Quick Method for the Analysis of Residues of numerous Highly Polar Pesticides in Food Commodities involving Simultaneous Extraction with Methanol and Determination via LC-MS/MS (QuPPe-AO-Method)

II. Food of Animal Origin

- **Version 2** (Jan 2016, Document History, see page 16)

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1. Scope and Short Description

A method is described for the residue analysis of very polar, non-QuEChERS-amenable, pesticides in food of animal origin. This first version of the method was tested for milk and eggs. More commodities will gradually follow.

Following water adjustment and addition of acidified methanol residues are extracted from the test portion via shaking. Following centrifugation, an aliquot of the raw extract is cleaned-up by simultaneous dilution with acetonitrile and dSPE with ODS sorbent, which leads to a precipitation or adsorption of a large portion of co-extractives. The cleaned-up extract is centrifuged and filtered and then subjected to determinative analysis via LC-MS/MS. Various LC-MS/MS methods for the simultaneous analysis of different combinations of pesticides are provided. Quantification is in most cases performed with the help of isotopically labeled analogues of the target analytes, which are used as internal standards (ISTDs). So far available, these ISTDs are added directly to the test portion at the beginning of the procedure to compensate for any factors having an influence on the recovery-rates such as volume-deviations, analyte losses during extraction and cleanup as well as matrix-effects during LC-MS/MS.

2. Apparatus and Consumables

2.1. Powerful sample homogenizer,

e.g. Braun MR 5550 hand blender (with chopper attachment)

2.2. 50 mL centrifuge tubes with screw caps,

for the extraction step (**5.2.1**), e.g.: a) disposable 50 mL centrifuge tubes (e.g. Sarstedt / Nümbrecht, Germany, 114x28 mm, PP, article-no. 62.548.004) or b) reusable 50 mL Teflon® centrifuge tubes with screw caps (e.g. Nalgene/Rochester, USA; Oak-ridge, article-no. 3114-0050).

2.3. 10 mL centrifuge tubes with screw caps,

For the d-SPE step (**5.2.6**), e.g.: disposable 10 mL PP-tubes by Simport/Beloeil (Canada), article-no. T550-10AT

2.4. Automatic pipettes,

suitable for handling volumes of 10 to 100 µl, 200 to 1000 µl and 1 to 10 ml.

2.5. 10 mL solvent-dispenser,

for the acidified methanol (**3.6**).

2.6. Centrifuge,

suitable for the centrifuge tubes employed in the procedure (**2.2**) and capable of achieving > 3000 g.

2.7. Syringe filters,

e.g. polyester filters 0.45 µm pore size.

2.8. Syringes

e.g. 2 or 5 mL disposable polypropylene syringes suitable for the above mentioned filters **2.7.**

2.9. Autosampler vials,

suitable for LC auto-samplers,

Use plastic vials if pesticides that tend to interact with glass-surfaces are present (e.g. Glyphosate and Ethephon)[1].

Note:

- *Such interaction with glass surfaces are more pronounced when solutions have low water content and low acidity.*

[1]The list of compounds requiring plastic vessels might not be comprehensive (this remark applies to the entire document)

2.10. Volumetric flask with stoppers,

for the preparation of stock and working solutions (3.11 - 3.16), e.g. 20 mL; 25 mL; 50 mL, 100 mL glass flasks. Use plastic flasks and stoppers if pesticides that tend to interact with glass-surfaces are present (e.g. Glyphosate and Ethephon).

2.11. LC-MS/MS instrumentation,

See latest version of QuPPe-PO-Method

3. Chemicals

Unless otherwise specified, use reagents of recognized analytical grade. Take every precaution to avoid possible contamination of water, solvents, sorbents, inorganic salts, etc.

3.1. Water (deionized)

3.2. Methanol (HPLC quality)

3.3. Acetonitrile (HPLC quality)

3.4. Formic acid (concentrated; > 95%)

3.5. Acetic Acid (concentrated; >98%)

3.6. Acidified methanol,

pipette 10 mL formic acid (**3.4**) in a 1000 mL volumetric flask and fill up to volume with methanol (**3.2**).

3.7. ODS (octadecylsilane) sorbent,

e.g. Polygoprep 30-300 µm Macherey-Nagel GmbH & Co KG/Düren (Germany), article-no. 711720.100).

3.8. Ammonium formate (p.a.)

for the LC-MS/MS eluent in Method M4

3.9. LC-MS/MS mobile phases

See latest version of QuPPe-PO-Method.

3.10. Pesticide Standards,

of known purity.

3.11. Pesticide stock solutions,

See latest version of QuPPe-PO-Method. Use plastic flasks and stoppers if pesticides that tend to interact with glass-surfaces are present (e.g. Glyphosate, Ethephon).

3.12. Pesticide working solutions / mixtures,

See latest version of QuPPe-PO-Method. Use plastic flasks and stoppers if pesticides that tend to interact with glass-surfaces are present (e.g. Glyphosate, Ethephon).

3.13. Internal Standards (ISTDs),

See latest version of QuPPe-PO-Method.

3.14. ISTD Stock solutions,

See latest version of QuPPe-PO-Method. Use plastic flasks and stoppers if pesticides that tend to interact with glass-surfaces are present (e.g. isotope labeled Glyphosate, Ethephon).

3.15. ISTD-working solution I (ISTD-WS I) for spiking samples prior to extraction,

See latest version of QuPPe-PO-Method. Use plastic flasks and stoppers if pesticides that tend to interact with glass-surfaces are present (e.g. isotope labeled Glyphosate, Ethephon).

3.16. ISTD-working solution II (ISTD-WS II) for preparation of calibration standards,

See latest version of QuPPe-PO-Method. Use plastic flasks and stoppers if pesticides that tend to interact with glass-surfaces are present (e.g. isotope labeled Glyphosate, Ethephon).

4. Disclaimer

This method refers to several trade name products and instruments which are commercially available and suitable for the described procedure. This information is given for the convenience of the users of this method and does not constitute an endorsement by the EURL of the products named. The application of this method may involve hazardous materials, operations and equipment. It is the responsibility of the users of this method to establish appropriate safety and health practices prior to use.

5. Procedure

5.1. Sample preparation

To obtain representative test-portions from the laboratory sample, proceed as required by the respective regulations and guidelines. Homogenize the eggs with a hand-blender (**2.1**) until a free flowing mixture is obtained. Proceed similarly with non-homogenized milk (e.g. if fat has separated).

5.2. Extraction / Centrifugation / Filtration

5.2.1. Weigh a representative portion (ma) of the sample homogenate (**5.1**) into a 50 mL centrifuge tube (**2.2**). In case of fresh milk and fresh eggs take 10 g ± 0.05 g of the homogenized sample.

5.2.2. Add water (**3.1**) to a content of ca. 10 g in total. In the case of cow milk (88 % water) add 1.2 g of water and in case of chicken eggs (76% water) add 2.4 g of water.

Notes:

- *Where no IL-ISTDs are used or where they are added after extract aliquotation, water adjustment is essential. Where the appropriate IL-ISTDs are employed before any aliquotation has taken place water adjustment is less critical and can be skipped for commodities containing ≥80% water.*

5.2.3. Add 10 mL acidified methanol (**3.6**) and 50 µL of the ISTD-WS I (**3.15**) containing isotopically labeled analogues of one or more of the analytes of interest (added ISTD mass = m_{ISTD}^{sample}).

Notes:

- *For screening purposes the ISTD can be alternatively added to an aliquot of the final sample extract (see **5.2.10**)*

5.2.4. Close the tube and shake vigorously for 1 min by hand or for 5-20 minutes by a mechanical shaker.

5.2.5. Centrifuge for 5 min at >3000 g.

5.2.6.Transfer a 2 mL aliquot into a 10 mL centrifuge tube with screw cap (**2.3**), which already contains the 2 mL of acetonitrile (**3.3**) and 100 mg of ODS sorbent (**3.7**).

5.2.7. Close the tube and shake vigorously for 1 min by hand.

5.2.8. Centrifuge (e.g. for 5 min at >3000 g).

5.2.9.Filter an aliquot (ca. 3-4 mL) of the extract through a syringe filter (**2.7**) into a sealable storage vessel.

5.2.10. Transfer, as required, one or more aliquots (e.g. 1 mL each) into auto-sampler vials (**2.9**) if necessary dilute the extract before measurement (see also hints in QuPPe-PO document).

Notes:

- *The cleaned-up extract will contain ca. **0.25 g sample equivalents per mL extract** (if 10 g sample are employed for extraction).*

- *Instead of adding the ISTD at the beginning of the procedure it can be added to an aliquot (e.g. 1 mL) of the final sample extract. This way the added amount of ISTD per sample can be drastically reduced (e.g. 40-fold if added to 1 mL extract). The ISTD added at this step will compensate for matrix effects including retention-time shifts. The quantitative result should however be considered as tentative. For more accuracy samples should be re-analyzed with the ISTD being added in step **5.2.3**.*

5.3. Blank extracts

Using suitable blank commodities (not containing any detectable residues of the analytes of interest), proceed sample preparation exactly as described under **5.2** but **SKIP THE ADDITION OF ISTDs.**

5.4. Recovery experiments

See latest version of QuPPe-PO-Method.

5.5. Preparation of calibration standards

5.5.1. **Solvent-based calibration standards**

An exemplary pipetting scheme for the preparation of solvent-based calibration standards is shown in **Table 1**. The calculation of the mass-fraction W_R of the pesticide in the sample, when ISTD is used, is shown in **5.7.1**.

Note:

- *Where solvent-based calibrations are used the use of IL-ISTDs for quantification is essential as the ISTD compensates for any matrix-related signal suppressions / enhancements.*

5.5.2.**Matrix matched calibration standards**

Transfer suitable aliquots of the blank extract **(5.3)** to auto-sampler vials and proceed as shown in **Table 1**.

The calculation of the mass-fraction W_R of the pesticide in the sample using matrix-matched calibration standards, with and without the use of ISTD, is shown in **5.7.1** and **5.7.2.1** respectively.

Table 1: Exemplary pipetting scheme for the preparation of calibration standards

		Calibration standards								
		Solvent based (5.5.1)			Matrix-matched (5.5.2)					
		using ISTD [1]			without ISTD [5]			using ISTD [1]		
Calibration levels in µg pesticide /mL OR in µg pesticide/ "ISTD-portion" [2]		**0.0125**	**0.025**	**0.0625**	**0.0125**	**0.025**	**0.0625**	**0.0125**	**0.025**	**0.0625**
Corresponding conc. in sample using 10 g test portions (mg/kg)		**0.05**	**0.1**	**0.25**	**0.05**	**0.1**	**0.25**	**0.05**	**0.1**	**0.25**
		Pipetting Volumes								
Blank extract (5.3)		-	-	-	875 µL	875 µL	875 µL	825 µL	825 µL	825 µL
1:1 (v/v) mix of water (3.1) and acidified MeOH (3.6)		925 µL	900 µL	825 µL	100 µL	75 µL	-	100 µL	75 µL	-
Pesticide working solutions (3.12) [3]	**0.5 µg/mL**	25 µL	50 µL	125 µL	25 µL	50 µl	125 µL	25 µL	50 µL	125 µL
ISTD-WS II (3.16) [2,4]		50 µL	50 µL	50 µL	-	-	-	50 µL	50 µL	50 µL
Total volume		**1000 µL**	**1000 µL**	**1000 µL**	**1000 µL**	**1000 µL**	**1000 µL**	**1000 µL**	**1000 µL**	**1000 µL**

[1] *When employing IL-ISTDs matrix-matching and volume adjustments are of less importance as the ISTD compensates for any matrix-related signal suppressions / enhancements. Also solvent-based calibrations can be used here. Important is that a) the mass ratio of pesticide and ISTD in the respective calibration standards and b) the ratio between the ISTD mass added to the sample (**5.2.3**) and the ISTD mass added to the calibration standard(s) (**5.5.1** and **5.5.2**) is known and recorded. For convenience the latter mass ratio should be kept constant throughout all calibration levels (e.g. at 40:1 when preparing calibration standards of 1 mL).*

[2] *One ISTD portion would correspond to the ISTD mass contained in 50 µL ISTD-WS II (the volume added to each calibration standard).*

[3] *The concentration of the pesticide working solution(s) should be sufficiently high to avoid excessive dilution of the blank extract which would result in matrix effect deviations.*

[4] *For calibration standards of 1 mL it is recommended to prepare the ISTD-WS II **(3.16)** by diluting 40-fold the ISTD-WS I **(3.15)**. The same volume and pipette as in **5.2.3** can then be used for the preparation of the calibration standards.*

[5] *Where IL-ISTDs are <u>not</u> available/employed, matrix-matching via matrix-matched standards (**Table 1**) or the standard additions approach (**5.5.3**) are particularly important to compensate for matrix effects in measurement. In both cases the final extract is assumed to contain 0.25 g sample/mL (when 10 g sample are used).*

5.5.3. **Standard-Additions-Approach**

Where no appropriate ISTDs are available the method of standard additions is a very effective approach for compensating matrix-induced enhancement or suppression phenomena. As this procedure involves a linear extrapolation it is mandatory that pesticide concentrations and detection signals show a linear relationship throughout the relevant concentration range. The procedure furthermore requires knowledge of the approximate (estimated) residue level in the sample ($w_{R(exp.)}$) as derived from a preliminary analysis.

Prepare 4 vials containing equal portions of the final extract. Three of them should be spiked with increasing amounts of the analyte. The amounts to be added should be chosen to be close to the expected amount of the analytes in the aliquots $m_{pest(\exp.)}^{aliquot}$. It is important to remain within the linear range. Prepare a working solution (**3.12**) of the analyte at a concentration level where e.g. 50 or 100 µL of the solution contain the smallest amount of analyte to be added. Below some examples of standard additions:

Example A: Vial 1) no addition; vial 2) 0.5 x $m_{pest(\exp.)}^{aliquot}$, vial 3) 1 x $m_{pest(\exp.)}^{aliquot}$, and vial 4) 1.5 x $m_{pest(\exp.)}^{aliquot}$,

Example B: Vial 1) no addition; vial 2) 1 x $m_{pest(\exp.)}^{aliquot}$, vial 3) 2 x $m_{pest(\exp.)}^{aliquot}$, and vial 4) 3 x $m_{pest(\exp.)}^{aliquot}$.

Adjust the volume within all vials by adding the corresponding solvent amounts.

Table 2 shows a pipetting scheme following Example A. The calculation of the mass fraction of the pesticide in the sample w_R is shown in **5.7.2.2**.

Table 2 : Exemplary pipetting scheme of a standard additions approach (for a sample extract containing 0.25 g sample equivalents per mL and an estimated residue level ($w_{R(approx)}$) of 0.4 mg/kg (corresponds to 0.1 µg/mL)

Additions	**Vial 1**	**Vial 2**	**Vial 3**	**Vial 4**
Volume of final sample extract	1000 µl (= 0.25 g sample)	1000 µl (= 0.25 g sample)	1000 µl (= 0.25 g sample)	1000 µl (= 0.25 g sample)
ISTD	none	none	none	none
Added volume of pesticide working solution containing 1 µg/ml (**3.12**)	-	50 µl	100 µl	150 µl
Resulting mass ($m_{pest}^{std\ add}$) of pesticide added to each vial		0.05 µg	0.1 µg	0.15 µg
Volume of solvent	150 µl	100 µl	50 µl	-
Final volume	**1150 µl**	**1150 µl**	**1150 µl**	**1150 µl**

5.6. LC-MS/MS Measurement Conditions

Any suitable LC and MS/MS conditions including those proposed in the QuPPe-PO-Method may be used.

For food of animal origin we have so far only tested method M 1.3, M 1.4 and M 4.

5.6.1. **Exemplary LC-MS/MS chromatograms (method M1.3)**

Figure 1: Chromatograms of Fosetyl, Maleic Hydrazide, HEPA, Ethephon, MPPA, Glyphosate, Glufosinate, N-Acetyl-Glufosinate, at 0.1 µg/mL in MeOH (with 1% formic acid).

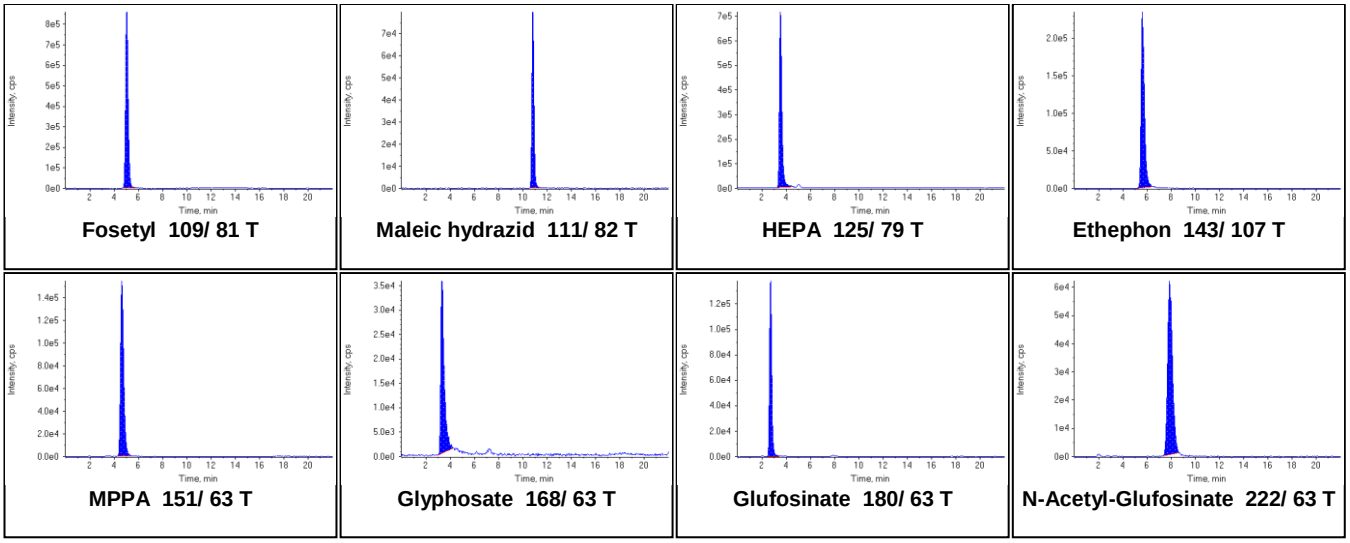


Figure 2: Chromatograms of Fosetyl, Maleic Hydrazide, HEPA, Ethephon, MPPA, Glyphosate, Glufosinate, N-Acetyl-Glufosinate, at 0.0125 µg/mL in whole cow's milk extract.

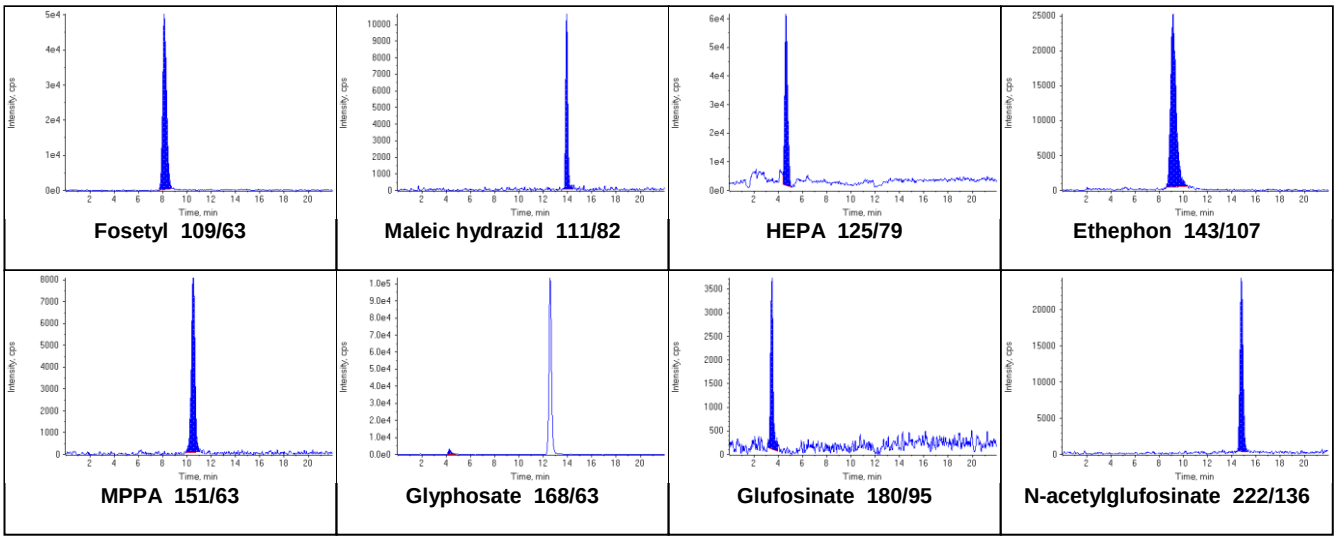
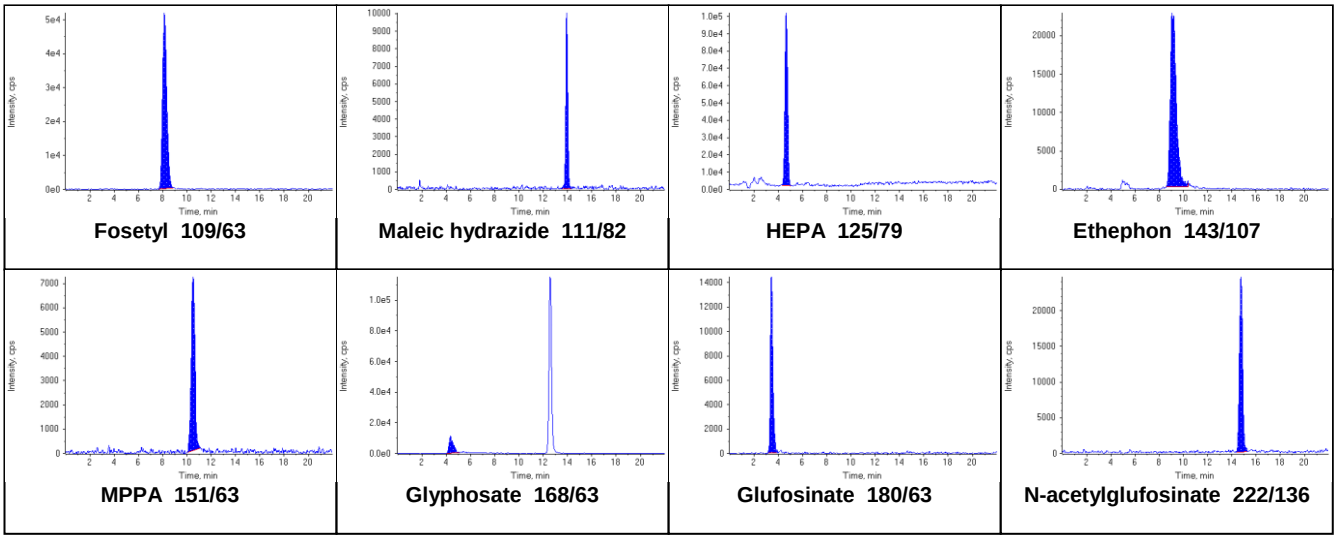


Figure 3: Chromatograms of Fosetyl-Al, Maleic Hydrazide, HEPA, Ethephon, MPPA, Glyphosate, Glufosinate, N-Acetyl-Glufosinate, at 0.0125 µg/mL in chicken eggs extract.



5.6.2. **Exemplary LC-MS/MS chromatograms (method M4)**

Figure 4: Chromatograms of Trimethylsulfonium cation, Nereistoxin, Mepiquat, Chlormequat, Daminozide, Cyromazine, Difenzoquat at 0.1 µg/mL in MeOH (with 1% formic acid).

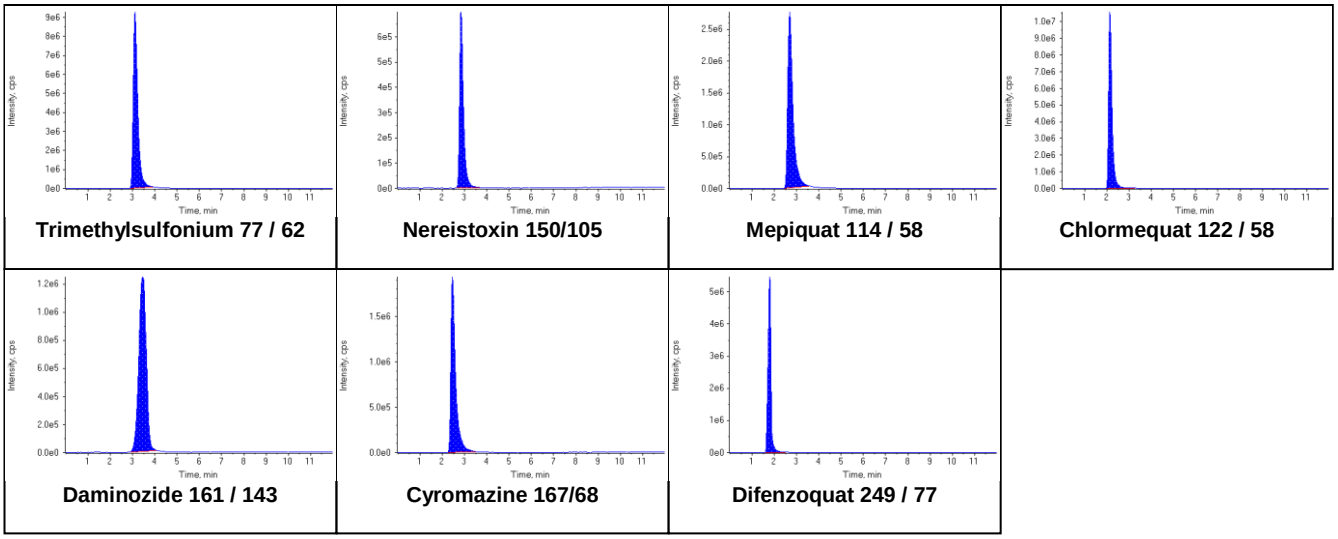


Figure 5: Chromatograms of Trimethylsulfonium cation, Nereistoxin, Mepiquat, Chlormequat, Daminozide, Cyromazine, Difenzoquat at 0.0125 µg/mL in whole cow's milk extract.

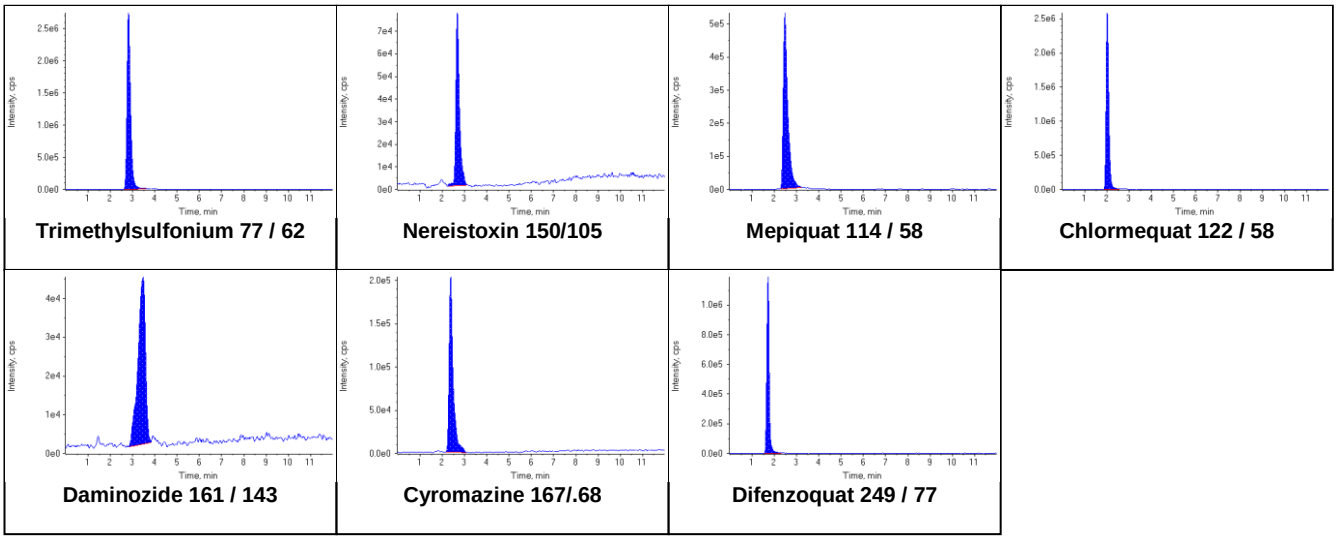
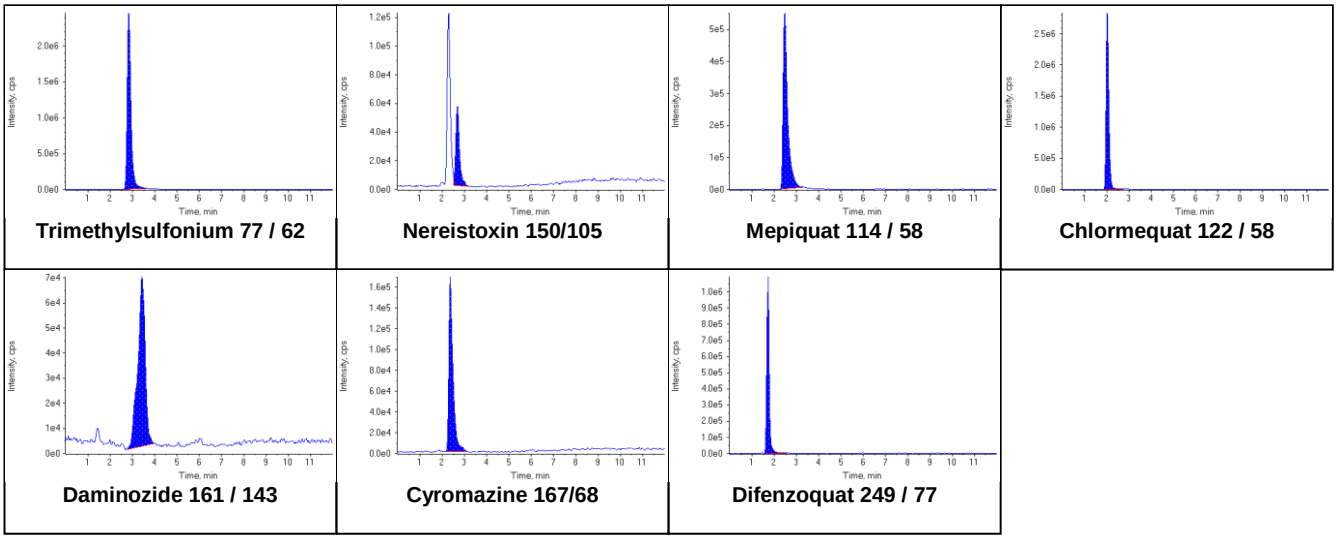


Figure 6: Chromatograms of Trimethylsulfonium cation, Nereistoxin, Mepiquat, Chlormequat, Daminozide, Cyromazine, Difenzoquat at 0.0125 µg/mL in chicken eggs extract.



5.7. Calibration and Calculations

5.7.1. **Using ISTD**

5.7.1.1. Where ISTD is added to the sample before any aliquotation:

Follow the latest version of QuPPe-PO-Method. To ensure similar concentration of the ISTD is sample extracts and calibration standards it is reasonable to prepare the calibration standards in such a way that the ratio m_{ISTD}^{sample} / $m_{ISTD}^{cal\ mix}$ equals 40 (to account for the final volume of the raw extract of 20 mL and the 1:1 dilution during cleanup). The absolute masses of the ISTD-WS I and II do not need to be necessarily known.

5.7.1.2. Where ISTD is added to an aliquot of the extract

Follow the latest version of QuPPe-PO-Method. When adding the ISTD to an aliquot of the extract (e.g. 1 mL) it is mandatory to know the exact concentration of matrix-equivalnts per mL extract. If water adjustment is done as described in **5.2.2,** the total volume of the raw extract can be assumed to be exactly 20 mL. Considering the 2-fold dilution during the cleanup step 1 mL sample extract will represent 1/40th of the test portion (m_a). The mass of the ISTD to be added to an aliquot ($m_{ISTD}^{aliquot}$) should be scaled according to the aliquot volume used ($V_{aliquot}$) with the ISTD mass ratio ($m_{ISTD}^{aliquot}$ / $m_{ISTD}^{cal\ mix}$) being important for the calculation.

5.7.2. **Not using ISTD**

If no appropriate ISTDs are used it is of high importance to properly compensate for matrix effects. For the compensation of matrix effects matrix-matched calibrations (**5.5.2**) and the standard additions approach (**5.5.3**) are recommended. In both cases the assumption is made that the total volume of the raw sample extract is exactly 20 mL, which is then diluted by a factor of 2. Adjustment of the water content (and extract volume) in the sample is thus paramount.

5.7.2.1. Calculations when employing matrix-matched calibration without ISTD

Follow the latest version of QuPPe-PO-Method.
In the formula multiply V_{end} by two to account for the 2-fold dilution in the cleanup step.

5.7.2.2. Calculations when employing the standard additions approach

The standard additions approach is the method of choice where no appropriate IL-ISTD is available. This approach typically compensates matrix effect better than matrix-matched calibrations (**5.5.2**). The mass fraction of the pesticide in the sample (w_R) is calculated via linear regression as shown in the latest version of QuPPe-PO-Method.

In the formula multiply V_{end} by two to account for the 2-fold dilution in the cleanup step.

5.8. Validation Data

5.8.1. **Method 1.3 ("Glyphosate & Co")**

Table 3 : Recoveries of analytes of method M1.3 in whole milk and eggs.

Compound	ISTD used	n	Recovery 0.1 mg/kg in whole cow milk		Recovery 0.1 mg/kg in chicken eggs	
			Recovery mean [%]	RSD [%]	Recovery mean [%]	RSD [%]
Fosetyl	Yes	5	99	1.9	104	1.9
Maleic hydrazide	Yes	5	106	3.6	107	4.5
HEPA	Yes	5	105	1.2	102	3.2
Ethephon	Yes	5	99	4.0	114	4.4
MPPA	Yes	5	103	4.7	100	7.7
Glyphosate	Yes	5	196	9.6	117	1.0
Glufosinate	Yes	5	94	8.5	100	4.1
N-Acetyl-Glufosinate	Yes	5	103	1.9	104	3.0

5.8.2. **Method 1.4 ("PerChloPhos")**

Table 4 : Recoveries of analytes of method M1.4 in whole milk.

Compound	ISTD used	n	Recovery 0.02 mg/kg in whole cow milk		Recovery 0.05 mg/kg in whole cow milk	
			Recovery mean [%]	RSD [%]	Recovery mean [%]	RSD [%]
Phosphonic acid	Yes	5	103	3.6	99	2.9
Perchlorate	Yes	5	104	3.3	97	2.4
Chlorat	Yes	5	102	3.7	97	2.6

5.8.3. **Method 4 ("Quats & Co")**

Table 5 : Recoveries of analytes of Method M4 in whole milk and eggs.

	ISTD used	n	Recovery 0.1 mg/kg in milk		Recovery 0.1 mg/kg in eggs	
			Recovery mean [%]	RSD [%]	Recovery mean [%]	RSD [%]
Trimethylsulfonium	No, (matrix matched)	5	99	0.7	90	1.1
Nereistoxin	Yes	5	98	1.8	98	2.8
Mepiquat	Yes	5	98	2.1	100	1.4
Chlormequat	Yes	5	102	1.5	98	1.7
Daminozide	Yes	5	90	3.7	91	7.2
Cyromazine	Yes	5	100	0.8	104	2.4
Difenzoquat	No, (matrix matched)	5	92	2.4	78	1.6

5.8.4. **Method 8 ("TDMs")**

Table 6 : Recoveries of analytes of Method M5 in whole milk

	ISTD used	n	Recovery 0.02 mg/kg		Recovery 0.2 mg/kg		Recovery 0.4 mg/kg	
			Recovery mean [%]	RSD [%]	Recovery mean [%]	RSD [%]	Recovery mean [%]	RSD [%]
1,2,4-Triazole* (TRZ)	No, (matrix matched)	5	-	-	87	7	96	7
Triazole acetic acid TAA)	Yes	5	89	6	92	2	89	2
Triazole ala-nine (TA)	Yes	5	85	21	88	4	100	7
Triazole lactic acid (TLA)	Yes	5	97	4	91	3	92	6

6. References

Anastassiades, M and Mack, D (2008); New Developments in the Analysis of Pesticides Typically not Covered by Multiresidue Methods; European Pesticide Residue Workshop, EPRW 2008, Berlin, oral presentation O1, Book of Abstracts

Kolberg DI, Mack D, Anastassiades M, Hetmanski MT, Fussell RJ, Meijer T, Mol HG. Anal Bioanal Chem. 404(8):2465-74 (2012); Development and independent laboratory validation of a simple method for the determination of paraquat and diquat in potato, cereals and pulses

Alder L. and Startin J. R. (2005); Determination of Chlormequat and Mepiquat in Foods by Liquid Chromatography/Mass Spectrometry or Liquid Chromatography/Tandem Mass Spectrometry: Interlaboratory Study; Journal of AOAC International Vol. 88, No. 6: 1762-1776

Vahl, M. et al. (1998); Analysis of Chlormequat residues in grain using liquid chromatography-mass spectrometry (LC-MS/MS); Fresenius J Anal Chem 361:817-820

Table 6: Document History

Action	When?	Version
Development of Method by the EURL-SRM	2012	-
Drafting of V1	2012-2013	V1
Placing of V1 in EURL-Website	Feb. 2013	
Adding of Validation Data method 1.4 “PerChloPhos” Adding validation data of method 8 triazole derivative metabolites (TDMs)	Jan. 2016	V2