | 0.019 | 108.5971<br>109.7326<br>110.6819<br>110.7630<br>98.2239 | 108 | 4.9 |
| 2.1 | 0.85 | 115.4613<br>116.5912<br>117.5196<br>112.1892<br>110.5947<br>114.9365<br>113.3790<br>116.8260<br>107.3998 | 114 | 2.8 |

| | | | | |
|---|---|---|---|---|
| | | 114.0506 | | |
| 8 | 3.2 | 112.6688<br>114.0702<br>113.0390<br>113.6891<br>113.2599 | 113 | 0.48 |

For the method to be deemed fit for purpose, SANTE/2020/12830, Rev.2 states that mean recoveries must be in the range 70-120% and the RSDs must be ≤ 20%. The data meet these requirements and are therefore acceptable.

**Selectivity and specificity**

There was no response ≥ 30% of the LOQ in the unfortified control media at the retention time of propamocarb hydrochloride, therefore, the method meets the requirements of SANTE/2020/12830, Rev.2 in this regard.

**Extract Stability**

Fortified control solutions were analysed on the day of preparation, and there was no evidence of degradation before or during analysis. During analysis of actual study samples, a Procedural Recovery sample was prepared alongside the samples and analysed on the same day. Any degradation of sample residues would be noticed as significant changes in the recovery values calculated from the Procedural Recovery. The data from the validation batches provide no evidence of stability issues in sample matrix under the circumstances used for its preparation and analysis.

**Standard Stability**

Stability of the analyte in the stored stock solution was assessed (LIMS run 65369) by comparing the response of a calibration standard prepared from a freshly prepared stock with a calibration standard freshly prepared from a stored stock solution. The mean peak area of the older standard was 2% lower than the mean peak area of the fresh standard. As the responses were within 10% of each other, the stock propamocarb hydrochloride standard has been shown to be stable in acetonitrile for at least 26 days when stored in a freezer.

**Conclusion**

Method FR/002722A v2 was assessed as being fit-for-purpose for this study.

## A 2.1.1.1.3.2 Method validation zoxamide technical

| | |
|---|---|
| Comments of zRMS: | The method was successfully validated according to SANTE/2020/12830 rev. 2. Method FR/002786-B was assessed as being fit-for-purpose for this study. |

| | |
|---|---|
| Reference: | KCP 10.2.1 |
| Report | Zoxamide Technical: *Pseudokirchneriella subcapitata* Growth Inhibition Test, Jarratt, N., 2023, Fera Science Ltd, Report No.: FR/002786 |

| | |
|---|---|
| Guideline(s): | SANTE/2020/12830, rev. 2 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

**Materials and methods**

The analytical method for the determination of Zoxamide concentration in OECD algal media was validated as part of Fera Study FR/002720.

Apparatus
Electronic pipette e.g. Gilson Repetman
Glass pipettes
Volumetric flasks
HPLC amber vials

Materials
Zoxamide reference material
Elga pure water (Produced on site), or equivalent
Acetonitrile (ACN), HPLC grade or equivalent
Methanol, HPLC grade or equivalent (MeOH)
OECD algal media, supplied by aquatics team

Preparation of matrix matched stock solution
Using an analytical balance and suitable glass volumetric flask, prepare a stock solution ca. 250 µg/mL of Zoxamide in ACN. Other concentrations may be used if appropriate.

Reagent Prep
OECD algal media : MeOH (9:1, v/v)
Measure 450 mL OECD media with a measuring cylinder and transfer it to a suitable container. Measure 50 mL of MeOH with a measuring cylinder and add it to the container. Cap and swirl to mix. Allow mixture to cool to room temperature before using it. Other volumes may be used as long as the ratio is maintained and volumes recorded.
Water : MeOH (9:1, v/v)
Follow the method above with Elga water instead of OECD algal media. Other volumes may be used as long as the ratio is maintained and volumes recorded.

**Results and discussions**

Method FR/002786-B was validated at 5 µg zoxamide/L (low-level validation, LLV), and at 200 µg zoxamide/L (high-level validation, HLV).

The data from LIMS run 65321 demonstrate that the analytical method satisfies the validation guidelines in SANTE/2020/12830 rev.2 as follows:

**Matrix-Effects**

An assessment of the effect of blank formation and OECD algal media (sample matrix) on the detector response was performed by comparing the slope (gradient) of the calibration function prepared with standards in solvent with that for the calibration with standards in matrix.

The matrix effects were calculated by comparison of the slopes of the 2 calibration curves using the equation:

Matrix effects (%) = 100 x mean peak area (matrix) / mean peak area (solvent) - 100

For zoxamide, the matrix effect was determined as -18.76%. As the matrix effect did not exceed ±20% in enhancement or suppression, it was not considered to be significant.

Study FR/002786 used technical grade zoxamide (no formulation). A matrix assessment of just OECD algal media was not carried out, however as no significant matrix effect was seen for blank formation and OECD media, it can be assumed the media on its own would not cause any significant matrix effects.

**Calibration**

The calibration curve consisted of four levels with duplicate injections, bracketing the samples. The calibration range covered 0.0013 – 0.07 µg zoxamide/mL. The LLV (= limit of quantification, LOQ) was performed at 5 µg zoxamide/L equivalent to 0.0045 µg/mL in extract when diluted according to method; 30% of this is 0.00135 µg zoxamide/mL. The lowest calibration level, 0.0013 µg/mL is, therefore, <30% of the LOQ. The highest concentration samples were diluted sufficiently to ensure that the highest calibration level was at least 20% above the concentration being measured. Therefore, the calibration range met or exceeded the requirements of SANTE/2020/12830 (≤ 30% of LOQ to ≥ 20% above the highest measured solution concentration). Inspection of the plots of the residuals from the unweighted linear regressions showed that they were reasonably evenly distributed, therefore, the fitted line was considered acceptable. Although not required by Sante, the $R^2$ for the graph was 0.9701. The calibration graphs meet the principles of SANTE/2020/12830/Rev.2.

**Limit of detection**

The lowest calibration level is 0.0013 µg zoxamide/mL, equivalent to 1.43 µg zoxamide/L in undiluted sample based on dilution scheme followed for the controls and LLV.

**Limit of quantification**

This is equal to the lowest validation level which satisfies the SANTE/2020/12830/Rev.2 criteria, i.e. 5 µg zoxamide/L.

**Selectivity and specificity**

There was no response ≥ 30% of the LOQ in the unfortified control media at the retention time of zoxamide, therefore, the method meets the requirements of SANTE/2020/12830/Rev.2 in this regard.

**Recovery and repeatability**

Three sets of fortified control samples were analysed, one set below the lowest expected sample concentration, one set above the highest expected sample concentration and one set at an intermediate concentration. Calculated per cent recoveries and per cent relative standard deviations (RSDs) in the fortified controls are shown below:

| Fortification level (µg zoxamide/mL) | Recovery (%) | Mean recovery (%) | RSD (%) |
|---|---|---|---|

| | | | |
|---|---|---|---|
| 5 | 81.61<br>79.37<br>76.40<br>77.60<br>74.93 | 77.98 | 3.33 |
| 200 | 88.22<br>83.18<br>83.92<br>85.97<br>83.18 | 84.89 | 2.57 |

For the method to be deemed fit for purpose, SANTE/2020/12830 states that mean recoveries must be in the range 70-120% and the RSDs must be ≤ 20%. The data meet these requirements and are therefore acceptable.

**Extract Stability**

Fortified control solutions were analysed on the day of preparation, and there was no evidence of degradation before or during analysis. During analysis of actual study samples, a Procedural Recovery sample was prepared alongside the samples and analysed on the same day. Any degradation of sample residues would be seen as significant changes in the recovery values calculated from the Procedural Recovery sample. The data from the validation batch provide no evidence of stability issues in sample matrix under the circumstances used for its preparation and analysis.

**Standard Stability**

Stability of the analyte in the stored stock solution was assessed (LIMS run 65362) by comparing the response of a calibration standard prepared from a freshly prepared stock with a calibration standard freshly prepared from a stock solution stored for 21 days. The mean peak area of the older standard was 10.59% lower than the mean peak area of the fresh standard. As the responses were not within 10% of each other, the stock zoxamide standard has been shown to not be stable in acetonitrile for 21 days when stored in a freezer. Fresh stocks should be prepared for each analytical run or a shorter storage period should be assessed.

**Conclusion**

Method FR/002786-B was assessed as being fit-for-purpose for this study.

### A 2.1.1.1.3.3 Method validation RH-163353

Please refer to A 2.1.1.1.2.2.

### A 2.1.1.1.3.4 Method validation RH-127450

| | |
|---|---|
| Comments of zRMS: | The method was successfully validated according to SANTE/2020/12830 rev. 1. |

| | |
|---|---|
| Reference: | KCA 4.1.2 |
| Report | RH-127450 in Algae Media Enforcement Analytical Method, Liu, Y., 2023, Stillmeadow Inc., Report No.: 26102-22 |
| Guideline(s): | OCSPP 830.1800<br>SANTE/2020/12830, rev. 1 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

**Materials and methods**

The purpose of this study was to validate an enforcement analytical method suitable for active ingredient analysis of the test substance, RH-127450 in Algae Media, within an algae media matrix. Confirmation of the identity of the test substance active ingredient was evaluated against a certified analytical grade standard and ultraviolet spectroscopy (UV/Vis). The concentration of the active ingredient, 3,5-dichloro-4-methyl-N-(3-methyl-2-oxopentan-3-yl) benzamide, was determined by high-performance liquid chromatography (HPLC). The method for active ingredient determination was validated by assessment of matrix effects, linearity, Limit of Detection (LOD), Limit of Quantification (LOQ), recovery and repeatability, selectivity and specificity and stability.

**Results and discussions**

| **Method Validation** | |
|---|---|
| **Specificity and Selectivity** | Confirmation of identity by UV/Visible Spectra retention time and absorbance |
| **Average Accuracy of Standards** | 100.12% |
| **Linearity (Correlation Coefficient)** | 1.0000* |
| **Precision (RSD from multiple injections of Check Standard)** | 0.44% (Check Standard C)<br>3.73% (Check Standard G) |
| **Percent Recovery of Spiked Samples** | 108% |
| **Limit of Detection** | 0.13 mg/L |
| **Limit of Quantification** | 0.370 mg/L |
| **Matrix Effect (<20%)** | 1.4020% |
| **Blank Matrix Samples (at least 2)** | Blanks values were 0. |

RSD - Relative Standard Deviation
* Correlation coefficient is the same for both the High Curve and the Low Curve for the calibration curve.

**Conclusion**

The validity criteria for the analytical method have been met according to SANTE/2020/12830 rev.1.

## A 2.1.1.1.3.5 Method validation RH-139432

| | |
|---|---|
| Comments of zRMS: | The method was successfully validated according to SANTE/2020/12830 rev. 1. |

| | |
|---|---|
| Reference: | KCA 4.1.2 |

| | |
|---|---|
| Report | RH-139432 in Algae Media Enforcement Analytical Method, Liu, Y., 2023, Stillmeadow Inc., Report No.: 26103-22 |
| Guideline(s): | OCSPP 830.1800<br>SANTE/2020/12830, rev. 1 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

**Materials and methods**

The purpose of this study was to validate an enforcement analytical method suitable for active ingredient analysis of the test substance, RH-139432 in Algae Media, within an algae media matrix. Confirmation of the identity of the test substance active ingredient was evaluated against a certified analytical grade standard and ultraviolet spectroscopy (UV/Vis). The concentration of the active ingredient, 3,5-dichloro-4-methylbenzamide, was determined by high-performance liquid chromatography (HPLC). The method for active ingredient determination was validated by assessment of matrix effects, linearity, Limit of Detection (LOD), Limit of Quantification (LOQ), recovery and repeatability, selectivity and specificity and stability.

**Results and discussions**

| **Method Validation** | |
|---|---|
| **Specificity and Selectivity** | Confirmation of identity by UV/Visible Spectra retention time and absorbance |
| **Average Accuracy of Standards** | 100.96% |
| **Linearity (Correlation Coefficient)** | 1.0000* |
| **Precision (RSD from multiple injections of Check Standard C)** | 0.59% for active ingredient analysis<br>0.788% for storage stability analysis |
| **Percent Recovery of Spiked Samples** | 102% |
| **Limit of Detection** | 0.09 mg/L |
| **Limit of Quantification** | 0.315 mg/L |
| **Matrix Effect (<20%)** | 0.3215% |
| **Blank Matrix Samples (at least 2)** | Blanks values were 0. |

RSD - Relative Standard Deviation
* Correlation coefficient is the same for both the High Curve and the Low Curve for the calibration curve.

**Conclusion**

The validity criteria for the analytical method have been met according to SANTE/2020/12830 rev.1.

## A 2.1.1.1.4 Non-target plants

## A 2.1.1.1.4.1 Method validation

| | |
|---|---|
| Comments of zRMS: | The method was successfully validated according to SANTE/2020/12830 rev. 2. |

| | |
|---|---|
| Reference: | KCP 10.6.2 |
| Report | GLOB2007bF: OECD Terrestrial Plant Test - Vegetative Vigour Test, Davies, C., 2023, Stockbridge Technology Centre Ltd., Report No.: STC/22/E1555 |
| Guideline(s): | SANTE/2020/12830, rev. 2 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

**Materials and methods**

The content of the active ingredients, propamocarb HCl and zoxamide, in the spray solution was determined using separate high performance liquid chromatography (HPLC) methods based on conditions provided by the Sponsor. The analytical methods were validated as part of Study STC/22/E1555 and also covers Study STC/22/E1556.

Method validation

The accuracy and precision of the analytical procedure was verified by the analysis of laboratory prepared aqueous solutions containing known weights of the test item.

During method development, it was confirmed that the batch of test item used to prepare the spray solution has significantly lower zoxamide content in the test item thus a different batch of test item (KS080523-1) was used for the method verification samples.

Five solutions were prepared at a level equivalent to approximately 110% of the highest treatment rate concentration. Test item (approximately 1.1 mL) was accurately weighed into separate 25 mL volumetric flasks. Purified water was used to disperse the test item and dilute the samples to volume.

Five solutions were also prepared at a level equivalent to approximately 20% of the highest treatment rate concentration supplied for analysis (nominal limit of quantification (LOQ)). Test item (approximately 0.2 mL) was accurately weighed into separate 25 mL volumetric flasks. Purified water was used to disperse the test item and dilute the samples to volume.

Each flask was shaken and sonicated for 20 minutes with intermittent shaking and stirred for 5 minutes prior to sampling. The stirring was continued as aliquots were removed for analysis. Aliquots (5 mL) of each verification sample were transferred to separate 100 mL volumetric flasks and diluted to volume with methanol.

The resulting solutions were then filtered (0.45 µm, PTFE) into separate glass vials prior to analysis of the filtrates by the HPLC methods.

An additional 3 samples at each level were also prepared using the original batch of test item (LCM22012601) used to prepare the spray solution and the preparation procedure is identical to the above.

**Results and discussions**

The data obtained from the analysis of the calibration standard solutions were used to prepare calibration curves by plotting the concentration of active ingredient versus the detector response (peak area) using least squares regression with no weighting. The detector was found to be linear over the range of standard solutions for propamocarb and zoxamide in methanol:water (95:5 v/v) with correlation coefficients of 1.0000.

The following recovery data was obtained from the analysis of the method verification samples:

| Matrix | Component | Verification level (mg/L) | n | Recovery (%) | RSD (%) |
|---|---|---|---|---|---|
| Water | propamocarb HCl | 3684<br>20581 | 5<br>5 | 105<br>107 | 0.9<br>0.4 |
| | zoxamide | 514.2<br>2798 | 5<br>5 | 99<br>101 | 1.1<br>0.7 |

RSD - relative standard deviation

The following recovery data was obtained from the analysis of the extra verification samples prepared using the original batch of test item used to prepare the spray solution:

| Matrix | Component | Verification level (mg/L) | n | Recovery (%) | RSD (%) |
|---|---|---|---|---|---|
| Water | propamocarb HCl | 3254<br>20922 | 3<br>3 | 106<br>110 | 3.8<br>0.2 |
| | zoxamide | 442.3<br>2736 | 3<br>3 | 49<br>53 | 5.2<br>0.5 |

RSD - relative standard deviation

This data served to confirm that the method used was valid for the analysis of the spray solution samples and also demonstrates the lower zoxamide content present in the original batch of test item.

No significant matrix effect was observed between the calibration solutions prepared using laboratory water and water supplied by the Test Facility as the calculated the matrix effect was ≤ 20%.

The specificity of the analytical method was confirmed by retention time match and by diode array analysis. In addition analysis of the dilution solvent confirmed that it did not contain components that would interfere with the analysis.

The following recovery data was obtained from the analysis of propamocarb HCl and zoxamide in the spray solution:

| Sample | Active ingredient | Actual content (mg/L) | Theoretical content (mg/L) | Recovery (%) |
|---|---|---|---|---|
| Trt F | propamocarb HCl<br>zoxamide | 18343<br>1293 | 17146<br>2470 | 107<br>52 |

**Conclusion**

The method was demonstrated to be suitable for the analysis of aqueous solutions containing GLOB2007bF.

The contents of the active ingredients in the provided spray solution were found to be within the range of 52 to 107% of the nominal value.

## A 2.1.1.1.5 Bumblebees

### A 2.1.1.1.5.1 Method validation

| | |
|---|---|
| Comments of zRMS: | The method was successfully validated according to SANTE/2020/12830 rev. 1. |

| | |
|---|---|
| Reference: | KCP 10.3.1.1 |
| Report | GLOB2007bF: Acute Contact and Oral Toxicity to Bumblebees (*Bombus terrestris* L.) in the Laboratory, Chwiesko, D., 2022, Ibacon GmbH, Report No.: 169561105 |
| Guideline(s): | SANTE/2020/12830, rev. 1 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

**Materials and methods**

The analytical method was developed in the performing laboratory. The method was implemented and validated in accordance with the criteria set forth by SANTE/2020/12830 Rev.1.
Method for Determination: LC-MS/MS-method

**Results and discussions**

Recovery Rate of Nominal Values:
<u>contact application solution (200 µg a.i./bumblebee)</u>:
Propamocarb: 113 %
Zoxamide: 112 %

<u>oral feeding solution (200 µg a.i./bumblebee):</u>
Propamocarb - A-sample: 92 %
Propamocarb - B-sample: 85 %
Zoxamide – A-sample: 88 %
Zoxamide - B-sample: 86 %

Limit of Detection:
Contact Test: 0.5 µg Propamocarb/L
1 µg Zoxamide/L
Oral Test: 0.5 µg Propamocarb/L
1 µg Zoxamide/L

Limit of Quantification:
<u>Contact Test Solution:</u>
25 g test item/L
corresponding to 3.4 µg Propamocarb/L after dilution by factor 2500000
corresponding to 5.8 µg Zoxamide/L after dilution by factor 250000

<u>Oral Test Feeding Solution:</u>

2.5 g test item/L
corresponding to 2.1 µg Propamocarb/L after dilution by factor 400000
corresponding to 3.7 µg Zoxamide/L after dilution by factor 40000

**Validity Criteria of the Analytical Part**

Selectivity and Specificity: No interference above 30% of LOQ was observed in control samples at retention time of target analytes.

Calibration:

<u>Calibration Range:</u>
Contact Test: 0.5 to 10 mg Propamocarb/L
corresponding to 15 % of LOQ to 147 % of higher fortification level
1 to 20 mg Zoxamide/L
corresponding to 17 % of LOQ to 171 % of higher fortification level

Oral Test: 0.5 to 10 mg Propamocarb/L
corresponding to 24 % of LOQ to 157 % of higher fortification level
1 to 20 mg Zoxamide/L
corresponding to 27 % of LOQ to 182 % of higher fortification level

<u>Correlation Coefficient:</u>
Contact Test:
Propamocarb: r =0.9998
Zoxamide: r = 0.9993

Oral Test:
Propamocarb: r = 0.9982 at least
Zoxamide: r = 0.9998 at least

<u>Calibration Curve (linear regression):</u>
Contact Test:
Propamocarb: y =106778 x + 45868
Zoxamide: y = 12985 x - 745

Oral Test:
Propamocarb: y = 139126 x + 14799
Zoxamide: y = 17496 x + 1121

Residual Plots: The regression residuals are randomly distributed and no trends are visible.

Matrix Effects:

Contact Test:

| | |
|---|---|
| 0.5 µg Propamocarb/L | Matrix Effect = 53 % |
| 10 µg Propamocarb/L | Matrix Effect = -44 % |
| 1 µg Zoxamide/L | Matrix Effect = 3 % |
| 20 µg Zoxamide/L | Matrix Effect = 0 % |

Significant matrix effect (≥ 20 %) was observed for standards prepared for the contact test for Propamocarb.

Oral Test:

| | |
|---|---|
| 0.5 µg Propamocarb/L | Matrix Effect = 1 % |
| 10 µg Propamocarb/L | Matrix Effect = -38 % |
| 1 µg Zoxamide/L | Matrix Effect = 0 % |
| 20 µg Zoxamide/L | Matrix Effect = -3 % |

Significant matrix effect (≥ 20 %) was observed for standards prepared for the oral test for Propamocarb.

Standard and Extract Stability: The stock solution of the reference item was prepared on the day of analysis. Samples were analysed on the day of sample processing. Therefore, stability of standards and extract were not assessed.

Recovery and Repeatability: The mean recovery rate and the relative standard deviation of fortified samples was:

| | **Propamocarb** | | |
|---|---|---|---|
| | mean value (%) | RSD (%) | n |
| **Contact Test** | | | |
| Lower Level (LOQ Level) | 88 % | 3 % | 5 |
| Higher Level | 96 % | 5 % | 5 |
| **Oral Test Feeding Solution** | | | |
| Lower Level (LOQ Level) | 95 % | 4 % | 7 |
| Higher Level | 92 % | 5 % | 6 |

| | **Zoxamide** | | |
|---|---|---|---|
| | mean value (%) | RSD (%) | n |
| **Contact Test** | | | |
| Lower Level (LOQ Level) | 96 % | 2 % | 5 |
| Higher Level | 96 % | 5 % | 5 |
| **Oral Test Feeding Solution** | | | |
| Lower Level (LOQ Level) | 92 % | 5 % | 7 |
| Higher Level | 86 % | 6 % | 8 |

**Conclusion**

All validity criteria for the analytical method have been met.

## A 2.1.1.1.6 Bees adult chronic

### A 2.1.1.1.6.1 Method validation

| Comments of zRMS: | The method was successfully validated according to SANTE/2020/12830 rev. 1. |
|---|---|

| | |
|---|---|
| Reference: | KCP 10.3.1.2 |
| Report | GLOB2007bF: Chronic Oral Toxicity Test on the Honey Bee (*Apis mellifera* L.) in the Laboratory, Schabio, S., 2023, Ibacon Gmbh, Report No.: 169561136 |
| Guideline(s): | SANTE/2020/12830, rev. 1 |
| Deviations: | No |

| | |
|---|---|
| GLP: | Yes |
| Acceptability: | Yes |

**Materials and methods**

The analytical method was developed in the performing laboratory. The method was implemented and validated in accordance with the criteria set forth by SANTE/2020/12830 Rev.1.

Method for Determination: LC-MS/MS-method

**Results and discussions**

Recovery Rate of Nominal Values:

Feeding Solution 500 ppm DAA8

| | |
|---|---|
| Propamocarb: | 80 % |
| Zoxamide: | 80 % |

Feeding Solution 8000 ppm DAA8

| | |
|---|---|
| Propamocarb: | 81 % |
| Zoxamide: | 85 % |

Limit of Detection: 0.5 µg Propamocarb/L
0.5 µg Zoxamide/L

Limit of Quantification: 0.5 g test item/L
corresponding to 3.4 µg Propamocarb/L after dilution by factor 50000
corresponding to 2.9 µg Zoxamide/L after dilution by factor 10000

**Validity Criteria of the Analytical Part**

Selectivity and Specificity: No interference above 30% of LOQ was observed in control samples at retention time of target analyte.

Calibration:

<u>Calibration Range:</u>

0.5 to 12 µg reference item/L

corresponding to 15 % of LOQ to 147 % of higher fortification level for Propamocarb

corresponding to 17 % of LOQ to 171 % of higher fortification level for Zoxamide

<u>Correlation Coefficient:</u>

Propamocarb: r = 1.0000
Zoxamide: r = 1.0000

<u>Calibration Curve:</u>

Propamocarb: $y = -342\ x^2 + 40966\ x + 993$ (quadratic regression)
Zoxamide: $y = 6777\ x + 654$ (linear regression)

Residual Plots: The regression residuals are randomly distributed and no trends are visible.

| | | |
|---|---|---|
| Matrix Effects: | 0.5 µg Propamocarb/L | Matrix Effect = 2 % |
| | 12 µg Propamocarb/L | Matrix Effect = 0 % |
| | No significant matrix effect (≥ 20 %) was observed. | |
| | 0.5 µg Zoxamide/L | Matrix Effect = 23 % |
| | 12 µg Zoxamide /L | Matrix Effect = -3 % |
| | Significant matrix effect (≥ 20 %) was observed. However, matrix-matched standards were used for determination of the active ingredient. | |
| Standard and Extract Stability: | The stock solution of the reference item was prepared on the day of analysis. Samples were analysed on the day of sample processing. Therefore, stability of standards and extract were not assessed. | |
| Recovery and Repeatability: | The mean recovery rate and the relative standard deviation of fortified samples was: | |

| | Propamocarb | | |
|---|---|---|---|
| | mean value | RSD | n |
| Lower Level (LOQ Level) | 86 % | 3 % | 4 |
| Higher Level | 85 % | 4 % | 5 |
| overall: | 85 % | 3 % | 9 |

| | Zoxamide | | |
|---|---|---|---|
| | mean value | RSD | n |
| Lower Level (LOQ Level) | 85 % | 2 % | 4 |
| Higher Level | 88 % | 2 % | 5 |
| overall: | 87 % | 6 % | 9 |

**Conclusion**

All validity criteria for the analytical method have been met.

## A 2.1.1.1.7 Bees larvae chronic

### A 2.1.1.1.7.1 Method validation

| | |
|---|---|
| Comments of zRMS: | The method was successfully validated according to SANTE/2020/12830 rev. 1. |

Reference: KCP 10.3.1.3

| | |
|---|---|
| Report | ~~Honey Bee Larvae Toxicity Test (*Apis mellifera*)~~ Effects of GLOB2007bF on honeybees (Apis mellifera L.) 22-day larval toxicity test with repeated exposure,<br>Colli, M., 2022, Biotecnologie Bt S.R.L., Report No.: BT127/22 |
| Guideline(s): | SANTE/2020/12830, rev. 1 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

**Materials and methods**

The HPLC-DAD analytical method for the determination of Zoxamide in aqueous matrix solution treated with **GLOB2007bF** was validated according to the guideline SANTE/2020/12830 Rev.1 (24/02/2021).

**Results and discussions**

**Summary of the validation parameters for Zoxamide**

| Validation parameters | Results | Validity criteria |
|---|---|---|
| **Matrix effect** | LIN 2 level: -6.37%<br>LIN 4 level: -9.11% | ± 20% |
| **Calibration**<br>1.5477 mg/L – 61.9089 mg/L (corresponding to 0.0031 g/L – 0.6879 g/L in matrix) | $R^2$ = 0.99980000<br>r = 0.99989999 | $R^2$ > 0.98<br>r > 0.99 |
| **Repeatability (Precision) at LOQ level** (Nominal = 0.011 g a.s./L) | RSD = 1.85% | ≤ 20% |
| **Repeatability (Precision) at REC HIGH level** (Nominal = 0.585 g a.s./L) | RSD = 0.65% | |
| **Recovery at LOQ level** (Nominal = 0.011 g a.s./L) | Recovery = 100.87% | 70-120% |
| **Recovery at REC HIGH level** (Nominal = 0.585. g a.s./L) | Recovery = 111.32% | |
| **LOD**<br>1.5477 mg/L | S/N = 2684.46 | S/N > 3 |
| **Selectivity and specificity** | 0.01% of LOQ | < 30% of LOQ |
| **Confirmatory analysis** | Match = 999.9982% | Match > 98% |

**Summary of the validation parameters for Propamocarb-HCl**

| Validation parameters | Quantitative analysis Results Q1 (189.2 m/z → 102.0 m/z) | Confirmatory analysis Results Q2 (189.2 m/z → 74.1 m/z) | Validity criteria |
|---|---|---|---|
| **Matrix effect** | LIN 2 level: 30.91%<br>LIN 4 level: 46.47% | n.d. | ± 20% |
| **Calibration**<br>3.6112 µg/L – 156.4870 µg/L (corresponding to 0.0193 g/L – 6.2595 g/L in matrix) | $R^2$ = 0.99963658<br>r = 0.99981827 | $R^2$ = 0.99966178<br>r = 0.99983088 | $R^2$ > 0.98<br>r > 0.99 |
| **LOD**<br>3.6112 µg/L<br>(Lowest calibration level) | S/N = 27.79 | n.d. | S/N > 3 |
| **Recovery at LOQ level**<br>(Nominal = 0.073 g a.s./L) | Recovery = 105.19% | Recovery = 104.14% | Recovery 70-120% |
| **Recovery at REC HIGH level**<br>(Nominal = 4.057 g a.s./L) | Recovery = 94.89% | n.d. | Recovery 70-120% |
| **Repeatability (Precision) at LOQ level**<br>(Nominal = 0.073 g a.s./L) | %RSD = 1.93% | %RSD = 2.15% | % RSD ≤ 20% |
| **Repeatability (Precision) at REC HIGH level**<br>(Nominal = 4.057 g a.s./L) | %RSD = 2.55% | n.d. | % RSD ≤ 20% |
| **Selectivity and specificity** | 1.08% of LOQ | 1.11% of LOQ | < 30 % of LOQ |

**Conclusion**

The analytical method was suitable to determine zoxamide and propamocarb-HCl in ultrapure water solutions. The method fulfils all requirements of SANTE/2020/12830, Rev.1 (24/02/2021) guideline concerning Linearity (Calibration and detector response), Accuracy (% mean recovery), Precision (% RSD) and LOQ.

| | |
|---|---|
| Comments of zRMS: | The method was successfully validated according to SANTE/2020/12830 rev. 2. |

| | |
|---|---|
| Reference: | KCA 8.3.1.3 |
| Report | Zoxamide technical: Honey Bee (*Apis mellifera* L.) Larval Toxicity Test following Repeated Exposure under laboratory conditions, Aguilar-Alberola, J., 2023, Eurofins Trialcamp S.L.U., Report No.: S23-106642 |
| Guideline(s): | SANTE/2020/12830, rev. 2 |
| Deviations: | No |

| | |
|---|---|
| GLP: | Yes |
| Acceptability: | Yes |

**Materials and methods**

In brief, sample extraction of larval diet containing 1.5 % acetone diet were performed by dilution of samples with acetonitrile/water (1:1; v/v). Quantification was performed by use of LC-MS/MS.

**Results and discussions**

The limit of quantification (LOQ) was 2.97 mg zoxamide /kg with a limit of detection (LOD) of 0.619 mg/kg.
The maximum storage interval from sampling until extraction was 23 days.
The storage temperature of the samples at the analytical test site was ≤ -18 °C with no exceedance.
Storage stability testing was not necessary because the interval from sampling until injection did not exceed 30 days for any analysed sample.
Results in mg/kg are reported without correction for the obtained concurrent recoveries, i.e. no adjustments to hypothetical concurrent recoveries of 100 % were made.
The following concentrations were detected in the samples:

| Sample Name | *Active Ingredient Nominal (mg/kg) | Calculated Concentration of Active Ingredient (mg/kg) | Recovery (%) | Mean Recovery (%) |
|---|---|---|---|---|
| S23-106642-L2-D3-T1-A | 32.466 | 28.004 | 86.3 | 95.6 |
| S23-106642-L2-D4-T1-A | 32.466 | 31.584 | 97.3 | |
| S23-106642-L2-D5-T1-A | 32.466 | 32.636 | 101 | |
| S23-106642-L2-D6-T1-A | 32.466 | 31.976 | 98.5 | |
| S23-106642-L2-D3-T5-A | 519.48 | 360.83 | 71.4[a] | 85.8 |
| S23-106642-L2-D3-T5-R | 519.48 | 381.24 | | |
| S23-106642-L2-D4-T5-A | 519.48 | 393.30 | 78.3[b] | |
| S23-106642-L2-D4-T5-R | 519.48 | 420.49 | | |
| S23-106642-L2-D5-T5-A | 519.48 | 509 | 98 | |
| S23-106642-L2-D6-T5-A | 519.48 | 496.66 | 95.6 | |

*Taken from study plan, [a] Mean of 69.5 and 73.4 %; [b] Mean of 75.7 and 80.9 %.

**Analytical performance**

Selectivity and Specificity

The analyte was determined in the final sample extracts by use of LC-MS/MS detection with evaluation of one (1) mass transition per analyte
A second mass transition was monitored for confirmation of peak identity but was not used for the quantification of target analyte.
Untreated samples for accompanying control sample work up, for determination of (concurrent) recoveries and, for preparation of matrix-matched calibration standards were supplied by the Test Site of the Analytical Phase.

The blank values at the expected retention time of the analyte resulting from reagents and/or the control sample material used for recovery determinations and for preparation of matrix-matched calibration standards did not exceed a level that would correspond to 30 % of the LOQ.
Correction for blank values was therefore not performed.
Example chromatograms representing control samples, the lowest calibration level, samples fortified at the LOQ and treated residue samples are included in Appendix D.

Matrix Effects

The effect of matrix on the detector response was assessed by comparing peak areas of matrix-matched standards of 90 % matrix amount with solvent standards at comparable (or the same) nominal concentrations. Matrix effects were calculated as follows:

| | |
|---|---|
| **Matrix effect (%)** | = [(100 · $A_{Matrix-Std}$ ) / ($A_{Solv-Std}$)] - 100 |
| $A_{Solv-Std}$ | Peak area of solvent standard |
| $A_{Matrix-Std}$ | Peak area of matrix-matched standard |

The matrix effects are summarised in the table below:

| **Matrix** | **Standard Concentration (ng/mL)** | **Matrix Effect for Zoxamide (%)** | **Matrix Effect for Zoxamide (%)** |
|---|---|---|---|
| | | **Quantification (*m/z* 336 → 187)** | **Mean Value** |
| Larval Diet containing 1.5 % acetone | 0.300 | -1.76 | (-) 0.010 |
| | 0.500 | 0.334 | |
| | 1.00 | 1.33 | |
| | 2.00 | -1.29 | |
| | 3.00 | 0.335 | |
| | 4.00 | -0.853 | |
| | 5.00 | 0.351 | |
| | 6.00 | 1.48 | |

(+) matrix enhancement; (-) matrix suppression

The matrix suppression or enhancement was < 20 % for the investigated matrix and thus deemed to be insignificant. However, matrix-matched standards were used for quantification throughout the analytical phase.

Calibration

The linearity of the detector response was demonstrated by single determination of matrix-matched calibration standards at a minimum of five (5) concentration levels ranging from 0.300 ng/mL to 6.00 ng/mL. This range corresponds to a mass fraction level of 0.619 mg/kg to 12.4 mg/kg and thus covers the range from no more than 30 % of the LOQ and at least + 20 % of the highest analyte concentration detected in any (diluted) sample extract. The calibration curve did not exceed two (2) orders of magnitude.
The calibration curves were linear and the regression residuals were randomly distributed. Furthermore, correlation coefficients (R) were ≥ 0.99. Linear regression was performed with 1/x-weighting.
Representative linear regression curves and residual plots are included in Appendix C.

Quantification

Quantification was performed using a calibration curve that fulfilled the above given criteria. Standard solutions were distributed over the whole acquisition batch. The linear regression equation was used for calculation of the analyte concentrations.
In order to still be within the linear calibration range, extracts of samples and recoveries at higher level were diluted by factors of 5and 100 with control sample extract.
Formula and an example calculation are part of the analytical method description given in Appendix A.

**Method Validation**

For the purpose of method validation, recoveries were conducted in accordance to SANTE/2020/12830, Rev. 2. prior sample analysis.
Five (5) recovery determinations at LOQ and five (5) recovery determinations at high level were performed for each analyte.
One (1) mass transition was evaluated and representative ion chromatograms along with the product ion mass spectrum are shown in Appendix B and D of the report. A second ion transition was included to the detection method but used for monitoring only. Recovery data are not reported for this mass transition.
The following recoveries were obtained:

| Matrix | Zoxamide Fortification level (mg/kg) | Recovery (%) | Mean Recovery (%) | Rel. Std. Dev. (%) | Replicates |
|---|---|---|---|---|---|
| **Zoxamide (Mass Transition *m/z* m/z 336 → 187)** | | | | | |
| Larval diet containing 1.5 % Acetone | 2.97 | 93.4, 93.6, 93.8, 96.1, 94.8 | 94.4 | 1 | 5 |
| | 677 | 102, 97.7, 98.1, 98.1, 97.8 | 98.7 | 2 | 5 |

No observable peak was detected in any control sample extract
Recoveries are without any blank correction

Concurrent Recoveries

The analytical performance in terms of accuracy and repeatability was assessed for each analytical set by fortification of control (untreated) test portions of the respective matrix and subsequent determination of the concurrent recoveries upon applying the analytical method.
Each analytical set included at least the following types and numbers of samples:

- 1 reagent blank (sample work-up without matrix)
- 1 control samples (concurrent control)
- 2 control samples fortified at the LOQ (concurrent recoveries)
- 2 control samples fortified at higher level (concurrent recoveries)

Concurrent recoveries were handled and stored in the same way and for the same period of time as the sample extracts that were prepared within the same analytical set.
The following table summarises the obtained recoveries:

| Zoxamide | | | | | |
|---|---|---|---|---|---|
| Matrix | Zoxamide Fortification level (mg/kg) | Concurrent Recovery (%) | Mean Recovery (%) | Rel. Std. Dev. (%) | Replicates |
| **Zoxamide (Mass Transition *m/z* m/z 336 → 187)** | | | | | |

| Zoxamide | | | | | |
|---|---|---|---|---|---|
| **Matrix** | **Zoxamide Fortification level (mg/kg)** | **Concurrent Recovery (%)** | **Mean Recovery (%)** | **Rel. Std. Dev. (%)** | **Replicates** |
| Larval diet containing 1.5 % Acetone | 2.97 | 96.1, 96.7, 94.5, 94.7, 102 | 96.8 | 3 | 5 |
| | 677 | 101, 96.7, 96.7, 97.6, 102 | 98.9 | 3 | 5 |

No observable peak was detected in any control sample extract
Recoveries are without any blank correction

One (1) mass transition was evaluated and representative ion chromatograms along with the product ion mass spectrum are shown in Appendix B of the report.

Accuracy is reflected by the mean recovery per level while precision is reflected by the corresponding relative standard deviation.
All mean values at fortification levels of 2.97 mg zoxamide/kg (LOQ) and 677 mg zoxamide/kg for one (1) mass transition are within 70 % - 120 % with relative standard deviations ≤ 20 % and thereby comply with the standard acceptance criteria of the guidance document SANTE/2020/12830, rev. 2.

Stability of Stock and Fortification Solutions

A stock solution was stored at typically 1 °C to 10 °C in the dark. After storage, a freshly prepared dilution of the stock solution was compared to a dilution of freshly prepared stock solution by fivefold injection (n = 5).

Results were derived from GLP data available at the Test Site and are summarised below.

| **Analyte** | **Solvent** | **Concentration (µg/mL)** | **Storage Period (Days)** | **Mean difference (in %) of stored stock solution compared to freshly prepared stock solution** |
|---|---|---|---|---|
| Zoxamide | Acetonitrile | 1010 | 51 | 8 |

The mean peak area of the standard solution was within ± 10 % of the mean peak area of the freshly prepared solution indicating that stock solution is stable when stored at 1 °C to 10 °C in the dark for 51 days. This was sufficient to cover the length of time it was used in this study (10 days).

Stability of Final Extracts

The interval from preparation of the final extracts to injection did not exceed 24 hours. Due to the shortness of the interval any effect on the results due to a possible instability of the analyte in final sample extracts are considered to be insignificant.

Storage Stability

The maximum storage period from sampling to analysis was 23 days within this study. Residues are regarded as stable if the samples are stored deep-frozen for up to 30 days between sampling and analysis (OECD (2007), Test No 506: Stability of Pesticide Residues in Stored Commodities). Therefore, the proof of stability of the analytes in/on fortified or incurred samples upon storage in deep frozen conditions was not conducted as part of this analytical phase.

**Conclusion**

The method was successfully validated for determination of zoxamide in larval diet containing 1.5 % acetone with an LOQ of 2.97 mg zoxamide/kg and up to 677 mg zoxamide/kg according to guidance document(s) SANTE/2020/12830, rev. 2.
With regard to selectivity, accuracy and precision, the analytical method was applied successfully for each analytical set when analysing the samples of the study.

## A 2.1.2 Methods for post-authorization control and monitoring purposes (KCP 5.2)

### A 2.1.2.1 Description of analytical methods for the determination of residues in plant matrices (KCP 5.2)

No new or additional studies have been submitted.

### A 2.1.2.2 Description of analytical methods for the determination of residues in animal matrices (KCP 5.2)

No new or additional studies have been submitted.

### A 2.1.2.3 Description of Methods for the Analysis of Soil (KCP 5.2)

No new or additional studies have been submitted.

### A 2.1.2.4 Description of Methods for the Analysis of Water (KCP 5.2)

No new or additional studies have been submitted.

### A 2.1.2.5 Description of Methods for the Analysis of Air (KCP 5.2)

No new or additional studies have been submitted.

### A 2.1.2.6 Description of Methods for the Analysis of Body Fluids and Tissues (KCP 5.2)

| | |
|---|---|
| Comments of zRMS: | LC-MS/MS determination was conducted with evaluation of two mass transitions (*m/z* 336→187 and *m/z* 336→159 for zoxamide and *m/z* 219→175 and *m/z* 219→147 for RH-1452). Due to enhanced sensitivity mass transition *m/z* 336→187 (zoxamide) and 219→175 (RH-1452) is proposed to be used for quantification but both mass transitions are applicable interchangeably for quantification and confirmation.<br>Matrix effects were ≥ ± 20 % and deemed to be significant for RH-1452 in both |

matrices.
Matrix effects were < ± 20 % and deemed to be insignificant for zoxamide in both matrices. Therefore, solvent standards were used for quantification throughout the study.
The LOQ of 0.01 mg/kg was confirmed for zoxamide and RH-1452 in bovine (liver) and porcine (urine).
The following recoveries were obtained:

| Zoxamide | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Mass Transition** | **Fortification Level (mg/kg)** | **Recovery (%)** | **Mean Recovery (%)** | **Rel. Std. Dev. (%)** | **Replicates** | **Overall Mean Recovery (%)** | **Overall Rel. Std. Dev. (%)** |
| **Bovine (liver)** | | | | | | | |
| m/z 336→187* | 0.01 | 89, 87, 83, 79, 79 | 84 | 5.6 | 5 | 84 | 4.8 |
| | 0.1 | 83, 83, 81, 90, 86 | 85 | 4.3 | 5 | | |
| m/z 336→159 | 0.01 | 91, 85, 84, 76, 78 | 83 | 7.2 | 5 | 84 | 5.6 |
| | 0.1 | 86, 85, 79, 88, 87 | 85 | 3.9 | 5 | | |
| **Porcine (urine)** | | | | | | | |
| m/z 336→187* | 0.01 | 103, 104, 103, 107, 108 | 105 | 2.3 | 5 | 105 | 2.3 |
| m/z 336→159 | 0.01 | 102, 103, 103, 110, 104 | 105 | 3.1 | 5 | 105 | 3.1 |
| **RH-1452** | | | | | | | |
| **Bovine (liver)** | | | | | | | |
| m/z 219→175* | 0.01 | 89, 90, 84, 78, 76 | 83 | 7.3 | 5 | 86 | 7.0 |
| | 0.1 | 89, 94, 90, 91, 81 | 89 | 5.6 | 5 | | |
| m/z 221→147 | 0.01 | 94, 86, 81, 71, 72 | 81 | 12 | 5 | 85 | 10 |
| | 0.1 | 90, 93, 89, 93, 81 | 89 | 5.5 | 5 | | |
| **Porcine (urine)** | | | | | | | |
| m/z 219→175* | 0.01 | 98, 110, 115, 95, 92 | 102 | 9.7 | 5 | 102 | 10 |
| m/z 221→147 | 0.01 | 90, 95, 95, 102, 106 | 98 | 6.6 | 5 | 98 | 6.6 |

No observable peak was detected in any control sample extract
Recoveries are without any blank correction
* Proposed to be used for quantification

The method was successfully validated for the determination of zoxamide and RH-1452 in bovine (liver) and porcine (urine) from the tested LOQ of 0.01 mg/kg up to 0.1 mg/kg (for bovine (liver) only) according to the guidance document SANTE/2020/12830, rev. 2 for risk assessment and/or monitoring.

| | |
|---|---|
| Reference: | KCA 4.2 |
| Report | Validation of an Analytical Method for Determination of Zoxamide in Body Fluids and Animal Tissues, Gustloff, C., 2023, Eurofins Agroscience Services Chem Gmbh, Report No.: S23-100691 |
| Guideline(s): | SANTE/2020/12830, rev. 2 |
| Deviations: | No |
| GLP: | Yes |
| Acceptability: | Yes |

**Materials and methods**

| | |
|---|---|
| Analytes | Zoxamide and RH-1452 |
| Matrices | Body fluids (urine) and animal tissues (liver) |

| | |
|---|---|
| Method Reference | S21-07039 (GLC-2110V) [1] and S21-07040 (GLC-2111V) [2] |
| Principle of the Analytical Procedure | Homogenisation: Dry ice, cutter for animal tissues (liver), Shaking for body fluids (urine)<br>**Zoxamide:**<br>Extraction: Acetonitrile and addition of water, high-speed homogeniser<br>(Ratio: 10 mL of extraction solvent per g of matrix)<br>Clean-up: Dispersive SPE with primary/secondary amine (PSA) and C18 for Bovine (liver) only<br>Concentration step: nitrogen stream<br>Reconstitution: Acetonitrile/0.1 % formic acid (1+1, v+v)<br>Sample concentration in final extract: 0.1 g sample per mL of extract<br>Quantification: LC-MS/MS<br>**RH-1452:**<br>Extraction: glycine buffer, high-speed homogeniser for bovine (liver), shaking for porcine (urine)<br>(Ratio: 25 mL of extraction solvent per g of matrix)<br>Liquid-liquid-partition: two times with ethyl acetate<br>Clean-up: ENVI-Carb cartridge<br>Concentration step: nitrogen stream<br>Reconstitution: Acetonitrile/water (2+8, v+v)<br>Sample concentration in final extract: 0.2 g sample per mL of extract<br>Quantification: LC-MS/MS |

**Results and discussions**

| | |
|---|---|
| Selectivity and Specificity | Demonstrated by validation of two (2) mass transitions<br>Analyte levels or chromatographic interferences of unknown compounds in a reagent blank and control sample extracts were below of what would correspond to an analyte level of 30 % of the LOQ. |
| Matrix Effects on Analyte Detection | Significant (≥ 20 %) for RH-1452 in bovine (liver) and porcine (urine).<br>Insignificant (< 20 %) for zoxamide in bovine (liver) and porcine (urine). |
| Calibration | Matrix-matched calibration standards<br>A minimum of five (5) concentration levels<br>Single determination<br>Injection of standard solutions spread over the whole acquisition batch<br>Concentration range: 0.30 ng/mL to 30 ng/mL for zoxamide and 0.60 ng/mL to 60 ng/mL for RH-1452<br>Corresponding mass fraction range: 0.003 mg/kg to 0.30 mg/kg<br>Coverage: 30 % of the LOQ to at least + 20 % of the highest analyte concentration level detected in a sample extract<br>The validated range does not exceed two (2) orders of magnitude |
| Quantification | Linear regression with 1/x weighting<br>Regression residuals randomly distributed<br>Coefficients of determination ($R^2$) ≥ 0.99 |

<table>
<tr><td>Accuracy<br>and Precision</td><td>Five (5) fortifications at 0.01 mg/kg (LOQ)<br>Five (5) fortifications at 0.1 mg/kg (10x LOQ) for bovine (liver).<br>Mean recoveries for the two (2) evaluated mass transitions comply with the standard acceptance criteria of the guidance document SANTE/2020/12830, rev. 2:
<table>
<tr><th>Concentration Level (mg/kg)</th><th>Range of Mean Recoveries (%)</th><th>Precision, Rel. Std. Dev. (%)</th></tr>
<tr><td>≤ 0.01</td><td>60 - 120</td><td>≤ 30</td></tr>
<tr><td>> 0.01 - ≤ 0.1</td><td>70 - 120</td><td>≤ 20</td></tr>
</table></td></tr>
<tr><td>LOQ</td><td>0.01 mg/kg (lowest validated fortification level)</td></tr>
<tr><td>LOD</td><td>30 % of the LOQ (lowest calibration standard)</td></tr>
<tr><td>Stability of Analyte(s) in Standard Solutions</td><td>Within ± 10 % for at least 13 days when prepared in acetonitrile/0.1 % formic acid (1+1, v+v) and stored at typically 1 °C to 10 °C in the dark for zoxamide as proven in study S21-07039 (GLC-2110V) [1].<br>Within ± 10 % for at least 11 days when prepared in water/acetonitrile (8+2, v+v) and stored at typically1 °C to 10 °C in the dark for RH-1452 as proven in study S21-07040 (GLC-2111V) [2].</td></tr>
<tr><td>Stability of Analytes in Sample Extracts</td><td>Recoveries within 70 % - 120 % in all matrix extracts for at least 7 days when stored at typically 1 °C to 10 °C in the dark.</td></tr>
</table>

**Conclusion**

The method was found to be valid according to the guidance document SANTE/2020/12830, rev. 2 for risk assessment and/or monitoring.

## A 2.1.2.7 A.2.A.9 Other Studies/ Information

No new or additional studies have been submitted.

## A 2.2 Analytical methods for propamocarb-HCl

Please refer to A.2.1.1.