REGISTRATION REPORT

Part B

Section 5

Analytical Methods

Detailed summary of the risk assessment

Product code: ARY-0469-04

Product name(s): ASAHI MAX

Chemical active substance(s):

Sodium 5-nitroguaiacolate, 3g/L
Sodium o-nitrophenolate, 6g/L

Sodium p-nitrophenolate, 9g/L

Central Zone

Zonal Rapporteur Member State: POLAND

CORE ASSESSMENT

Applicant: Asahi Chemical Europe s.r.o

Submission date: June 2022, update February 2023

MS Finalisation date: April 2023 (initial Core Assessment)

June 2023 (final Core Assessment)

Version history

When	What
June 2022	Initial version of dRR for submission to zRMS
February 2023	Dossier updated on request of zRMS.
April 2023	Initial zRMS assessment. The report in the dRR format has been prepared by the Applicant, therefore all comments, additional evaluations and conclusions of the zRMS are presented in grey commenting boxes. Minor changes are introduced directly in the text and highlighted in grey. Not agreed or not relevant information are ~~struck through and shaded for transparency~~.
June 2023	Final report (Core Assessment updated following the commenting period) No additional information or assessments after the commenting period.

Table of Contents

5 Analytical methods

5.1 Conclusion and summary of assessment

zRMS conclusion:

In EFSA Scientific Report (2008) 191, 1-130 – "Conclusion on the peer review of sodium 5-nitroguaiacolate, sodium o-nitrophenolate and sodium p-nitrophenolate" it is stated that *Adequate methods are available to monitor all compounds given in the respective residue definitions in food/feed of plant origin and environmental matrices. The methods available determine the three compounds concurrently.*
A HPLC-MS/MS method with column switching is available to monitor residues in food/feed of plant origin with LOQ 0.01 mg/kg (sugar beet, oil seed rape, tomatoes) for each individual compound.
Since residues in foodstuff of animal origin will not reach a level of significance, no analytical methods are required for the determination of Na 5-NG, Na p-NP and Na o-NP residues in matrices of animal origin.
Adequate HPLC-MS/MS methods with column switching are available to monitor residues of Na 5- NG, Na o-NP and Na p-NP in soil with LOQ of 0.01 mg/kg; in drinking, surface and ground water with a LOQ of 0.1µg/L and in air with a LOQ of 1.25µg/m3 for each individual compound.
Analytical methods for the determination of residues in body fluids and tissues are not required as Na 5-NG, Na o-NP and Na p-NP are not classified as toxic or highly toxic.

According to the EFSA Journal 2015;13(12):4356:
An analytical method using HPLC-MS/MS was validated for the monitoring of sodium 5-nitroguaiacolate, sodium o-phenolate and sodium p-nitrophenolate with, for each compound, a limit of quantification (LOQ) of 0.01 mg/kg in high water content and high oil content commodities (EFSA, 2008). This method is supported by an independent laboratory validation (ILV) and a confirmatory method was not deemed necessary.
During the Member States consultation, EURLs indicated to EFSA that a single residue method was validated for enforcement of sodium 5-nitroguaiacolate, sodium o-phenolate and sodium p-nitrophenolate in dry commodities. However, as this statement is not supported by data, it cannot be evaluated in the present assessment.
Hence analytical methods for monitoring of sodium 5-nitroguaiacolate, sodium o-nitrophenolate and sodium p-nitrophenolate in high acid content commodities, dry commodities and hops (dried) are still required.

To address the data gap related to validated analytical enforcement method, the validation data of a method for the determination of sodium nitrocompounds in high acid content commodities, dry commodities and hops (dried) was submitted. The data gap was considered satisfactorily addressed (please refer EFSA Journal 2020;18(3):6060).

<u>Body fluids and tissues</u>
According to the SANTE/2020/12830: "*Analytical methods for monitoring residues in body fluids and tissues are required for detection of active substances and/or metabolites in humans and animals after possible intoxications or for biomonitoring purposes, regardless of their toxicological classification."*
Therefore, an analytical method for the residues of Na 5-NG, Na o-NP and Na p-NP in body fluids and tissues is required. Applicant has been requested by the zRMS to submit the additional analytical method.

Applicant submitted new analytical method for determination of residues of Na 5-NG, Na o-NP and Na p-NP in body fluids and tissues (Guserle, R., 2020 (P 5263 G (433-001)) – "*Development and validation of a method for the determination of 5-nitroguaiacol, ortho-nitrophenol and para-nitrophenol expressed as sodium 5-nitroguaiacolate, sodium ortho-nitrophenolate and sodium para-nitrophenolate, respectively, in blood, urine and meat matrix".*
The limit of quantification was established at 0.1 mg/kg for meat and 0.05 mg/L for blood and urine, but according to the SANTE/2020/12830, Rev.1, 24. February 2021, the LOQ should be lower - 0.01 mg/L for body fluids and 0.01 mg/kg for body tissues.

In our opinion, it is necessary to supply the method for determining the residues of Na 5-NG, Na o-NP and Na p-NP in body fluids and tissues with lower LOQ of 0.01 mg/L at the renewal of the active substance and/or re-evaluation of plant production product.

<u>Nectar</u>
At the request of the zRMS, the Applicant submitted a new nectar residue study in the framework of this application. Analytical method has been validated to determine of residues of sodium 5-nitroguaiacolate, sodium o-nitrophenolate and sodium p-nitrophenolate in nectar by Kugel (2020). The analytical method is acceptable according to SANCO/3029/99 rev. 4 and fits for purpose the requirements of SANTE/2020/12830, Rev.1 for determination of Na 5-NG, Na o-NP and Na p-NP in nectar.

For the detailed description please see Appendix 2.

Sufficiently sensitive and selective analytical methods are available for the active substances sodium 5-nitroguaiacolate (Na 5-NG), sodium *ortho*-nitrophenolate (Na *o*-NP) and sodium *para*-nitrophenolate (Na *p*-NP) ~~and relevant impurities~~ in the plant protection product.

Data gap:
- an analytical method for the determination relevant impurities specified for sodium o-nitrophenolate and sodium p-nitrophenolate.

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

Commodity/crop	Supported / Not supported
Cereals / wheat	Supported
Root crops / sugar beet	Supported
Pulses & oliseeds / oilseed rape	Supported
The representative formulated product for the evaluation was 'Atonik', a soluble concentrate (SL) containing 1 g/l Na 5-NG 2 g/l Na o-NP and 3 g/l Na p-NP. The EFSA peer review concluded that, at the authorised application rates, significant residues are not expected in edible parts of the investigated crops. In sugar beet leaves, however, two major compounds remain unidentified and further information on their possible structure was requested by EFSA. Meanwhile, in the absence of this information, the residue definition in sugar beet leaves is deemed tentative (EFSA, 2009, 2015, 2020)	
Rotational crops	Studies not available and not required as residues not expected in rotational crops (DT90 = 7.5 days _ 100 days = trigger value) (EFSA, 2009)
Processed commodities	Studies not available and not required as a no-residue situation is expected in fruit crops, root crops and pulses/oilseeds (EFSA, 2015)

Data gap:
- an analytical method for the determination of sodium 5-nitroguaiacolate, sodium o-nitrophenolate and sodium p-nitrophenolate in body fluids with lower LOQ of 0.01 mg/L is required according to SANTE/2020/12830, Rev.1, 24. February 2021 and should be provided at the renewal of the active substances and/or re-evaluation of plant production product.

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)

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

Methods of Analysis
Analytical methods for the active substance (Annex IIA, point 4.1)

Report:	IIIA 5.2.1/01, Lien, T.P. (2008)
Title:	Development and Validation of an Analytical Method for Determination of the Content of Sodium 5-nitroguaiacolate, Sodium ortho-nitrophenolate and Sodium para-nitrophenolate in the Formulation ATONIK PLUS 1.8%. eurofins-GAB GmbH, Niefern, Germany
Document No:	S08-02059
Guidelines:	SANCO/3030/99 rev.4
GLP	Yes

	Sodium 5-nitroguaiacolate	**Sodium *o*-nitrophenolate**	**Sodium *p*-nitrophenolate**
Technical as (principle of the method)	HPLC-UV	HPLC-UV	HPLC-UV
Impurities in technical as (principle of the method)	HPLC-UV	HPLC-UV	HPLC-UV
Plant protection product (principle of the method)	HPLC-UV	HPLC-UV	HPLC-UV

Residue definitions for monitoring purposes

		Sodium 5-nitroguaiacolate	**Sodium *o*-nitrophenolate**	**Sodium *p*-nitrophenolate**
Food of plant origin		5-nitroguaiacole	*o*-nitrophenol	*p*-nitrophenol
Food of animal origin		Not necessary because no MRL needs to be set for products of animal origin.	Not necessary because no MRL needs to be set for products of animal origin.	Not necessary because no MRL needs to be set for products of animal origin.
Soil		5-nitroguaiacole	*o*-nitrophenol	*p*-nitrophenol
Water	surface	5-nitroguaiacole	*o*-nitrophenol	*p*-nitrophenol
	drinking/ground	5-nitroguaiacole	*o*-nitrophenol	*p*-nitrophenol
Air		5-nitroguaiacole	*o*-nitrophenol	*p*-nitrophenol
Body fluids		5-nitroguaiacole	*o*-nitrophenol	*p*-nitrophenol

No new studies submitted within a frame of this application. Validated analytical method for the determination of the active substances in the plant protection product conducted on ASAHI MAX has previously been reviewed by zRMS Greece and is provided in support of this assessment in Appendix 2 (A 2.1.2.1).

Conclusion

No interferences likely to affect the chromatographic peaks of o-Nitrophenol, p-nitrophenol and 5-nitroguaiacol have been found.

The detector response to o-Nitrophenol, p-nitrophenol and 5-nitroguaiacol have been found to be linear in the appropriate ranges injected with determination coefficient, r^2, >0.999 for each compound.

Accuracy and repeatability of the method for each compound was acceptable with recovery values ranging from 100.2 to 102.8% and precision (RSD) from 0.31 to 0.32%. All values are within the acceptance criteria listed in Sanco/3030/99.

The method has been successfully validated for the analysis of sodium 5-nitroguaiacolate, sodium ortho-nitrophenolate and sodium para-nitrophenolate in ATONIK PLUS and shows that each compound can be measured in the presence of the other two active ingredients.

Comments of zRMS Greece to the RR Part B5 Southern Zone assessment, 05.09.2013:	Method principle and validation data are sufficiently described. HPLC-UV method for the determination of sodium nitrocompounds (sodium 5-nitroguaiacolate, sodium o-nitrophenolate and sodium p-nitrophenolate) in the formulation ATONIK PLUS is acceptable. The study is acceptable and analytical method suitable for the determination of fluopicolide and fluoxastrobin in the formulation and complies with SANCO/3030/99 rev. 4.
Comments of zRMS PL	PL agrees with the assessment of zRMS Greece.

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

No methods are available for the determination of relevant impurities in ASAHI MAX. There are relevant impurities specified for sodium *o*-nitrophenolate and sodium *p*-nitrophenolate in technical material but these are unlikely to be formed during storage of the formulation and therefore the maximum concentration in the technical material is not expected to increase and does not need to be monitored. Furthermore, the maximum concentration of any relevant impurity is 0.32 g/kg or 0.032%, w/w (2,6 dinitrophenol in sodium *o*-nitrophenolate) and would be at very low concentrations in ASAHI MAX. For this example with sodium *o*-nitrophenolate being at 0.2% w/w in ASAHI MAX, the relevant impurity in ASAHI MAX would be at a maximum of 0.00032 x 0.6 = 0.000192%, w/w. The development and validation of methods for formulated material with such low analytical determination limits would be technically challenging and are considered not necessary.

zRMS PL comments:

Monitoring methods for all relevant impurities are required, regardless if they form during storage or not in the formulation.
Analytical method need to be provided to determine the content of the relevant impurities specified for sodium o-nitrophenolate and sodium p-nitrophenolate in the PPP in accordance with the data requirements set out in Reg. (EU) 284/2013. The method should be validated in accordance with SANCO/3030/99 rev. 5 (including confirmation of impurity identity) at a level appropriate to the maximum impurity content in the PPP.

Applicant has been requested by the ZRMS PL for additional clarification.

Applicant: The Netherlands (CTGb) had been established as the Rapporteur member state for AIR of nitrophenolate compounds in EU. At the time of submission of the supplementary dossier the method/study for determination of impurities was not available, and therefore has not yet been submitted to Ctgb of RMS Netherlands during the EU Active substance renewal. The method will be submitted within the ongoing EU renewal during CTGb data-call. According to last information from CTGb: "*due to a high workload the concept RAR will not be finalized before Q3 2023*".

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

No new studies submitted within a frame of this application. No methods are required for co-formulants or components of co-formulants.

5.2.1.4 Applicability of existing CIPAC methods (KCP 5.1.1)

No CIPAC method available.

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 sodium 5-nitroguaiacolate, sodium o-nitrophenolate and sodium p-nitrophenolate for the generation of pre-

authorization data is given in the following table.

Table 5.2-1: Validated methods for the generation of pre-authorization data

Component of residue definition: Sodium 5-Nitroguaiacolate, Sodium *o*-Nitrophenolate and Sodium *p*-Nitrophenolate				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
Tomato (high water), oilseed rape (high oil), sugar beet (sugar beet leaves and tops)	Primary	LOQ 0.01mg/kg	HPLC-MS/MS validated for each sodium nitrocompound	Krainz A., 2004 / EU agreed
Soil (toxicology, ecotoxicology)	Primary	LOQ 0.01mg/kg	HPLC-MS/MS validated for each sodium nitrocompound	Tribolet R, 2004/ EU agreed
Drinking water, ground water, surface water (toxicology, environmental fate)	Primary	LOQ 0.1 µg /L	HPLC-MS/MS validated for each sodium nitrocompound	Tribolet R, 2004/ EU agreed
Air (toxicology)	Primary	LOQ 1.25 µg /m^3	HPLC-MS/MS validated for each sodium nitrocompound	Krainz A., 2004 / EU agreed

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

Table 5.3-1: Validated methods for the generation of post-authorization data

Component of residue definition: Sodium 5-Nitroguaiacolate, Sodium *o*-Nitrophenolate and Sodium *p*-Nitrophenolate				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
Strawberry (high acid matrix), cucumber (high water), split peas (dry/high starch matrix), sunflower (high oil)	Primary	LOQ 0.01 mg/kg	HPLC-MS/MS validated for each sodium nitrocompound	Lefresne S., 2013 / EU agreed
Hop (dried)	Primary	LOQ 0.1 mg/kg for dried hops	HPLC-MS/MS validated for each sodium nitrocompound	Lefresne S., 2013 / EU agreed

5.3.1 Analysis of the plant protection product (KCP 5.2)

No new studies submitted within a frame of this application.

5.3.2 Description of analytical methods for the determination of residues of sodium 5-nitroguaiacolate, sodium o-nitrophenolate and sodium p-nitrophenolate (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.

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 sodium 5-nitroguaiacolate, sodium o-nitrophenolate and sodium p-nitrophenolate in plant matrices is given in the following tables.

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

Component of residue definition: Sodium 5-Nitroguaiacolate, Sodium *o*-Nitrophenolate and Sodium *p*-Nitrophenolate				
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 validated for each sodium nitrocompound	Krainz A., 2004 / EU agreed Lefresne S., 2013 / EU agreed*
	ILV	0.01 mg/kg		Maldonado Ribeiro Lopez N., 2005a / EU agreed Taoudi M., 2016 / EU agreed**
High acid content	Primary	0.01 mg/kg	HPLC-MS/MS validated for each sodium nitrocompound	Lefresne S., 2013 / EU agreed*
	ILV	0.01 mg/kg		Taoudi M., 2016 / EU agreed**
High oil content	Primary	0.01 mg/kg	HPLC-MS/MS validated for each sodium nitrocompound	Krainz A., 2004 / EU agreed Lefresne S., 2013 / EU agreed
	ILV	0.01 mg/kg		Maldonado Ribeiro Lopez N., 2005b / EU agreed Taoudi M., 2016 / EU agreed**
High protein/high	Primary	0.01 mg/kg	HPLC-MS/MS validated	Lefresne S., 2013 / EU agreed*

Component of residue definition: Sodium 5-Nitroguaiacolate, Sodium *o*-Nitrophenolate and Sodium *p*-Nitrophenolate				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing / EU agreed**
starch content (dry)	ILV	0.01 mg/kg	for each sodium nitrocompound	Taoudi M., 2016 / EU agreed**
Difficult (if required, depends on intended use)	Primary	0.01 mg/kg	HPLC-MS/MS validated for each sodium nitrocompound	Lefresne S., 2013 / EU agreed*
	ILV	0.01 mg/kg		Taoudi M., 2016 / EU agreed**

* + ** Final samples were analyzed by highly specific LC-MS/MS technique, monitoring two mass transitions (MRMs) for all analytes.

These studies have been evaluated in the Evaluation Report prepared under Art8 of Regulation (EC) No 396/2005 by the NL (May 2020) and the analytical methods have been considered acceptable. The conclusion of this assessment is presented in EFSA REASONED OPINION EFSA Journal 2020;18(3):6060

Table 5.3-3: Statement on extraction efficiency

	Method for products of plant origin
Not required, because:	residues expected ≤ LOQ

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

EFSA is of the opinion that MRLs for nitrocompounds in animal commodities are currently not required. Nevertheless, it is highlighted that this conclusion does not take into consideration the possible intake of sugar beet leaves for which uncertainties on the nature and magnitude of residues were identified in section 1. Therefore, when authorising a GAP on sugar beet, Member States are recommended to recalculate the dietary burden following submission of the missing data for sugar beet leaves and re-assess the need to establish MRLs in livestock commodities EFSA 2015).
The previous assessment of residues in livestock (EFSA, 2015) is still valid (EFSA 2020).

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 Sodium 5-Nitroguaiacolate, Sodium o-Nitrophenolate and Sodium p-Nitrophenolate in soil is given in the following table.

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

Component of residue definition: Sodium 5-Nitroguaiacolate, Sodium *o*-Nitrophenolate and Sodium *p*-Nitrophenolate			
Method type	**Method LOQ**	**Principle of method (*i.e.* GC-MS or HPLC-UV)**	**Author(s), year**
Primary	0.01 mg/kg	HPLC-MS/MS validated for each sodium nitrocompound	Tribolet R, 2004/EU agreed Egron C.. 2016*
ILV	0.01 mg/kg	HPLC-MS/MS validated for each sodium nitrocompound	Lefresne S. 2014a* Egron C.. 2016*

*data submitted within the Confirmatory data in 2017 but not yet evaluated

zRMS comments:

The analytical method has been independently validated for the determination of 5-nitroguaiacol, ortho-nitrophenol and para-nitrophenol in soil in accordance to the guidance document SANTE/2020/12830, Rev.1, 24. February 2021 with the LOQ of 0.01 mg/kg.
The details of the evaluation of additional study of Egron C., 2016 are referred in 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 Sodium 5-Nitroguaiacolate, Sodium o-Nitrophenolate and Sodium p-Nitrophenolate in surface, ground and drinking water is given in the following tables.

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

Component of residue definition: Sodium 5-Nitroguaiacolate, Sodium *o*-Nitrophenolate and Sodium *p*-Nitrophenolate				
Matrix type	**Method type**	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year**
Drinking water	Primary	LOQ 0.1 µg /L	HPLC-MS/MS validated for each sodium nitrocompound	Tribolet R, 2004/EU agreed
	ILV	LOQ 0.1 µg /L		Lefresne S. 2014*
Surface water	Primary	LOQ 0.1 µg /L	HPLC-MS/MS validated for each sodium nitrocompound	Tribolet R, 2004/EU agreed
	ILV	LOQ 0.1 µg /L		Lefresne S. 2014*
Ground water	Primary	LOQ 0.1 µg /L	HPLC-MS/MS validated for each sodium nitrocompound	Tribolet R, 2004/EU agreed Lefresne S. 2014*

*data submitted within the Confirmatory data in 2017 but not yet evaluated

zRMS comments:

The analytical method has been independently validated for the determination of 5-nitroguaiacol, ortho-nitrophenol and para-nitrophenol in surface water in accordance to the guidance document SANTE/2020/12830, Rev.1, 24. February 2021 with the LOQ of 0.1 µg/L.
The details of the evaluation of additional study of Lefresne S. 2014 are referred in 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 Sodium 5-Nitroguaiacolate, Sodium o-Nitrophenolate and Sodium p-Nitrophenolate in air is given in the following tables.

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

Component of residue definition: Sodium 5-Nitroguaiacolate, Sodium *o*-Nitrophenolate and Sodium *p*-Nitrophenolate			
Method type	**Method LOQ**	**Principle of method (i.e. GC-MS or HPLC-UV)**	**Author(s), year / missing**
Primary	LOQ 1.25 µg /m^3	HPLC-MS/MS validated for each sodium nitrocompound	Krainz A., 2004 / EU agreed
Confirmatory	Not required		
Independent laboratory validation	Not required		

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

Analytical methods for the determination of residues in body fluids and tissues are not required as Na 5-NG, Na *o*-NP and Na *p*-NP are not classified as toxic or highly toxic (EFSA 2008).

zRMS comments:

According to the EFSA 2008 analytical methods for body fluids and tissues for Na 5-NG, Na o-NP and Na p-NP are not required.
In Regulation (EU) No 283/2013 it is stated that "…methods, with a full description, shall be submitted for the analysis in body fluids and tissues for the active substance and relevant metabolites" and this is a requirement of SANTE/2020/12830. According to the SANTE/2020/12830: "*Analytical methods for monitoring residues in body fluids and tissues are required for detection of active substances and/or metabolites in humans and animals after possible intoxications or for biomonitoring purposes, regardless of their toxicological classification."*

Therefore, an analytical method for the residues of Na 5-NG, Na o-NP and Na p-NP in body fluids and tissues is required. Applicant has been requested by the zRMS to submit the additional analytical method.

Applicant submitted new analytical method for determination of residues of Na 5-NG, Na o-NP and Na p-NP in body fluids and tissues (Guserle, R., 2020 (P 5263 G (433-001)) – "*Development and validation of a method for the determination of 5-nitroguaiacol, ortho-nitrophenol and para-nitrophenol expressed as sodium 5-nitroguaiacolate, sodium ortho-nitrophenolate and sodium para-nitrophenolate, respectively, in blood, urine and meat matrix".*
The limit of quantification was established at 0.1 mg/kg for meat and 0.05 mg/L for blood and urine, but according to the SANTE/2020/12830, Rev.1, 24. February 2021, the LOQ should be lower - 0.01 mg/L for body fluids and 0.01 mg/kg for body tissues.

In our opinion, it is necessary to supply the method for determining the residues of Na 5-NG, Na o-NP and Na p-NP in body fluids and tissues with lower LOQ=0.01 mg/L at the renewal of the active substance and/or re-evaluation of plant production product.

The details of the evaluation of new and additional studies are referred in Appendix 2.

5.3.2.8 Other studies/ information

~~No new studies submitted within a frame of this application.~~
Analytical method for the determination of sodium 5-nitroguaiacolate, sodium *o*-nitrophenolate and sodium *p*-nitrophenolate in nectar by Kugel (2020) was submitted in support of the evaluation.

Samples of nectar were extracted with acetonitrile and water (2:8). After centrifugation quantification was performed by use of LC-MS/MS detection.
The stability was demonstrated for 5-Nitroguaiacol, o-Nitrophenol and p-Nitrophenol expressed as Sodium 5-Nitroguaiacolate, Sodium o-Nitrophenolate and Sodium p-Nitrophenolate in nectar upon storage at ≤ -18 °C in the dark for at least 232 days.
The limit of quantification (LOQ) of the analytical method for the determination of Sodium 5-Nitroguaiacolate, Sodium o-Nitrophenolate or Sodium p-Nitrophenolate is 0.01 mg/kg with a limit of detection (LOD) set at ≤ 0.003 mg/kg (30% of the LOQ).

The analytical method is considered valid and acceptable according to SANCO/3029/99 rev. 4 for determination of Na 5 NG, Na o NP and Na p NP in nectar.
In addition, based on the study results, the analytical method also fits for purpose the requirements of SANTE/2020/12830, Rev.1 for determination of Na 5 NG, Na o NP and Na p NP in nectar.
For the detailed description please see Appendix 2.

zRMS comments:

At the request of the zRMS, the Applicant submitted a new nectar residue study in the framework of this application. The analytical method has been successfully validated for the determination of 5-nitroguaiacol, ortho-nitrophenol and para-nitrophenol in nectar with the LOQ of 0.01 mg/kg for each analyte and meets all criteria of guidelines SANTE/2020/12830, Rev.1, 24. February 2021.
The details of the evaluation of new study are referred in Appendix 2.

5.3.2.9 Overall conclusion

Monitoring analytical methods for determination of residues of Na 5-NG, Na *o*-NP and Na *p*-NP in plant matrices, soil, water and air in compliance with SANCO/825/00 rev. 8.1 are available and are described in Tab. 5.3-9 (see below). Plant matrices were extracted by agitation with acidified acetonitrile and a second time with acetonitrile. Soil samples were extracted with methanol / water (80/20) and the pH of the extracts was adjusted with hydrochloric acid. After clean up by SPE, analysis was carried out by HPLC-MS/MS. Water samples were diluted with methanol and the pH adjusted with hydrochloric acid. Further clean-up was conducted using SPE and analysis was performed by LC-MS/MS. Impinger solutions were treated with methanol and the pH adjusted with hydrochloric acid. Samples were further cleaned-up by SPE and analysed by LC-MS/MS. Body fluids and tissues were extracted with acidified acetonitrile after addition of water and further cleaned-up following the Quechers procedure. Analysis was performed using LC-MS/MS.

Table 5.3-9: Overall summary of validation data for analytical methods to be used for enforcement for determination of Na 5-NG, Na o-NP and Na p-NP residues in plant matrices, soil, water and air

Matrix	Method	Fortification level	Recovery rate [%]		%RSD	LOQ	Reference
			Range	Mean			
Strawberry Na 5-NG	Primary	0.01 mg/kg	86 - 98	92	6	0.01 mg/kg	Lefresne S., 2013 / EU agreed
	Primary	0.1 mg/kg	82 - 87	86	3		
	Confirmatory	0.01 mg/kg	87 - 96	92	4		
	Confirmatory	0.1 mg/kg	79 - 86	85	4		
Strawberry Na *o*-NP	Primary	0.01 mg/kg	72 - 99	84	12	0.01 mg/kg	Lefresne S., 2013 / EU agreed
	Primary	0.1 mg/kg	77 - 110	88	15		
	Confirmatory	0.01 mg/kg	66 - 97	85	15		
	Confirmatory	0.1 mg/kg	82 - 108	92	11		
Strawberry Na *p*-NP	Primary	0.01 mg/kg	85 - 100	90	7	0.01 mg/kg	Lefresne S., 2013 / EU agreed
	Primary	0.1 mg/kg	90 - 96	93	2		
	Confirmatory	0.01 mg/kg	77 - 102	89	11		
	Confirmatory	0.1 mg/kg	83 - 95	88	6		
Cucumber Na 5-NG	Primary	0.01 mg/kg	69 - 79	73	5	0.01 mg/kg	Lefresne S., 2013 / EU agreed
	Primary	0.1 mg/kg	80 - 90	86	5		
	Confirmatory	0.01 mg/kg	66 - 79	72	7		
	Confirmatory	0.1 mg/kg	80 - 91	85	5		
Cucumber Na *o*-NP	Primary	0.01 mg/kg	81 - 93	88	5	0.01 mg/kg	Lefresne S., 2013 / EU agreed
	Primary	0.1 mg/kg	87 - 99	91	5		
	Confirmatory	0.01 mg/kg	84 - 102	90	8		
	Confirmatory	0.1 mg/kg	93 - 104	96	5		

Matrix	Method	Fortification level	Recovery rate [%]		%RSD	LOQ	Reference
			Range	Mean			
Cucumber Na *p*-NP	Primary	0.01 mg/kg	67 - 82	75	8	0.01 mg/kg	Lefresne S., 2013 / EU agreed
	Primary	0.1 mg/kg	74 - 87	83	6		
	Confirmatory	0.01 mg/kg	71 - 97	82	12		
	Confirmatory	0.1 mg/kg	76 - 89	83	7		
Split peas Na 5-NG	Primary	0.01 mg/kg	72 - 84	76	6	0.01 mg/kg	Lefresne S., 2013 / EU agreed
	Primary	0.1 mg/kg	66 - 77	70	6		
	Confirmatory	0.01 mg/kg	67 - 82	72	8		
	Confirmatory	0.1 mg/kg	67 - 75	70	5		
Split peas Na *o*-NP	Primary	0.01 mg/kg	72 - 84	78	6	0.01 mg/kg	Lefresne S., 2013 / EU agreed
	Primary	0.1 mg/kg	66 - 79	72	8		
	Confirmatory	0.01 mg/kg	67 - 94	82	13		
	Confirmatory	0.1 mg/kg	71 - 81	76	6		
Split peas Na *p*-NP	Primary	0.01 mg/kg	72 - 80	74	5	0.01 mg/kg	Lefresne S., 2013 / EU agreed
	Primary	0.1 mg/kg	67 - 77	71	5		
	Confirmatory	0.01 mg/kg	67 - 82	76	8		
	Confirmatory	0.1 mg/kg	73 - 82	76	4		
Sunflower seeds Na 5-NG	Primary	0.01 mg/kg	102 - 113	106	4	0.01 mg/kg	Lefresne S., 2013 / EU agreed
	Primary	0.1 mg/kg	96 - 105	101	3		
	Confirmatory	0.01 mg/kg	105 - 114	109	4		
	Confirmatory	0.1 mg/kg	99 - 106	103	3		
Sunflower seeds Na *o*-NP	Primary	0.01 mg/kg	78 - 87	83	5	0.01 mg/kg	Lefresne S., 2013 / EU agreed
	Primary	0.1 mg/kg	73 - 83	80	5		
	Confirmatory	0.01 mg/kg	82 - 103	93	11		
	Confirmatory	0.1 mg/kg	70 - 89	80	11		
Sunflower seeds Na *p*-NP	Primary	0.01 mg/kg	95 - 100	98	3	0.01 mg/kg	Lefresne S., 2013 / EU agreed
	Primary	0.1 mg/kg	82 - 91	87	4		
	Confirmatory	0.01 mg/kg	84 - 97	91	6		
	Confirmatory	0.1 mg/kg	78 - 88	82	5		
Hops Na 5-NG	Primary	0.10 mg/kg	76 - 93	84	8	0.10 mg/kg	Lefresne S., 2013 / EU agreed
	Primary	1.0 mg/kg	93 - 94	94	0.4		
	Confirmatory	0.10 mg/kg	77 - 93	84	8		

Matrix	Method	Fortification level	Recovery rate [%]		%RSD	LOQ	Reference
			Range	Mean			
	Confirmatory	1.0 mg/kg	94 - 97	95	1		
Hops Na *o*-NP	Primary	0.10 mg/kg	71 - 85	79	8	0.10 mg/kg	Lefresne S., 2013 / EU agreed
	Primary	1.0 mg/kg	86 - 91	89	3		
	Confirmatory	0.10 mg/kg	65 - 87	75	11		
	Confirmatory	1.0 mg/kg	83 - 92	87	4		
Hops Na *p*-NP	Primary	0.10 mg/kg	67 - 81	73	8	0.10 mg/kg	Lefresne S., 2013 / EU agreed
	Primary	1.0 mg/kg	94 - 98	97	2		
	Confirmatory	0.10 mg/kg	65 - 85	74	11		
	Confirmatory	1.0 mg/kg	95 - 101	99	2		
Strawberry Na 5-NG	Primary - ILV	0.01 mg/kg	95.4 - 112	106	5.4	0.01 mg/kg	Taoudi M., 2016 / EU agreed
	Primary - ILV	0.1 mg/kg	91.8 - 104	97.9	5.0		
	Confirmatory	0.01 mg/kg	96.6 - 110	102	4.9		
	Confirmatory	0.1 mg/kg	88.8 - 104	95.1	5.4		
Strawberry Na *o*-NP	Primary - ILV	0.01 mg/kg	94.2 - 111	103	6.9	0.01 mg/kg	Taoudi M., 2016 / EU agreed
	Primary - ILV	0.1 mg/kg	104 - 110	108	2.1		
	Confirmatory	0.01 mg/kg	95.4 - 112	102	5.6		
	Confirmatory	0.1 mg/kg	99.0 - 110	104	3.9		
Strawberry Na *p*-NP	Primary - ILV	0.01 mg/kg	93.6 - 106	101	4.5	0.01 mg/kg	Taoudi M., 2016 / EU agreed
	Primary - ILV	0.1 mg/kg	69.0 – 100.8	82.7	13.5		
	Confirmatory	0.01 mg/kg	96.6 - 118	106	7.1		
	Confirmatory	0.1 mg/kg	63.9 – 96.6	82.9	12.9		
Split peas Na 5-NG	Primary - ILV	0.01 mg/kg	61.8 – 82.2	73.1	9.0	0.01 mg/kg	Taoudi M., 2016 / EU agreed
	Primary - ILV	0.1 mg/kg	56.4 – 89.7	81.9	17.2		
	Confirmatory	0.01 mg/kg	60.6 – 89.4	72.9	13.9		
	Confirmatory	0.1 mg/kg	57.3 – 88.2	78.8	15.3		
Split peas Na *o*-NP	Primary - ILV	0.01 mg/kg	58.4 – 82.8	72.0	12.1	0.01 mg/kg	Taoudi M., 2016 / EU agreed
	Primary - ILV	0.1 mg/kg	78.9 – 91.8	84.7	5.8		
	Confirmatory	0.01 mg/kg	57.0 – 81.6	69.7	12.3		
	Confirmatory	0.1 mg/kg	80.1 – 92.4	85.1	5.4		
Split peas Na *p*-NP	Primary - ILV	0.01 mg/kg	54.4 – 80.4	69.6	12.9	0.01 mg/kg	Taoudi M., 2016 / EU agreed
	Primary - ILV	0.1 mg/kg	61.2 – 78.9	73.2	9.5		

Matrix	Method	Fortification level	Recovery rate [%]		%RSD	LOQ	Reference
			Range	Mean			
	Confirmatory	0.01 mg/kg	53.8 – 84.6	72.4	~~53.8 – 84.6~~		
	Confirmatory	0.1 mg/kg	63.6 – 83.1	75.7	~~63.6 – 83.1~~		
Hops Na 5-NG	Primary - ILV	0.10 mg/kg	99.9 - 112	104	4.5	0.10 mg/kg	Taoudi M., 2016 / EU agreed
	Primary - ILV	1.0 mg/kg	83.0 - 112	97.9	10.2		
	Confirmatory	0.10 mg/kg	98.4 - 108	102	3.2		
	Confirmatory	1.0 mg/kg	84.3 - 108	97.5	8.3		
Hops Na *o*-NP	Primary - ILV	0.10 mg/kg	96.6 - 109	101	4.8	0.10 mg/kg	Taoudi M., 2016 / EU agreed
	Primary - ILV	1.0 mg/kg	89.7 – 99.6	94.8	4.0		
	Confirmatory	0.10 mg/kg	93.6 - 123	106	9.6		
	Confirmatory	1.0 mg/kg	83.7 - 116	101	11.0		
Hops Na *p*-NP	Primary - ILV	0.30 mg/kg	103 - 115	106	4.4	0.10 mg/kg	Taoudi M., 2016 / EU agreed
	Primary - ILV	0.30 mg/kg	84.0 - 107	97.3	8.2		
	Confirmatory	3.0 mg/kg	95.6 - 122	105	9.1		
	Confirmatory	3.0 mg/kg	93.0 - 118	101	8.9		
Soil Na 5-NG	Primary	0.01 mg/kg	73.2 – 96.6	83.1	10.6	0.01 mg/kg	Tribolet R, 2004 / EU agreed
	Primary	0.1 mg/kg	70.3 – 80.7	75.3	5.2		
	Primary	0.01 mg/kg	75 – 83	78	4	0.01 mg/kg	Egron C.. 2014
	Primary	0.1 mg/kg	65 – 84	77	10		
	Confirmatory	0.01 mg/kg	74 – 82	78			
	Confirmatory	0.1 mg/kg	66 – 86	78			
Soil Na *o*-NP	Primary	0.01 mg/kg	79.0 – 107.1	95.6	11.4	0.01 mg/kg	Tribolet R, 2004 / EU agreed
	Primary	0.1 mg/kg	82.7 – 89.4	85.9	3.1		
	Primary	0.01 mg/kg	76 – 82	78	3	0.01 mg/kg	Egron C.. 2014
	Primary	0.1 mg/kg	64 – 83	74	10		
	Confirmatory	0.01 mg/kg	80 – 103	89	10		
	Confirmatory	0.1 mg/kg	68 – 83	76	8		
Soil Na *p*-NP	Primary	0.01 mg/kg	73.1 – 86.6	81.1	6.7	0.01 mg/kg	Tribolet R, 2004 / EU agreed
	Primary	0.1 mg/kg	69.5 – 80.7	74.5	5.4		
	Primary	0.01 mg/kg	73 – 89	79	8	0.01 mg/kg	Egron C.. 2014
	Primary	0.1 mg/kg	78 – 97	91	9		

Matrix	Method	Fortification level	Recovery rate [%]		%RSD	LOQ	Reference
			Range	Mean			
	Confirmatory	0.01 mg/kg	76 – 92	82	8		
	Confirmatory	0.1 mg/kg	80 – 98	91	8		
Drinking water Na 5-NG	Primary	0.1 µg/L	72.7 – 94.2	85.1	9.5	0.1 µg/L	Tribolet R, 2004 / EU agreed
	Primary	1.0 µg/L	80.6 – 93.3	89.4	5.6		
Drinking water Na *o*-NP	Primary	0.1 µg/L	80.2 – 102.0	93.3	8.7	0.1 µg/L	Tribolet R, 2004 / EU agreed
	Primary	1.0 µg/L	81.9 – 97.8	92.2	6.5		
Drinking water Na *p*-NP	Primary	0.1 µg/L	81.5 – 91.7	89.0	4.8	0.1 µg/L	Tribolet R, 2004 / EU agreed
	Primary	1.0 µg/L	80.5 – 93.1	90.3	6.1		
Ground water Na 5-NG	Primary	0.1 µg/L	74.6 – 93.8	85.5	11.5	0.1 µg/L	Tribolet R, 2004 / EU agreed
	Primary	1.0 µg/L	82.7 – 101.9	89.0	9.7		
Ground water Na *o*-NP	Primary	0.1 µg/L	76.9 – 89.3	84.2	7.3	0.1 µg/L	Tribolet R, 2004 / EU agreed
	Primary	1.0 µg/L	86.2 – 105.1	92.1	10.1		
Ground water Na *p*-NP	Primary	0.1 µg/L	76.8 – 95.3	86.7	9.7	0.1 µg/L	Tribolet R, 2004 / EU agreed
	Primary	1.0 µg/L	80.4 – 97.5	86.8	8.8		
Surface water Na 5-NG	Primary	0.1 µg/L	78.0 – 105.0	90.0	13.7	0.1 µg/L	Tribolet R, 2004 / EU agreed
	Primary	1.0 µg/L	77.9 – 97.6	86.5	10.3		
Surface water Na *o*-NP	Primary	0.1 µg/L	85.7 – 100.5	97.3	8.0	0.1 µg/L	Tribolet R, 2004 / EU agreed
	Primary	1.0 µg/L	79.6 – 98.2	87.5	9.5		
Surface water Na *p*-NP	Primary	0.1 µg/L	81.5 – 96.1	89.6	7.2	0.1 µg/L	Tribolet R, 2004 / EU agreed
	Primary	1.0 µg/L	77.3 – 93.3	84.9	9.1		
Surface water Na 5-NG	Primary – ILV	0.1 µg/L	73 – 93	83	9	0.1 µg/L	Lefresne S. 2014
	Primary – ILV	1.0 µg/L	69 – 98	84	16		
	Confirmatory	0.1 µg/L	76 – 98	86	9		
	Confirmatory	1.0 µg/L	68 – 97	84	16		
Surface water Na *o*-NP	Primary – ILV	0.1 µg/L	91 – 114	105	9	0.1 µg/L	Lefresne S. 2014
	Primary – ILV	1.0 µg/L	83 – 90	86	4		
	Confirmatory	0.1 µg/L	86 – 108	94	9		
	Confirmatory	1.0 µg/L	77 – 95	84	9		
Surface water Na *p*-NP	Primary – ILV	0.1 µg/L	79 – 100	86	11	0.1 µg/L	Lefresne S. 2014
	Primary – ILV	1.0 µg/L	82 – 103	93	10		

Matrix	Method	Fortification level	Recovery rate [%]		%RSD	LOQ	Reference
			Range	Mean			
	Confirmatory	0.1 µg/L	76 – 100	83	12		
	Confirmatory	1.0 µg/L	74 – 100	88	12		
Air Na 5-NG	Primary (20°C, ~ 45% rH)	1.41 µg/m^3	87.8 – 92.0	89.4	2.0	1.41 µg/m^3	Krainz A., 2004 / EU agreed
	Primary (20°C, ~ 45% rH)	14.1 µg/m^3	97.8 – 111.5	106.7	5.0		
	Primary (37°C, > 80% rH)	1.41 µg/m^3	97.7 – 107.2	101.3	4.1		
	Primary (37°C, > 80% rH)	14.1 µg/m^3	101.6 – 105.0	103.6	1.5		
Air Na *o*-NP	Primary (20°C, ~ 45% rH)	1.45 µg/m^3	88.1 – 93.1	87.4	4.2	1.45 µg/m^3	Krainz A., 2004 / EU agreed
	Primary (20°C, ~ 45% rH)	14.5 µg/m^3	89.0 – 100.5	97.2	4.7		
	Primary (37°C, > 80% rH)	1.45 µg/m^3	102.6 – 105.3	103.5	1.2		
	Primary (37°C, > 80% rH)	14.5 µg/m^3	96.2 – 102.1	100.1	2.4		
Air Na *p*-NP	Primary (20°C, ~ 45% rH)	1.45 µg/m^3	86.2 – 98.2	89.9	5.4	1.45 µg/m^3	Krainz A., 2004 / EU agreed
	Primary (20°C, ~ 45% rH)	14.5 µg/m^3	95.9 – 111.4	106.3	5.9		
	Primary (37°C, > 80% rH)	1.45 µg/m^3	100.8 – 104.6	102.1	1.8		
	Primary (37°C, > 80% rH)	14.5 µg/m^3	102.9 – 105.9	104.8	1.1		

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** **Company Report No.** **Source (where different from company)** **GLP or GEP status** **Published or not**	Vertebrate study Y/N	Owner
KCP 5.1.2/01	Lien, T.P.	2008	Development and Validation of an Analytical Method for Determination of the Content of Sodium 5-nitroguaiacolate, Sodium ortho-nitrophenolate and Sodium para-nitrophenolate in the Formulation ATONIK PLUS 1.8% eurofins-GAB GmbH, Niefern, Germany ALS Report No. S08-02059 GLP, Unpublished	N	Asahi
KCP 5.1.2/02	Krainz, A	2004	Development and Validation of a Residue Analytical Method for Sodium 5-Nitroguaiacolate, Sodium o-Nitrophenolate and Sodium p-Nitrophenolate (as Active Ingredients in Atonik Formulated Product) in Tomato (Fruits), Sugar Beet (Roots and Tops with Leaves) and Oil Seed Rape Report No 850917 RCC Ltd, Switzerland GLP Unpublished	N	Asahi Chemical Europe s.r.o.
KCP 5.1.2/03	Tribolet R	2004	Development and validation of a residue analytical method for sodium 5-nitroguaiacolate, sodium *o*-nitrophenolate and sodium *p*-nitrophenolate (as active ingredients in Atonik formulated product) in soil Report No 815343 RCC Ltd, Switzerland GLP Unpublished	N	Asahi Chemical Europe s.r.o.
KCP 5.1.2/04	Tribolet R	2004	Development and Validation of a Residue Analytical Method for Sodium 5-Nitroguaiacolate, Sodium *o*-Nitrophenolate and Sodium *p*-Nitrophenolate (as active Ingredients in Atonik formulated Product) in Drinking, Ground and Surface Water Report No 815321 RCC Ltd, Switzerland GLP Unpublished	N	Asahi Chemical Europe s.r.o.
KCP 5.1.2/05	Krainz, A	2004	Development and Validation of a Residue Analytical Method for Sodium 5-Nitroguaiacolate, Sodium *o*-Nitrophenolate and Sodium *p*-Nitrophenolate (as active Ingredients in Atonik formulated Product) in Air Report No 815332 RCC Ltd, Switzerland GLP Unpublished	N	Asahi Chemical Europe s.r.o.

Data point	Author(s)	Year	**Title** **Company Report No.** **Source (where different from company)** **GLP or GEP status** **Published or not**	Vertebrate study Y/N	Owner
KCP 5.1.2/06	Lefresne, S.	2013	Validation of the analytical method for the determination of sodium 5-nitroguaiacolate, sodium o-nitrophenolate and sodium p-nitrophenolate residues in plants (strawberry, cucumber, split peas, sunflower seeds and hops) GIRPA France Report No. ARYST-VAL-13.01 GLP Unpublished	N	Asahi Chemical Europe s.r.o.
KCP 5.1.2/07	Maldonado Ribeiro Lopez N	2005a	Independent Laboratory Validation of a residue analytical method for Na o-nitrophenolate, Na p-nitrophenolate and Na 5-nitroguaiacolate in tomato fruits samples. Bioagri Laboratorios. Final report PE-2254.034.076.03 GLP Unpublished	N	Asahi Chemical Europe s.r.o.
KCP 5.1.2/08	Taoudi, M.	2016	Independent Laboratory Validation study for study ARYST-VAL-13-1'Validation of the analytical method for the determination of sodium 5-nitroguaiacolate, sodium o-nitrophenolate and sodium p-nitrophenolate residues in plants (strawberry, cucumber, split peas, sunflower seeds and hops) GIRPA France Report No. ARYST-VAL-13.1' Battelle Ltd. UK Report No. MD/15/01 GLP Unpublished	N	Asahi Chemical Europe s.r.o.
KCP 5.1.2/09	Maldonado Ribeiro Lopez N	2005b	Independent laboratory validation of a residue analytical method for sodium ortho-nitrophenolate, sodium para-nitrophenolate and 5-nitroguaiacolate in tomato fruits samples Bioagri Laboratorios. Final report PE-2254.034.077.03 GLP Unpublished	N	Asahi Chemical Europe s.r.o.
KCP 5.1.2/10	Egron, C.	2014	Independent Laboratory Validation (ILV) of the analytical method RCC Study number 815343 for the determination of sodium 5-nitroguaiacolate, sodium o-nitrophenolate and sodium p-nitrophenolate in soil GIRPA France Report No. B13-A1-NOP-31 GLP Unpublished	N	Asahi Chemical Europe s.r.o.
KCP 5.1.2/11	Lefresne, S.	2014	Independent Laboratory Validation (ILV) of the analytical method RCC Study number 815321 for the determination of sodium 5-nitroguaiacolate, sodium o-nitrophenolate and sodium p-nitrophenolate in surface water GIRPA France Report No. B13-A1-NOP-32 GLP Unpublished	N	Asahi Chemical Europe s.r.o.

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
CA 4.2 (d)/01	Guserle, R.	2020	Development and validation of a method for the determination of 5-nitroguaiacol, ortho-nitrophenol and para-nitrophenol expressed as sodium 5-nitroguaiacolate, sodium ortho-nitrophenolate and sodium para-nitrophenolate, respectively, in blood, urine and meat matrix EAG Laboratories GmbH, Ulm, Germany Laboratory report P 5263 G (433-001) GLP Unpublished	N	Asahi Chemical Europe s.r.o.
KCP 5.2	Kugel, D.	2020	Determination of Residues of 5-Nitroguaiacol, *o*-Nitrophenol and *p*-Nitrophenol in Nectar after four Applications of ATONIK containing Sodium 5-Nitroguaiacolate, Sodium *o*-Nitrophenolate and Sodium *p*-Nitrophenolate in *Phacelia tanacetifolia* at 4 Sites in Central and Southern Europe in 2019.Company Report No S19-03993 (634-96002) Eurofins Agroscience Services Ecotox GmbH, Niefern-Öschelbronn, Germany GLP, Unpublished	N	Asahi Chemical Europe r.s.o.

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

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
KCP 5.1.2/02	Krainz, A	2004	Development and Validation of a Residue Analytical Method for Sodium 5-Nitroguaiacolate, Sodium o-Nitrophenolate and Sodium p-Nitrophenolate (as Active Ingredients in Atonik Formulated Product) in Tomato (Fruits), Sugar Beet (Roots and Tops with Leaves) and Oil Seed Rape Report No 815343 RCC Ltd, Switzerland GLP Unpublished	N	Asahi Chemical Europe s.r.o.
KCP 5.1.2/03	Tribolet R	2004	Development and validation of a residue analytical method for sodium 5-nitroguaiacolate, sodium *o*-nitrophenolate and sodium *p*-nitrophenolate (as active ingredients in Atonik formulated product) in soil Report No 850917 RCC Ltd, Switzerland GLP Unpublished	N	Asahi Chemical Europe s.r.o.
KCP 5.1.2/04	Tribolet R	2004	Development and Validation of a Residue Analytical Method for Sodium 5-Nitroguaiacolate, Sodium *o*-Nitrophenolate and Sodium *p*-Nitrophenolate (as active Ingredients in Atonik formulated Product) in Drinking, Ground and Surface Water	N	Asahi Chemical Europe s.r.o.

Data point	Author(s)	Year	**Title** **Company Report No.** **Source (where different from company)** **GLP or GEP status** **Published or not**	**Vertebrate study Y/N**	**Owner**
			Report No 815321 RCC Ltd, Switzerland GLP Unpublished		
KCP 5.1.2/05	Krainz, A	2004	Development and Validation of a Residue Analytical Method for Sodium 5-Nitroguaiacolate, Sodium *o*-Nitrophenolate and Sodium *p*-Nitrophenolate (as active Ingredients in Atonik formulated Product) in Air Report No 815332 RCC Ltd, Switzerland GLP Unpublished	N	Asahi Chemical Europe s.r.o.
KCP 5.1.2/07	Maldonado Ribeiro Lopez N	2005a	Independent Laboratory Validation of a residue analytical method for Na o-nitrophenolate, Na p-nitrophenolate and Na 5-nitroguaiacolate in tomato fruits samples. Bioagri Laboratorios. Final report PE-2254.034.076.03 GLP Unpublished	N	Asahi Chemical Europe s.r.o.
KCP 5.1.2/09	Maldonado Ribeiro Lopez N	2005b	Independent laboratory validation of a residue analytical method for sodium ortho-nitrophenolate, sodium para-nitrophenolate and 5-nitroguaiacolate in tomato fruits samples Bioagri Laboratorios. Final report PE-2254.034.077.03 GLP Unpublished	N	Asahi Chemical Europe s.r.o.

List of data submitted by the applicant and not relied on

Data point	Author(s)	Year	**Title** **Company Report No.** **Source (where different from company)** **GLP or GEP status** **Published or not**	**Vertebrate study Y/N**	**Owner**
-	-	-	-	-	-

List of data relied on not submitted by the applicant but necessary for evaluation

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
-	-	-	-	-	-

Appendix 2 Detailed evaluation of submitted analytical methods

A 2.1 Analytical methods for Sodium 5-Nitroguaiacolate, Sodium *o*-Nitrophenolate and Sodium *p*-Nitrophenolate

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

No new or additional studies have been submitted.

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 the active substance in the plant protection product (KCP 5.2)

Method plus validation

The following validated analytical method for the determination of the active substances in the plant protection product conducted on ATONIK PLUS has previously been reviewed by zRMS Greece in 2013 and was confirmed to be sufficient and acceptable.

Report:	IIIA 5.2.1/01, Lien, T.P. (2008)
Title:	Development and Validation of an Analytical Method for Determination of the Content of Sodium 5-nitroguaiacolate, Sodium ortho-nitrophenolate and Sodium para-nitrophenolate in the Formulation ATONIK PLUS 1.8%. eurofins-GAB GmbH, Niefern, Germany
Document No:	S08-02059
Guidelines:	SANCO/3030/99 rev.4
GLP	Yes

Principle of the method

Active ingredient contents of ATONIK PLUS 1.8% (0.3% 5NG, 0.6% oNP, 0.9% pNP) are determined by high performance liquid chromatography after dissolution of 500 mg test sample in a mixture of 10% phosphoric acid : methanol (approximately 1:49, v/v) followed by addition of water to a known aliquot to give a 1:1 final solution of acidic methanol : water. The separation of phenols is achieved using reverse phase chromatography with ultraviolet detection and external standardisation. Quantities of phenols present are expressed as the respective sodium salt by multiplying the measured values of free compound by 1.158 for the *o*- and *p*-nitrophenol, and by 1.130 for the 5-nitroguaiacol.

The method was validated using ATONIK PLUS 1.8% (Batch number 039D8) and water as a blank formulation for spiking purposes.

The method was validated for specificity, linearity, accuracy and precision.

Validation Findings

Recovery (accuracy)

Samples of blank formulation (water) were spiked at two levels with a mixture of each sodium nitro compound and prepared according to the specified method. Each sample was injected twice. The results for each compound are shown in Table 5.2.1-1 to Table 5.2.1-3.

Table 5.2.1-1: Recovery of sodium 5-nitroguaiacolate

Determinations	Fortification level of Na 5-nitroguaiacolate (mg/L / % w/w)	Na 5-nitroguaiacolate measured (mg/L / % w/w)	Na 5-nitroguaiacolate recovery (%)
1 (Low 1)	20.2 mg/L / 0.199%	20.3 mg/L / 0.200%	100.5
		20.2 mg/L / 0.199%	100.0
2 (Low 2)	20.2 mg/L / 0.196%	20.2 mg/L / 0.196%	100.0
		20.2 mg/L / 0.196%	100.0
3 (Low 3)	20.2 mg/L / 0.198%	20.3 mg/L / 0.199%	100.5
		20.3 mg/L / 0.199%	100.5
Mean		-	**100.3**
RSD (%)		-	**0.27***

4 (High 1)	40.2 mg/L / 0.386%	40.3 mg/L / 0.385%	100.2
		40.3 mg/L / 0.385%	100.2
5 (High 2)	40.2 mg/L / 0.388%	40.4 mg/L / 0.390%	100.5
		40.4 mg/L / 0.390%	100.5
6 (High 3)	40.2 mg/L / 0.386%	40.3 mg/L / 0.387%	100.2
		40.5 mg/L / 0.389%	100.7
Mean		-	**100.4**
RSD (%)		-	**0.21****
Overall Mean			**100.3**
Overall RSD (%)			**0.22%**

* RSD is below calculated acceptable Horwitz RSDr of 3.4 % for a concentration of 0.2%
** RSD is below calculated acceptable Horwitz RSDr of 3.1 % for a concentration of 0.4%

Table 5.2.1-2: Recovery of sodium ortho-nitrophenolate

Determinations	**Fortification level of sodium o-nitrophenolate (mg/L / % w/w)**	**Sodium o-nitrophenolate measured (mg/L / % w/w)**	**Sodium o-nitrophenolate recovery (%)**
1 (Low 1)	40.2 mg/L / 0.396%	41.3 mg/L / 0.407%	102.7
		41.3 mg/L / 0.407%	102.7
2 (Low 2)	40.2 mg/L / 0.390%	41.3 mg/L / 0.401%	102.7
		41.2 mg/L / 0.400%	102.5
3 (Low 3)	40.2 mg/L / 0.394%	41.4 mg/L / 0.406%	103.0
		41.4 mg/L / 0.406%	103.0
Mean		-	102.8
RSD (%)		-	0.19*
4 (High 1)	80.4 mg/L / 0.767%	81.5 mg/L / 0.778%	101.4
		81.5 mg/L / 0.778%	101.4
5 (High 2)	80.4 mg/L / 0.776%	81.5mg/L / 0.789%	101.4
		81.4 mg/L / 0.786%	101.2
6 (High 3)	80.4 mg/L / 0.772%	81.4 mg/L / 0.781%	101.2
		81.9 mg/L / 0.786%	101.9
Mean		-	101.4
RSD (%)		-	0.25**
Overall Mean			102.1
Overall RSD (%)			0.72%

* RSD is below calculated acceptable Horwitz RSDr of 3.1 % for a concentration of 0.4%
** RSD is below calculated acceptable Horwitz RSDr of 2.8 % for a concentration of 0.8%

Table 5.2.1-3: Recovery of sodium para-nitrophenolate

Determinations	**Fortification level of sodium p-nitrophenolate (mg/L / % w/w)**	**Sodium p-nitrophenolate measured (mg/L / % w/w)**	**Sodium p-nitrophenolate recovery (%)**
1 (Low 1)	60.0 mg/L / 0.592%	60.4 mg/L / 0.596%	100.7
		60.3 mg/L / 0.595%	100.5
2 (Low 2)	60.0 mg/L / 0.583%	60.2 mg/L / 0.584%	100.3
		60.3 mg/L / 0.585%	100.5
3 (Low 3)	60.0 mg/L / 0.588%	60.4 mg/L / 0.592%	100.7
		60.4 mg/L / 0.592%	100.7
Mean			**100.6**
RSD (%)			**0.16***
4 (High 1)	120.0 mg/L / 1.145%	120.1 mg/L / 1.146%	100.1
		120.0 mg/L / 1.145%	100.0
5 (High 2)	120.0 mg/L / 1.158%	120.4 mg/L / 1.162%	100.3
		120.2 mg/L/ 1.160%	100.2
6 (High 3)	120.0 mg/L / 1.152%	120.0 mg/L / 1.152%	100.0
		120.6 mg/L / 1.157%	100.5
Mean		**-**	**100.2**
RSD (%)		**-**	**0.19****
Overall Mean			**100.4**
Overall RSD (%)			**0.26%**

* RSD is below calculated acceptable Horwitz RSDr of 2.9 % for a concentration of 0.6%
** RSD is below calculated acceptable Horwitz RSDr of 2.6 % for a concentration of 1.15%

Specificity
Sodium 5-nitroguaiacolate, sodium ortho-nitrophenolate and sodium para-nitrophenolate (as the free phenols in the HPLC system) were identified in the injected samples by comparison with the corresponding certified reference standards.
The extent of potential interference was investigated by preparing final extracts from blank formulation (water) and analysing these under the HPLC conditions. There were no background peaks or components observed confirming that there is no interference for the measurement of the sodium nitro compounds and that the three active ingredients can be resolved.
Example chromatograms are included in the study report.

Linearity
Calibration curves were produced for each compound using standards over suitable ranges for each of Sodium 5-nitroguaiacolate, sodium ortho-nitrophenolate and sodium para-nitrophenolate. Linearity was tested over a low and high range for each compound. Calibration curves were provided in the report.

Sodium 5-nitroguaiacolate (measured as 5-nitroguaiacol)
Detector response to 5-Nitroguaiacol is found to be linear in the range 2.0 to 30.2 ng injected (low range) with a determination coefficient (r^2) of 0.9997.
Detector response to 5-Nitroguaiacol is found to be linear in the range 30.2 to 503.0 ng injected (high range) with a determination coefficient (r^2) of 0.9998.

Sodium ortho-nitrophenolate (measured as o-nitrophenol)
Detector response to o-nitrophenol is found to be linear in the range 3.0 to 60.4 ng injected (low range) with a determination coefficient (r^2) of 0.9994.
Detector response to 5-Nitroguaiacol is found to be linear in the range 60.4 to 1006.0 ng injected (high range) with a determination coefficient (r^2) of 0.9995.

Sodium para-nitrophenolate (measured as p-nitrophenol)
Detector response to p-nitrophenol is found to be linear in the range 6.0 to 90.1 ng injected (low range) with a determination coefficient (r^2) of 0.9996.
Detector response to 5-Nitroguaiacol is found to be linear in the range 90.1 to 1501.0 ng injected (high range) with a determination coefficient (r^2) of 0.9997.

Repeatability (precision)
Six separate samples of ATONIK PLUS 1.8% were weighed out at the nominal level (500 mg) and prepared according to the specified method. Each sample was injected twice. The results for each compound are shown in Table 5.2.1-4 to Table 5.2.1-6.

Table 5.2.1-4: Precision of sodium 5-nitroguaiacolate in ATONIK PLUS 1.8%

Determinations	Weight of ATONIK PLUS 1.8% (mg/ 50 mL)	5-nitroguaiacol measured (mg/L)	Factor (F)	Na 5-nitroguaiacolate (mg/L)	Na 5-nitroguaiacolate content (%)
1	509.6	28.6	1.130	32.3	0.317
		28.7		32.4	0.318
2	500.2	28.1		31.8	0.318
		28.1		31.8	0.318
3	210.1	28.7		32.4	0.318
		28.8		32.5	0.319
4	508.1	28.6		32.3	0.318
		28.6		32.3	0.318
5	505.1	28.4		32.1	0.318
		28.5		32.2	0.319
6	503.0	28.2		31.9	0.317
		28.3		32.0	0.318
Mean					**0.318**
RSD (%)					**0.31***

* RSD is below calculated acceptable Horwitz RSDr of 3.2 % for a concentration of 0.318%

Table 5.2.1-5: Precision of sodium ortho-nitrophenolate in ATONIK PLUS 1.8%

Determinations	Weight of ATONIK PLUS 1.8% (mg/ 50 mL)	o-nitrophenol measured (mg/L)	Factor (F)	Na o-nitrophenolate (mg/L)	Na o-nitrophenolate content (%)
1	509.6	57.6	1.158	66.7	0.654
		57.6		66.7	0.654
2	500.2	56.5		65.4	0.654
		56.5		65.4	0.654
3	210.1	57.5		66.6	0.653
		57.7		66.8	0.655
4	508.1	57.2		66.2	0.651
		57.0		66.0	0.649
5	505.1	56.8		65.8	0.651
		56.9		65.9	0.652
6	503.0	56.6		65.5	0.651
		56.7		65.7	0.653
Mean					**0.653**
RSD (%)					**0.31***

* RSD is below calculated acceptable Horwitz RSDr of 2.9 % for a concentration of 0.318%

Table 5.2.1-6: Precision of sodium para-nitrophenolate in ATONIK PLUS 1.8%

Determinations	Weight of ATONIK PLUS 1.8% (mg/ 50 mL)	p-nitrophenol measured (mg/L)	Factor (F)	Na p-nitrophenolate (mg/L)	Na p-nitrophenolate content (%)
1	509.6	82.7	1.158	95.8	0.940
		82.7		95.8	0.940
2	500.2	81.0		93.8	0.938
		81.2		94.0	0.940
3	210.1	82.7		95.8	0.939
		83.1		96.2	0.943
4	508.1	82.6		95.7	0.942
		82.5		95.5	0.940
5	505.1	81.9		94.8	0.938
		82.3		95.3	0.943
6	503.0	81.2		94.0	0.934
		81.5		94.4	0.938
Mean					**0.940**
RSD (%)					**0.32***

* RSD is below calculated acceptable Horwitz RSDr of 2.7 % for a concentration of 0.318%

Conclusion

No interferences likely to affect the chromatographic peaks of o-Nitrophenol, p-nitrophenol and 5-nitroguaiacol have been found.

The detector response to o-Nitrophenol, p-nitrophenol and 5-nitroguaiacol have been found to be linear in the appropriate ranges injected with determination coefficient, r^2, >0.999 for each compound.

Accuracy and repeatability of the method for each compound was acceptable with recovery values ranging from 100.2 to 102.8% and precision (RSD) from 0.31 to 0.32%. All values are within the acceptance criteria listed in Sanco/3030/99.

The method has been successfully validated for the analysis of sodium 5-nitroguaiacolate, sodium ortho-nitrophenolate and sodium para-nitrophenolate in ATONIK PLUS and shows that each compound can be measured in the presence of the other two active ingredients.

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

No new or additional studies have been submitted.

A 2.1.2.3 Description of analytical methods for the determination of residues in animal matrices (KCP 5.2)

No new or additional studies have been submitted.

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

Complete validation data according to SANCO/825/00 rev. 8.1 for the method are included in the study by Egron, C. (2016)

Comments of zRMS:	The analytical method has been independently validated for the determination of 5-nitroguaiacol, ortho-nitrophenol and para-nitrophenol in soil in accordance to the guidance documents SANCO/825/00, rev. 8.1 with the LOQ of 0.01 mg/kg. Quantification was performed by use of LC-MS/MS detection. All mean recoveries were in the range of 70 – 110% with relative standard deviations of ≤20% for all analytes at each level. The method meets all criteria of guidelines SANTE/2020/12830, Rev.1, 24. February 2021 to determine concentrations of 5-nitroguaiacol, ortho-nitrophenol and para-nitrophenol in soil at the LOQ level of 0.01 mg/kg. The method is acceptable.

Author(s) (year):	Egron, C. (2016)
Title:	Independent laboratory validation (ilv) of the analytical method RCC study number 815343 for the determination of sodium 5-nitroguaiacolate, sodium o-nitrophenolate and sodium p-nitrophenolate in soil
Laboratory report / project Number (Doc. No.):	B13-A1-NOP-31
Testing facility:	FREDON Pays de la Loire / GIRPA, Beaucouze Cedex, France
Published:	No
Test guideline used:	SANCO/825/00, rev. 8.1 (2010)
Deviations:	None
Previous evaluation:	No, not previously submitted
GLP:	Yes; certified by Groupe Interministeriel des Produits Chimiques, Paris
Acceptability/Reliability:	Yes

Dates of experimental work: 27 November 2013 to 22 February 2014.

Principle of the method
Soil samples were extracted with methanol/water (80/20). The extract was filtered over celite and diluted with water. The extract was treated with sodium hydroxide and concentrated under reduced pressure. After adjusting the pH to 2 with hydrochloric acid, the extract was purified on an Isolut ENV$^+$ cartridge. The eluate was diluted with water and methanol containing 0.2% formic acid. Quantification was carried out using HPLC-MS/MS.

Table 5.4-1: Chromatographic conditions – Tribolet, R. (2004)

HPLC-system	HPLC-MS/MS
Column 1	Inertsil Phenyl; 5 µm (60 mm x 2mm)
Mobile phase (isocratic):	Methanol / water (1/1) + 0.1% formic acid
Column 2	Inertsil ODS-3, 3 µm (150 x 2.1 mm)
Mobile phase (isocratic):	Methanol / water (8/2) + 0.1% formic acid
Column switch:	1.5 – 2.3 min
Column temperature:	28°C
Monitored ions	168 → 153 for 5-NG 138 → 108 for *o*-NP 138 → 108 for *p*-NP
Retention time	~ 5.0 minutes: 5-NG ~ 5.7 minutes: *o*-NP ~ 5.1 minutes: *p*-NP

Table 5.4-2: Chromatographic conditions – Doc. No. B13-A1-NOP-31

HPLC-system	HPLC-MS/MS
Column 1	Pursuit XRs Diphenyl (100 mm x 2 mm ID; PD 3 µm)

Column 2	Prodigy C18 (150 mm x 2 mm ID; PD 3 µm)
Mobile phase (isocratic):	A: Water / acetic acid (100/0.1) + 5 mM ammonium acetate B: Methanol / acetic acid (100/0.1) + 5 mM ammonium acetate A:B = 1/1
Column switch:	3.75 – 8.5 min
Monitored ions	168 → 153 and 168 → 123 for 5-NG 138 → 108 and 138 → 92 for *o*-NP 138 → 108 and 138 → 92 for *p*-NP
Retention time	~ 12.2 – 12.4 minutes: 5-NG ~ 15.8 – 16.1 minutes: *o*-NP ~ 12.8 – 12.9 minutes: *p*-NP

Validation

Specificity

The analysis of blank samples in comparison with the analysis of standard solutions and spiked samples showed no significant interference (i.e. < 30% LOQ) at the retention time of the analytes.

Linearity

The linearity of the method was demonstrated using calibration standards in methanol/water (1/1) containing 1% formic acid (n = 9, Tribolet, R. 2004) or matrix-matched standards (n = 7, B13-A1-NOP-31). Linear calibration functions were calculated by regression analysis. The coefficients of determination R^2 obtained were > 0.99. Please refer to the following tables.

Table 5.4-3: Linearity in soil – Tribolet, R. (2004)

Analyte and transition	Calibration range*	Equation	R^2 **
Na 5-NG; 168 → 153	0.005 – 0.2 µg/mL	$y = 3287830*x^{1/0.9881}$	0.9986
Na *o*-NP; 138 → 108	0.005 – 0.2 µg/mL	$y = 348546*x^{1.0054}$	0.9985
Na *p*-NP; 138 → 108	0.005 – 0.2 µg/mL	$y = 5006863*x^{0.9921}$	0.9992

* concentrations are expressed as the phenol forms

** the regression coefficients r would be > 0.999 for all analytes if linear calibration had been used

Table 5.4-4: Linearity in soil – Doc. No. B13-A1-NOP-31

Analyte and transition	Calibration range*	Equation	R^2
Na 5-NG; 168 → 153	3 – 50 µg/L (0.003 – 0.25 mg/kg)	y = 215649.96x + 102154.91	0.9940
Na 5-NG; 168 → 123		y = 35566.40x + 18457.09	0.9928
Na *o*-NP; 138 → 108	3 – 30 µg/L (0.003 – 0.15 mg/kg)	y = 17727.23x + 1545.6	0.9976
Na *o*-NP; 138 → 92		y = 1799.70x + 668.8	0.9960
Na *p*-NP; 138 → 108	3 – 50 µg/L (0.003 – 0.25 mg/kg)	y = 104183.36x + 127031.25	0.9999
Na *p*-NP; 138 → 92		y = 10864.50x + 12945.93	0.9996

* concentrations are expressed as the phenol forms

Accuracy and precision

The recovery rates and relative standard deviations obtained from all fortified samples are shown in the following Tables.

Table 5.4-5: Accuracy and precision – Tribolet, R. (2004)

Matrix and transition	Fortification level [mg/kg] 1)	Recovery range [%]	Mean recovery [%]	RSD [%]
Na 5-NG				
Soil *m/z* 168→153	0.01 (n = 5)	73.2 – 96.6	83.1	10.6
	0.1 (n = 5)	70.3 – 80.7	75.3	5.2
	Overall (n = 10)	70.3 – 96.6	79.2	9.6
Na *o*-NP				
Soil *m/z* 138→108	0.01 (n = 5)	79.0 – 107.1	95.6	11.4
	0.1 (n = 5)	82.7 – 89.4	85.9	3.1
	Overall (n = 10)	79.0 – 107.1	90.7	9.9
Na *p*-NP				
Soil *m/z* 138→108	0.01 (n = 5)	73.1 – 86.6	81.1	6.7
	0.1 (n = 5)	69.5 – 80.7	74.5	5.4
	Overall (n = 10)	69.5 – 86.6	77.8	7.3

1) fortification levels are expressed as the phenol forms of the analytes

Table 5.4-6: Accuracy and precision – Doc. No. B13-A1-NOP-31

Matrix and transition	Fortification level [mg/kg] 1)	Recovery range [%]	Mean recovery [%]	RSD [%]
Na 5-NG				
Soil *m/z* 168→153	0.01 (n = 5)	75 – 83	78	4
	0.1 (n = 5)	65 – 84	77	10
	Overall (n = 10)	65 – 84	78	7
Soil *m/z* 168→123	0.01 (n = 5)	74 – 82	78	4
	0.1 (n = 5)	66 – 86	78	10
	Overall (n = 10)	66 – 86	78	7
Na *o*-NP				
Soil *m/z* 138→108	0.01 (n = 5)	76 – 82	78	3
	0.1 (n = 5)	64 – 83	74	10
	Overall (n = 10)	64 – 83	76	7
Soil *m/z* 138→92	0.01 (n = 5)	80 – 103	89	10
	0.1 (n = 5)	68 – 83	76	8
	Overall (n = 10)	68 – 103	82	12
Na *p*-NP				
Soil *m/z* 138→108	0.01 (n = 5)	73 – 89	79	8
	0.1 (n = 5)	78 – 97	91	9
	Overall (n = 10)	73 – 97	85	11
Soil *m/z* 138→92	0.01 (n = 5)	76 – 92	82	8
	0.1 (n = 5)	80 – 98	91	8
	Overall (n = 10)	76 – 98	87	9

1) fortification levels are expressed as the phenol forms of the analytes

The mean recovery at each fortification level as well as the overall mean recovery was in the range of 70 – 110% with the relative standard deviation below 20% for each analyte. Analysis of control samples showed no significant interference (< 30% LOQ) with the determination of the analytes.

Limit of quantification (LOQ) and limit of detection (LOD)
The lowest fortification level with acceptable mean recovery and precision was 0.01 mg/kg for each analyte (expressed as their phenol forms).
The LOD was defined as 30% of the LOQ in the study by Egron, C. (2016), i.e. 0.003 mg/kg for each analyte (expressed as their phenol forms).

Matrix effects
Matrix effects were assessed in the study by Egron, C. (2016), by comparison of calibration graphs obtained from standard solutions in solvent and matrix-matched standards and were found to be non-significant for all analytes.

Conclusion
The method is valid and acceptable according to SANCO/825/00 rev. 8.1 for the determination of Na 5-NG, Na *o*-NP and Na *p*-NP in soil.

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

The independent method validation to the study by Tribolet, R. (2004), Doc. No. 815321 was conducted in surface water, not in drinking water. As surface water is a more difficult matrix compared to drinking water, this is considered acceptable.

Comments of zRMS:	The analytical method has been independently validated for the determination of 5-nitroguaiacol, ortho-nitrophenol and para-nitrophenol in surface water in accordance to the guidance document SANCO/825/00, rev. 8.1 with the LOQ of 0.1 µg/L. Quantification was performed by use of LC-MS/MS detection. All mean recoveries were in the range of 70 – 110% with relative standard deviations of ≤20% for all analytes at each level. The method meets all criteria of guidelines SANCO/825/00 rev. 8.1 and SANTE/2020/12830, Rev.1, 24. February 2021 to determine concentrations of 5-

	nitroguaiacol, ortho-nitrophenol and para-nitrophenol in water at the LOQ level of 0.1 µg/L. According to the SANTE/2020/12830, Rev.1, 24. February 2021: *Provided that a method has been successfully validated for surface water at the LOQ required for drinking water (≤0.1 µg/L), no separate validation in drinking water is required.* The method is acceptable.

Author(s) (year):	Lefresne, S. (2014)
Title:	Independent laboratory validation (ilv) of the analytical method RCC Study number 815321 for the determination of sodium 5-nitroguaiacolate, sodium o-nitrophenolate and sodium p-nitrophenolate in surface water
Laboratory report / project Number (Doc. No.):	B13-A1-NOP-32 (435-002)
Testing facility:	FREDON Pays de la Loire / GIRPA, Beaucouze Cedex, France
Published:	No
Test guideline used:	SANCO/825/00, rev. 8.1 (2010)
Deviations:	None
Previous evaluation:	No, not previously submitted
GLP:	Yes; certified by Groupe Interministeriel des Produits Chimiques, Paris
Acceptability/Reliability:	Yes

Dates of experimental work: 27 November 2013 to 28 November 2013.

Principle of the method

There were no deviations from the primary method.

Table 5.5-2: Chromatographic conditions

HPLC-system	HPLC-MS/MS
Column 1	Pursuit XRs Diphenyl (100 mm x 2 mm ID; PD 3 µm)
Column 2	Prodigy C18 (150 mm x 2 mm ID; PD 3 µm)
Mobile phase (isocratic):	A: Water / acetic acid (100/0.1) + 5 mM ammonium acetate B: Methanol / acetic acid (100/0.1) + 5 mM ammonium acetate A/B = 1/1
Column switch:	3.75 – 8.5 min
Monitored ions	168 → 153 and 168 → 123 for 5-NG 138 → 108 and 138 → 92 for *o*-NP 138 → 108 and 138 → 92 for *p*-NP
Retention time	~ 13.4 – 14.2 minutes: 5-NG ~ 18.9 – 20.4 minutes: *o*-NP ~ 13.8 – 15.2 minutes: *p*-NP

Validation

Specificity

The analysis of blank samples in comparison with the analysis of standard solutions and spiked samples showed no significant interference (i.e. < 30% LOQ) at the retention time of the analytes.

Linearity

The linearity of the method was demonstrated using calibration standards in methanol / water (1/1) containing 0.1% formic acid (n = 9). Linear calibration functions were calculated by regression analysis. The coefficients of determination R^2 obtained were > 0.99. Please refer to the following table.

Table 4.2 (b) - 7: Linearity

Analyte	Calibration range*	Equation	R^2
Na 5-NG; 168 → 153	3 – 25 µg/L (0.03 – 0.25 µg/L)	y = 111775.37x + 12717.98	0.9904
Na 5-NG; 168 → 123		y = 201986.07x – 6392.57	0.9960
Na *o*-NP; 138 → 108		y = 8929.81x + 3214.43	0.9933
Na *o*-NP; 138 → 92		y = 915.24x + 593.84	0.9906
Na *p*-NP; 138 → 108		y = 58302.98x + 10013.56	0.9956
Na *p*-NP; 138 → 92		y = 6166.86x – 371.54	0.9923

* concentrations are expressed as the phenol forms

Accuracy and precision
The recovery rates and relative standard deviations obtained from all fortified samples are shown in the following Table.

Table 4.2 (b) - 8: Accuracy and precision

Matrix and transition	Fortification level [µg/L] [1)]	Recovery range [%]	Mean recovery [%]	RSD [%]
Na 5-NG				
Surface water 168 → 153	0.1 (n = 5)	73 – 93	83	9
	1.0 (n = 5)	69 – 98	84	16
	Overall (n = 10)	69 – 98	84	12
Surface water 168 → 123	0.1 (n = 5)	76 – 98	86	9
	1.0 (n = 5)	68 – 97	84	16
	Overall (n = 10)	68 – 98	85	13
Na *o*-NP				
Surface water 138 → 108	0.1 (n = 5)	91 – 114	105	9
	1.0 (n = 5)	83 – 90	86	4
	Overall (n = 10)	83 – 114	96	12
Surface water 138 → 92	0.1 (n = 5)	86 – 108	94	9
	1.0 (n = 5)	77 – 95	84	9
	Overall (n = 10)	77 – 108	89	10
Na *p*-NP				
Surface water 138 → 108	0.1 (n = 5)	79 – 100	86	11
	1.0 (n = 5)	82 – 103	93	10
	Overall (n = 10)	79 – 103	90	11
Surface water 138 → 92	0.1 (n = 5)	76 – 100	83	12
	1.0 (n = 5)	74 – 100	88	12
	Overall (n = 10)	74 – 100	85	12

1) fortification levels are expressed as the phenol forms of the analytes

The mean recovery at each fortification level as well as the overall mean recovery was in the range of 70 - 110% with the relative standard deviation below 20% for each analyte. Analysis of control samples showed no significant interference (< 30% LOQ) with the determination of the analytes.

Limit of quantification (LOQ) and limit of detection (LOD)
The lowest fortification level with acceptable mean recovery and precision was 0.1 µg/L for each analyte (expressed as their phenol forms).
The LOD was defined as one third of the LOQ, i.e. 0.033 µg/L for each analyte (expressed as their phenol forms).

Matrix effects
The sample preparation included an SPE clean-up step after which the analyte was present in methanol and the concentration of the matrix was significantly reduced. Furthermore, the mean recovery at each fortification level as well as the overall mean recovery was in the range of 70 - 110% with the relative standard deviation below 20% for each analyte and no interferences were detected in blank samples. Thus, there were no matrix effects.

Conclusion
The method is valid and acceptable according to SANCO/825/00 rev. 8.1 for the determination of Na 5-NG, Na *o*-NP and Na *p*-NP in water.

A 2.1.2.6 Description of Methods for the Analysis of Air (KCP 5.2)

No new or additional studies have been submitted

A 2.1.2.7 Description of Methods for the Analysis of Body Fluids and Tissues (KCP 5.2)

No new or additional studies have been submitted.

Comments of zRMS:	The analytical method has been successfully validated for the determination of 5-nitroguaiacol, ortho-nitrophenol and para-nitrophenol expressed as sodium 5-nitroguaiacolate, sodium ortho-nitrophenolate and sodium para-nitrophenolate, respectively, in blood, urine and meat matrix in accordance to the guidance documents SANCO/825/00, rev. 8.1 and SANCO/3029/99, rev. 4 with the LOQ of 0.1 mg/kg for meat and 0.05 mg/L for blood and urine. Quantification was performed by use of LC-MS/MS detection. All mean recoveries were in the range of 70 – 110% with relative standard deviations of ≤20% for all analytes and matrices at each level. The method meets all criteria of guidelines SANCO/825/00 rev. 8.1 to determine concentrations of 5-nitroguaiacol, ortho-nitrophenol and para-nitrophenol in body tissues and body fluid at the LOQ level of 0.1 mg/kg and 0.05 mg/L, respectively, but according to the SANTE/2020/12830, Rev.1, 24. February 2021, the LOQ should be lower - 0.01 mg/L for body fluids and 0.01 mg/kg for body tissues.

Data point addressed:	CA 4.2 (d)/01
Author(s) (year):	Guserle, R. (2020)
Title:	Development and validation of a method for the determination of 5-nitroguaiacol, ortho-nitrophenol and para-nitrophenol expressed as sodium 5-nitroguaiacolate, sodium ortho-nitrophenolate and sodium para-nitrophenolate, respectively, in blood, urine and meat matrix
Laboratory report / project Number (Doc. No.):	P 5263 G (433-001)
Testing facility:	EAG Laboratories GmbH, Ulm, Germany
Published:	No
Test guideline used:	SANCO/825/00, rev. 8.1, ENV/JM/MONO(2007)17, SANCO/3029/99 rev. 4 (2000)
Deviations:	None
Previous evaluation:	No, not previously submitted
GLP:	Yes; certified by LUBW Landesanstalt für Umwelt, Messungen und Naturschutz Baden-Württemberg, Karlsruhe
Acceptability/Reliability:	Yes

Principle of the method

Samples of blood, meat and urine were extracted with acidified acetonitrile after addition of water. A salt mixture containing magnesium sulphate, sodium chloride and sodium citrate was added and the extract was shaken. After centrifugation, an aliquot of the acetonitrile phase was cleaned-up by adding primary secondary amine (PSA) material and $MgSO_4$. After further centrifugation an aliquot of the supernatant was diluted with acetonitrile/water/acetic acid (50/50/0.2, v/v/v). Quantification was carried out by HPLC-MS/MS.

Chromatographic conditions

HPLC-system	HPLC-MS/MS
Column	Phenomenex Synergi Hydro RP (100 mm x 3 mm), 2.5 µm
Pre-column	Phenomenex C_{18} 4x3 mm
Mobile phase (gradient)	A: Water + 5mM ammonium acetate + 0.1 % acetic acid B: Methanol + 5mM ammonium acetate + 0.1 % acetic acid
Column temperature:	60°C
Monitored ions	168 → 123 and 168 → 153 for 5-NG 138 → 108 and 138 → 92 for *o*-NP 138 → 108 and 138 → 92 for *p*-NP
Retention time	~ 1.4 minutes: 5-NG ~ 2.0 minutes: *o*-NP ~ 1.4 minutes: *p*-NP

Validation

Specificity

The analysis of blank samples in comparison with the analysis of standard solutions and spiked samples showed no significant interference (i.e. < 30% LOQ) at the retention time of the analytes.

Linearity

The linearity of the method was demonstrated using matrix-matched calibration standards (n = 7). Linear calibration functions were calculated by regression analysis. The correlation coefficients R obtained were > 0.99. Please refer to the following tables.

Table 4.2 (d) - 1: Linearity

Analyte and transition	Calibration range	Equation	R
Blood			
Na 5-NG; 168 → 123	0.50 – 20.0 ng/mL (0.015 – 0.60 mg/L)	$y = 2.03*10^5x – 1.11*10^4$	0.9997
Na 5-NG; 168 → 153		$y = 7.06*10^5x – 5.57*10^4$	0.9997
Na *o*-NP; 138 → 108		$y = 1.61*10^5x – 1.79*10^3$	0.9994
Na *o*-NP; 138 → 92		$y = 1.25*10^4x + 3.7$	0.9991
Na *p*-NP; 138 → 108		$y = 5.59*10^5x – 2.3*10^4$	0.9996
Na *p*-NP; 138 → 92		$y = 3.24*10^4x – 2.67*10^3$	0.9995
Urine			
Na 5-NG; 168 → 123	0.50 – 20.0 ng/mL (0.015 – 0.60 mg/L)	$y = 2.05*10^5x – 1.45*10^4$	0.9998
Na 5-NG; 168 → 153		$y = 7.06*10^5x – 4.92*10^4$	0.9998
Na *o*-NP; 138 → 108		$y = 1.58*10^5x – 1.4*10^3$	0.9997
Na *o*-NP; 138 → 92		$y = 1.24*10^4x + 53.4*10^4$	0.9999
Na *p*-NP; 138 → 108		$y = 5.42*10^5x – 4.22*10^4$	0.9997
Na *p*-NP; 138 → 92		$y = 3.17*10^4x – 3.79*10^3$	0.9998
Tissues			
Na 5-NG; 168 → 123	0.50 – 20.0 ng/mL (0.03 – 1.2 mg/kg)	$y = 3.72*10^5x – 2.42*10^4$	0.9995
Na 5-NG; 168 → 153		$y = 1.27*10^6x – 8.29*10^4$	0.9996
Na *o*-NP; 138 → 108		$y = 2.9*10^5x – 1.36*10^4$	0.9997
Na *o*-NP; 138 → 92		$y = 2.27*10^4x - 1.42*10^3$	0.9994
Na *p*-NP; 138 → 108		$y = 1.02*10^6x – 9.35*10^4$	0.9997
Na *p*-NP; 138 → 92		$y = 5.83*10^4x – 7.44*10^3$	0.9997

Accuracy and precision

The recovery rates and relative standard deviations obtained from all samples fortified with each analyte are shown in the following Table.

Table 4.2 (d) - 2: Accuracy and precision – body fluids

Matrix and transition	Fortification level [mg/L]	Recovery range [%]	Mean recovery [%]	RSD [%]
Na 5-NG				
Blood *m/z* 168→153	0.05 (n = 5)	105 – 109	107	2
	0.5 (n = 5)	100 – 110	105	4
	Overall (n = 10)	100 – 110	106	3
Blood *m/z* 168→123	0.05 (n = 5)	105 – 111	108	2
	0.5 (n = 5)	100 – 109	104	4
	Overall (n = 10)	100 – 111	106	3
Urine *m/z* 168→153	0.05 (n = 5)	105 – 110	108	2
	0.5 (n = 5)	98 – 104	101	2
	Overall (n = 10)	98 – 110	105	4
Urine *m/z* 168→123	0.05 (n = 5)	106 – 109	108	1
	0.5 (n = 5)	99 – 103	101	2
	Overall (n = 10)	99 – 109	105	4
Na *o*-NP				
Blood *m/z* 138→108	0.05 (n = 5)	104 – 110	107	2
	0.5 (n = 5)	99 – 108	105	4
	Overall (n = 10)	99 – 110	106	3
Blood *m/z* 138→92	0.05 (n = 5)	106 – 109	108	1
	0.5 (n = 5)	99 – 110	105	4
	Overall (n = 10)	99 – 110	107	3
Urine *m/z* 138→108	0.05 (n = 5)	103 – 108	105	1
	0.5 (n = 5)	93 – 100	97	3
	Overall (n = 10)	93 – 108	101	5
Urine	0.05 (n = 5)	97 – 106	102	4

m/z 138→92	0.5 (n = 5)	92 – 100	96	4
	Overall (n = 10)	92 – 106	99	5
Na *p*-NP				
Blood *m/z* 138→108	0.05 (n = 5)	104 – 106	105	1
	0.5 (n = 5)	101 – 108	105	3
	Overall (n = 10)	101 – 108	105	2
Blood *m/z* 138→92	0.05 (n = 5)	104 – 109	107	2
	0.5 (n = 5)	100 – 107	105	3
	Overall (n = 10)	100 – 109	106	3
Urine *m/z* 138→108	0.05 (n = 5)	104 – 109	107	2
	0.5 (n = 5)	98 – 103	101	2
	Overall (n = 10)	98 – 109	104	4
Urine *m/z* 138→92	0.05 (n = 5)	96 – 107	104	5
	0.5 (n = 5)	95 – 100	98	2
	Overall (n = 10)	95 – 107	101	5

Table 4.2 (d) - 3: Accuracy and precision – tissues

Matrix	Fortification level [mg/kg]	Recovery range [%]	Mean recovery [%]	RSD [%]
Na 5-NG				
Meat *m/z* 168→123	0.1 (n = 5)	105 – 110 100 - 112	108 106	2 4
	1.0 (n = 5)	98 – 104 102	101 100	2
	Overall (n = 10)	98 – 110 112	105 103	4
Meat *m/z* 168→153	0.1 (n = 5)	106 – 109 100-113	108 106	1 5
	1.0 (n = 5)	99 – 103 98-102	101 99	2
	Overall (n = 10)	99 – 103 98-113	105 103	4 5
Na *o*-NP				
Meat 138→108	0.1 (n = 5)	103 – 108 99-111	105	2 5
	1.0 (n = 5)	93 – 100 98-102	97 99	3 2
	Overall (n = 10)	93 – 108 98-111	101 102	5 4
Meat *m/z* 138→92	0.1 (n = 5)	97 – 106 101-108	102 105	4 3
	1.0 (n = 5)	92 – 100 97-101	96 99	4 2
	Overall (n = 10)	92 – 106 97-108	99 102	5 4
Na *p*-NP				
Meat 138→108	0.1 (n = 5)	104 – 109 101-111	107 106	2 4
	1.0 (n = 5)	98 – 103 97-100	101 98	2
	Overall (n = 10)	98 – 109 97-111	104 102	4 5
Meat *m/z* 138→92	0.1 (n = 5)	96 – 110 101-112	104 106	5 4
	1.0 (n = 5)	95 – 100 98-101	98 100	2
	Overall (n = 10)	95 – 110 98-112	101 103	5 4

The mean recovery at each fortification level as well as the overall mean recovery was in the range of 70 – 110% with the relative standard deviation below 20%. Analysis of control samples showed no significant interference (< 30% LOQ) with the determination of the analytes.

Limit of quantification (LOQ) and limit of detection (LOD)

The lowest fortification level with acceptable mean recovery and precision was 0.05 mg/L for body fluids and 0.1 mg/kg for tissues for all analytes (expressed as sodium salts).

The LOD was defined as 30% of the LOQ, i.e. 0.015 mg/L for body fluids and 0.03 mg/kg for tissues for all analytes (expressed as sodium salts).

Matrix effects

Matrix effects were assessed by comparison of calibration graphs from standards in solvent and matrix-matched standards and were found to be non-significant for all analytes.

Matrix	Method	Fortification level	Recovery rate [%]		%RSD	LOQ	Reference
			Range	Mean			
Blood Na 5-NG	Primary	0.05 mg/L	105 – 109	107	2	0.05 mg/L	Guserle, R. (2020), CA 4.2 (d)/01
	Primary	0.5 mg/L	100 – 110	105	4		
	Confirmatory	0.05 mg/L	105 – 111	108	2		
	Confirmatory	0.5 mg/L	100 – 109	104	4		
Urine Na 5-NG	Primary	0.05 mg/L	105 – 110	108	2	0.05 mg/L	Guserle, R. (2020), CA 4.2 (d)/01
	Primary	0.5 mg/L	98 – 104	101	2		
	Confirmatory	0.05 mg/L	106 – 109	108	1		
	Confirmatory	0.5 mg/L	99 – 103	101	2		
Blood Na *o*-NP	Primary	0.05 mg/L	104 – 110	107	2	0.05 mg/L	Guserle, R. (2020), CA 4.2 (d)/01
	Primary	0.5 mg/L	99 – 108	105	4		
	Confirmatory	0.05 mg/L	106 – 109	108	1		
	Confirmatory	0.5 mg/L	99 – 110	105	4		
Urine Na *o*-NP	Primary	0.05 mg/L	103 – 108	105	1	0.05 mg/L	Guserle, R. (2020), CA 4.2 (d)/01
	Primary	0.5 mg/L	93 – 100	97	3		
	Confirmatory	0.05 mg/L	97 – 106	102	4		
	Confirmatory	0.5 mg/L	92 – 100	96	4		
Blood Na *p*-NP	Primary	0.05 mg/L	104 – 106	105	1	0.05 mg/L	Guserle, R. (2020), CA 4.2 (d)/01
	Primary	0.5 mg/L	101 – 108	105	3		
	Confirmatory	0.05 mg/L	104 – 109	107	2		
	Confirmatory	0.5 mg/L	100 – 107	105	3		
Urine Na *p*-NP	Primary	0.05 mg/L	104 – 109	107	2	0.05 mg/L	Guserle, R. (2020), CA 4.2 (d)/01
	Primary	0.5 mg/L	98 – 103	101	2		

Matrix	Method	Fortification level	Recovery rate [%]		%RSD	LOQ	Reference
			Range	Mean			
	Confirmatory	0.05 mg/L	96 – 107	104	5		
	Confirmatory	0.5 mg/L	95 – 100	98	2		
Meat Na 5-NG	Primary	0.1 mg/kg	105 – 110	108	2	0.1 mg/kg	Guserle, R. (2020), CA 4.2 (d)/01
	Primary	1.0 mg/kg	98 – 104	101	2		
	Confirmatory	0.1 mg/kg	106 – 109	108	1		
	Confirmatory	1.0 mg/kg	99 – 103	101	2		
Meat Na *o*-NP	Primary	0.1 mg/kg	103 – 108	105	2	0.1 mg/kg	Guserle, R. (2020), CA 4.2 (d)/01
	Primary	1.0 mg/kg	93 – 100	97	3		
	Confirmatory	0.1 mg/kg	97 – 106	102	4		
	Confirmatory	1.0 mg/kg	92 – 100	96	4		
Meat Na *p*-NP	Primary	0.1 mg/kg	104 – 109	107	2	0.1 mg/kg	Guserle, R. (2020), CA 4.2 (d)/01
	Primary	1.0 mg/kg	98 – 103	101	2		
	Confirmatory	0.1 mg/kg	96 – 110	104	5		
	Confirmatory	1.0 mg/kg	95 – 100	98	2		

Conclusion
The method is valid and acceptable according to SANCO/825/00 rev. 8.1 for the determination of Na 5-NG, Na *o*-NP and Na *p*-NP in body fluids and tissues.

Overall conclusion:
Monitoring analytical method for determination of residues of Na 5-NG, Na *o*-NP and Na *p*-NP in body fluids and tissue in compliance with SANCO/825/00 rev. 8.1 is available and is described in table below. Body fluids and tissues were extracted with acidified acetonitrile after addition of water and further cleaned-up following the Quechers procedure. Analysis was performed using LC-MS/MS.

Overall summary of validation data for analytical methods to be used for enforcement for determination of Na 5-NG, Na *o*-NP and Na *p*-NP residues in body fluids and tissue.

A 2.1.2.8 Other Studies/ Information

No new or additional studies have been submitted.

Analytical method for the determination of sodium 5-nitroguaiacolate, sodium *o*-nitrophenolate and sodium

p-nitrophenolate in nectar was submitted in support of the evaluation.

Comments of zRMS:	The analytical method has been successfully validated for the determination of 5-nitroguaiacol, ortho-nitrophenol and para-nitrophenol in nectar in accordance to the guidance documents SANCO/825/00, rev. 8.1 and SANCO/3029/99, rev. 4 with the LOQ of 0.01 mg/kg for each analyte. Quantification was performed by use of LC-MS/MS detection. All mean recoveries were in the range of 70 – 110% with relative standard deviations of ≤20% for all analytes at each level. The method meets all criteria of guidelines SANTE/2020/12830, Rev.1, 24. February 2021 to determine concentrations of 5-nitroguaiacol, ortho-nitrophenol and para-nitrophenol in nectar at the LOQ level of 0.01 mg/kg. The method is acceptable.

Data point addressed:	KCP 5.2
Author(s) (year):	Kugel, D. (2020)
Title:	Determination of Residues of 5-Nitroguaiacol, o-Nitrophenol and p-Nitrophenol in Nectar after four Applications of ATONIK containing Sodium 5-Nitroguaiacolate, Sodium o-Nitrophenolate and Sodium p-Nitrophenolate in Phacelia tanacetifolia at 4 Sites in Central and Southern Europe in 2019
Laboratory report / project Number (Doc. No.):	S19-03993 (634-96002)
Testing facility:	Eurofins Agroscience Services Ecotox GmbH, Niefern-Öschelbronn, Germany
Published:	No
Test guideline used:	SANTE/11956/2016 rev. 9, OECD No. 509 (2009), SANCO/825/00, rev. 8.1 (2010), SANCO/3029/99, rev. 4 (2000), OECD Testing and assessment No. 72 and Series on Pesticides No. 39, ENV/JM/MONO(2007)17
Deviations:	None
Previous evaluation:	No
GLP:	Yes; certified by LUBW Landesanstalt für Umwelt Baden-Württemberg, Karlsruhe
Acceptability/Reliability:	Yes

Principle of the method:
Samples of nectar were extracted with acetonitrile and water (2:8). After centrifugation quantification was performed by use of LC-MS/MS detection.

Chromatographic conditions

HPLC-system:	Shimadzu LC-30 AD HPLC pump with autosampler SIL-30ACMP
Column:	Phenomenex Kinetex C18 (50 mm x 2.1 mm ID x 1.7 µm)
Column oven temp.:	40°C
Injection volume:	40 µL
Mobile phase (gradient)	Eluent A: Water + 10 mM ammonium acetate Eluent B: Methanol
Divert valve:	0.0 min to 1.7 min to waste; 1.7 min to 3.3 min to MS; 3.3 min to 5.0 min to waste
Monitored ions	168 → 153 and 168 → 123 for 5-NG 138 → 108 and 138 → 92 for Na *o*-NP 138 → 108 and 138 → 92 for Na *p*-NP
Retention time:	5-NG: approx. 2.3 min *o*-NP: approx. 2.4 min *p*-NP: approx. 2.1 min

Validation
Selectivity/Specificity:
The analysis of untreated samples in comparison with the analysis of standard solutions and spiked samples showed no significant interference (i.e. < 30% LOQ) at the retention times of Na 5-NG, Na *o*-NP and Na *p*-NP. Final samples were analyzed by the highly specific LC-MS/MS technique, monitoring two mass transitions (MRMs) for all analytes. Representative mass spectrums, chromatograms of standards at the

lowest calibrated level, matrix blanks and samples fortified at the lowest fortification level for each analyte are provided in the study report.

Calibration:
The linearity of the detector response was demonstrated by single determination of solvent calibration standards at seven concentration levels ranging from 0.12 ng/mL to 10 ng/mL (expressed as sodium salts). This range corresponds to a fortification level of 0.003 mg/kg to 0.25 mg/kg (expressed as each sodium salt) 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 curves obtained for both mass transitions were linear since correlation coefficients (R) were > 0.995. Linear regression was performed with 1/x-weighting. Please refer to the following table:

Table A2-1: Linearity of detector response

Analyte and transition	Calibration range	Equation	R^2
Na 5-NG; 168 → 153	0.12 to 10 ng/mL (0.003 – 0.25 mg/kg)	y = 9.11E+05 x + 3.68E+04	0.9994
Na 5-NG; 168 → 123		y = 2.58E+05 x + 8.55E+03	0.9996
Na *o*-NP; 138 → 108		y = 8.67E+04 x + 4.45E+03	0.9974
Na *o*-NP; 138 → 92		y = 8.39E+03 x + 486	0.9987
Na *p*-NP; 138 → 108		y = 1.04E+06 x + 6.23E+03	0.9998
Na *p*-NP; 138 → 92		y = 6.65E+04 x + 462	0.9998

Accuracy and precision:
The recovery rates and relative standard deviations obtained from five samples fortified at LOQ and 10 x LOQ with Na 5-NG, Na *o*-NP and Na *p*-NP are shown in the following Tables.

Table A2-2: Accuracy and precision

Analyte and transition	Fortification level [mg/kg]	Recovery range [%]	Mean recovery [%]	RSD [%]
Na 5-NG 168 → 153	0.01 (n = 5)	82 – 90	85	4
	0.10 (n = 5)	94 – 103	99	3
	Overall (n = 10)	82 – 103	92	8
Na 5-NG 168 → 123	0.01 (n = 5)	79 – 88	85	4
	0.10 (n = 5)	89 – 102	94	5
	Overall (n = 10)	79 – 102	90	7
Na *o*-NP 138 → 108	0.01 (n = 5)	67 – 95	83	14
	0.10 (n = 5)	82 – 103	92	8
	Overall (n = 10)	67 – 103	87	12
Na *o*-NP 138 → 92	0.01 (n = 5)	70 – 92	79	13
	0.10 (n = 5)	82 – 90	87	4
	Overall (n = 10)	70 – 92	83	10
Na *p*-NP 138 → 108	0.01 (n = 5)	89 – 94	92	2
	0.10 (n = 5)	87 – 93	90	3
	Overall (n = 10)	87 – 94	91	2
Na *p*-NP 138 → 92	0.01 (n = 5)	91 – 93	92	1
	0.10 (n = 5)	89 – 94	90	2
	Overall (n = 10)	89 – 94	91	2

The mean recovery at each fortification level and the overall mean recovery for Na 5-NG, Na *o*-NP and Na *p*-NP in each matrix were in the range of 70-110% and the relative standard deviation was less than or equal to 20%.

Limit of quantification / limit of detection:
The limit of quantification (LOQ) of the method was defined as the lowest analyte concentration in a sample at which the methodology has been successfully validated. A LOQ of 0.01 mg/kg was confirmed for Na 5-NG, Na *o*-NP and Na *p*-NP. The LOD was set at 0.003 mg/kg which is 30% of the LOQ.

Matrix effects:
The effect of matrix on the LC-MS/MS response was assessed by comparing mean peak areas of matrix-matched standards of 90% matrix amount with solvent standards at identical nominal concentrations. Mean matrix effects were <±20% and deemed to be insignificant for all analytes. Therefore, solvent standards were used for quantification throughout the analytical phase.

Stability of Stock and Fortification Solutions:
The stock solutions prepared in acetonitrile were stored at typically 1°C to 10°C for 19 days in the dark, which was sufficient to cover the length of time they were used in this study. After this time freshly prepared dilutions of the stored stock solutions were compared to freshly prepared dilutions of freshly prepared stock solutions by single injection. One mass transition per analyte was evaluated. Results obtained are summarised in the table below:

Table A2-3: Stability of Stock and Fortification Solutions

Analyte and transition	Concentration level [µg/mL]	Storage period [days]	Difference [%] of stored stock solution compared to freshly prepared solution
5-NG 168 → 153	1130	19	- 4
	100	19	- 16
	0.5	19	- 13
o-NP 138 → 108	1158	19	8
	100	19	5
	0.5	19	18
p-NP 138 → 108	1158	19	4
	100	19	0
	0.5	19	- 9

The peak areas of the stored diluted stock solutions were within ± 20% of the peak areas of the freshly prepared diluted stock solutions indicating that stock solutions are stable when stored at 1°C to 10°C in the dark for 19 days.
Fortification solutions were prepared in the same solvent as the stock solutions and were also stored at typically 1°C to 10°C in the dark. Therefore, investigation of the stability of fortification solutions was not necessary.

Stability of Solvent Calibration Solutions
The calibration solutions prepared in water/acetonitrile (4:1 v/v) were stored at typically 1°C to 10°C for 7 days in the dark, which was sufficient to cover the length of time they were used in this study. After this time solvent calibration solutions were compared by single injection to a freshly prepared solvent calibration. One mass transition per analyte was evaluated. Results obtained are summarised in the table below:

Table A2-4: Stability of Solvent Calibration Solutions

Analyte and transition	Concentration level [ng/mL]	Storage period [days]	Difference [%] of stored solution compared to freshly prepared calibration solution
5-NG 168 → 153	10.0	7	- 7
	4.0	7	- 2
	1.0	7	4
	0.4	7	- 5
p-NP 138 → 108	10.0	7	- 7
	4.0	7	- 5
	1.0	7	- 3
	0.4	7	- 5
o-NP 138 → 108	10.0	7	- 1
	4.0	7	9
	1.0	7	10
	0.4	7	16

The peak areas of the store solvent calibration solutions were within ± 20% of the freshly prepared calibration indicating that solvent standards solutions are stable when stored at 1°C to 10°C in the dark for 7 days.

Stability of Analyte in Sample Extracts:
Following the first analysis, the final extracts of samples fortified at the 10x LOQ level together with one control sample extract were stored at typically 1°C to 10°C in the dark for 7 days. After this period, the final extracts were re-analysed against freshly prepared calibration standards One mass transition per analyte was evaluated. The results obtained are summarised in the table below.

Table A2-5: Stability of Analyte in Sample Extracts

Analyte and transition	Concentration level [ng/mL]	Storage period [days]	Recovery 1st injection [%]	Recovery 2nd injection [%]	Difference [%] of recoveries
5-NG 168 → 153	0.1	7	88	85	3
			93	89	
			83	92	
			91	93	
			82	93	
o-NP 138 → 108	0.1	7	82	70	0
			67	86	
			96	90	
			88	92	
			92	88	
p-NP 138 → 108	0.1	7	90	89	- 2
			91	87	
			89	91	
			95	94	
			93	91	

The mean recovery values of the re-analysed extracts were in the range of 70 - 120% and within 120% of the original result. Therefore, extracts are considered to be stable when stored at 1°C to 10°C for 7 days in the dark.

Storage Stability Samples:
The residues levels detected in the storage samples (3 replicates) allow the monitoring of the stability of the analytes upon storage. The values were as follows:

Table A2-6: Storage Stability Samples

Analyte and transition	Nominal fortification level [mg/kg]	Storage period [days]	Mean Recovery ± RSD [%]	Storage period [days]	Mean Recovery ± RSD [%]	Storage period [days]	Mean Recovery ± RSD [%]
5-NG 168 → 153	0.128	0	95 ± 2	113	100 ± 2	232	103 ± 3
o-NP 138 → 108	0.128	0	82 ± 7	113	86 ± 1	232	89 ± 3
p-NP 138 → 108	0.128	0	99 ± 1	113	98 ± 3	232	103 ± 4

The average amount of analyte recovered relative to the initial recovery at day 0 was ≥ 70% at any testing intervals (113 and 232 days), which can be seen as criterion for sufficient storage stability.

Conclusion:
The analytical method is considered valid and acceptable according to SANCO/3029/99 rev. 4 for determination of Na 5-NG, Na *o*-NP and Na *p*-NP in nectar.

In addition, based on the study results, the analytical method also fits for purpose the requirements of SANTE/2020/12830, Rev.1 for determination of Na 5-NG, Na *o*-NP and Na *p*-NP in nectar.