Quick Method for the Analysis of Numerous Highly Polar Pesticides in Foods of Plant Origin via LC-MS/MS Involving Simultaneous Extraction with Methanol (QuPPe-Method)

II. Food of Animal Origin (QuPPe-AO-Method)

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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. Following water adjustment and addition of acidified methanol and EDTA 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 for liquid samples,

e.g. Stephan UM 5 or Retsch Grindomix GM 300 or Vorwerk-Thermomix TM31. For liquid samples (e.g. milk, eggs): it is also possible to use a less powerful blender, e.g. Braun MR 5550 hand blender with chopper attachment.

2.2. LC-Plastic tub,

for filling-in liquid nitrogen to immerge the samples prior to milling (**5.1**), see latest version of QuPPe-PO-Method.

2.3. 50 mL centrifuge tubes with screw caps,

for the extraction step. see latest version of QuPPe-PO-Method.

2.4. 10 mL centrifuge tubes with screw caps,

for the d-SPE step, see latest version of QuPPe-PO-Method.

2.5. Automatic pipettes,

see latest version of QuPPe-PO-Method.

2.6. 10 mL solvent-dispenser,

see latest version of QuPPe-PO-Method.

2.7. Centrifuge,

see latest version of QuPPe-PO-Method.

2.8. Syringes

see latest version of QuPPe-PO-Method.

2.9. Syringe filters,

see latest version of QuPPe-PO-Method.

2.10. Ultrafiltration filters,

with 5 kDa molecular weight cutoff suitable for centrifuges, e.g. Vivaspin® 6 mL 5 kDa entailing Polyethersulfone membranes or alternatively Amicon® Ultra-15 10K entailing Ultracel® low binding regenerated cellulose.

2.11. Autosampler vials,

see latest version of QuPPe-PO-Method.

Notes: The use of **plastic vials** is highly recommended as several of the compounds covered by this method (e.g. Phosphonate, Nicotine, Paraquat, Diquat, Streptomycin and Glyphosate)[1] tend to interact with glass-surfaces. Such interactions with glass surfaces are typically more pronounced in solutions consisting of aprotic solvents (e.g. acetonitrile). Increasing water content and/or acidity typically reduces such interactions. Percent losses due to such interactions are typically higher at low concentrations.

2.12. Volumetric flask with stoppers,

see latest version of QuPPe-PO-Method. Mind to use plastic containers (see note under **2.11**).

2.13. 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 (LC-MS quality)

3.3. Acetonitrile (LC-MS quality)

3.4. Formic acid (concentrated; ≥ 98%)

3.5. Acetic Acid (concentrated; ≥98%)

3.6. Acidified methanol,

see latest version of QuPPe-PO-Method.

3.7. Acidified methanol with 30% water,

for fat extraction, prepared by pipetting 10 mL of formic acid (**3.4**) into a 1000 mL volumetric flask, followed by 300 mL water (**3.1**) and filling up to volume with methanol (**3.2**).

3.8. C18-sorbent (ODS sorbent),

see latest version of QuPPe-PO-Method.

3.9. Ammonium formate (p.a.)

3.10. Ethylenediaminetetraacetic acid tetrasodium tetrahydrate (purity ≥ 99%),

e.g. 34103-M EMD Millipore/Merck (CAS No.: 13235-36-4)

3.11. 10% aqueous EDTA solution,

prepared by weighing 15,85 g ethylenediaminetetraacetic acid tetrasodium tetrahydrate into a 100 mL Erlenmeyer flask with stopper, dissolving it in 80 mL water and filling up to 100 mL with water. This solution contains 10 % (w/v) EDTA tetraanion.

[1] The list of compounds requiring plastic vessels is not comprehensive.

3.12. LC-MS/MS mobile phases

see latest version of QuPPe-PO-Method.

3.13. Pesticide Standards,

of known purity.

3.14. Dry ice,

see latest version of QuPPe-PO-Method.

3.15. Pesticide stock solutions,

see latest version of QuPPe-PO-Method. Mind to use plastic containers (see note under **2.11**).

3.16. Pesticide working solutions / mixtures,

see latest version of QuPPe-PO-Method. Mind to use plastic containers (see note under **2.11**).

3.17. Internal Standards (ISs),

of known purity.

3.18. IS Stock solutions,

see latest version of QuPPe-PO-Method. Mind to use plastic containers (see note under **2.11**).

3.19. IS-working solution I (IS-WSln 1) for spiking samples prior to extraction,

see latest version of QuPPe-PO-Method. Mind to use plastic containers (see note under **2.11**).

3.20. IS-working solution II (IS-WSln 2) for preparation of calibration standards,

see latest version of QuPPe-PO-Method. Mind to use plastic containers (see note under **2.11**).

4. Disclaimer

This method refers to several trade names of 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. Any consumables and chemicals used in the procedure should be periodically checked, e.g. through reagent blank tests, for any relevant levels of the analytes of interest.

5. Procedure

5.1. Sample preparation

To obtain representative test-portions from the laboratory sample, proceed as required by the respective regulations and guidelines.

Eggs are deshelled and homogenized by a hand-blender (**2.1**) until a free flowing mixture is obtained. Proceed similarly with **non-homogenized milk** (e.g. if fat has separated). Homogenized milk can be used as such.

Animal tissues (muscle, kidney and liver) are preferably milled cryogenically (e.g. using dry ice). This is done to reduce analyte degradation and particle sizes, with the latter resulting in improved homogeneity and residue accessibility. One possibility for cryogenic milling is to cut large units coarsely to ca 3x3 cm pieces, freeze them and then

mill them for ca. 1-2 minutes with a powerful mill. Then add dry ice (ca. 150-200 g per 500 g sample) and continue milling until barely any carbon dioxide fumes are observed. Alternatively fill a plastic or polystyrene container with a ca. 5-15 cm thick layer of liquid nitrogen and immerse the sample pieces into the liquid nitrogen. When completely frozen transfer the material into a powerful knife mill and grind at high speed until it gets a snow-like consistency. If necessary, crush large units with a hammer before milling. If the material starts defrosting during milling, add some more liquid nitrogen or dry ice and continue milling as described above. Place the homogenate immediately in the freezer.

Isolated and pre-homogenized animal fat, such as commercial butterfat or rendered lard may be used as such. Trimmed adipose tissue should be homogenized. This can be done either at room temperature using a high speed knife mill or cryogenically by cutting the fat in small pieces (e.g. 2x2 cm) freezing it out and homogenizing it with a powerful knife mill. For this place the frozen fat pieces into the mill, add dry ice (ca. 4:1 ratio) and mill until a free-flowing powder is obtained. Alternatively, immerse the fat pieces into liquid nitrogen and mill with a knife mill to obtain a free flowing powder. Fill the milled material into a suitable vessel or bag and freeze immediately.

5.2. Extraction / Centrifugation / Filtration

The general **analytical procedure at a glance** is shown in **Figure 1** for liver and milk and in **Figure 2** for animal fat.

5.2.1. Weighing of analytical portions

Weigh a representative analytical portion (ma) of the sample homogenate (**5.1**) into a 50 mL centrifuge tube (**2.2**). In case of animal tissues (e.g. liver, muscle) as well as milk and egg weigh 10 g ± 0.1 g of the homogenized sample. In case of animal fat weigh 5 g ± 0.05 g.

5.2.2. Adjustment of water content

Add water (**3.1**) to the analytical portion (**5.2.1**), to reach a total water content of ca. 10 g per portion.

The amount of water to be added to the analytical portion is shown in **Table 1.** No extra water is added in the case of animal fat.

Notes: Where no ISs are used or where they are added after extract aliquotation, water adjustment to 10 g is essential for minimizing the volumetric error to acceptable levels. Where the appropriate ISs are employed before any aliquotation, water adjustment is less critical and may be skipped for commodities containing >80% natural moisture, or for commodities containing >70% natural moisture if the analytical procedure involved the addition of 1 mL aqueous EDTA solution (see below). The water contained in the aqueous solution EDTA solution added during the extraction step (**5.2.3**) is also considered in the overall water content. Keep in mind that the water volume adjustments in **Table 1** are approximate.

Table 1: *Adjustment of water content for various matrixes of animal origin according to their natural water content. Further commodities will be added soon.*

Commodity	Sample weight	Typical natural water content in g/100 g	Water to be added	Volume EDTA solution	Water add. may be skipped *	IS-WSln1 added e.g.	Extra Formic acid	Extraction Solution
Cow's milk (whole fat)	10 g	85	0.5 mL	1 mL	Yes	100 µL	100 µL	10 mL MeOH + 1% FA **(3.6)**
Cow's milk (1.5% fat)	10 g	90	-	1 mL	Yes	100 µL	100 µL	
Liver	10 g	70	2 mL	1 mL	**No**	100 µL	100 µL	
Animal fat	5 g	-	-	-	Not applicable	100 µL	none	10 mL MeOH:Water (7:3) + 1% FA **(3.7)**

* if suitable IS is used before aliquotation

5.2.3. **Extraction**

a) Liver and Milk:

Add 10 mL acidified methanol (**3.6**) and an appropriate small volume (e.g. 100 µl) of the **IS-WSln-1** (**3.19**) containing isotopically labeled analogues of the analytes of interest (added IS mass = m_{IS}^{sample}).

In the case of **liver and milk add an extra amount of 100 µL formic acid** (**3.4**). Close the tube and shake for a few seconds to distribute the acid and allow proteins to coagulate.

Add 10% aqueous EDTA solution (**3.11**) and shake either for 1 min by hand or for 2-15 min by an automatic shaker.

Notes: Where no ISs are used the aim should be to reach a total volume of the liquid phase as close as possible to 20 mL, which corresponds to 0.5 g / 0.25 g sample per mL extract if 10 g / 5 g sample are used. This volume will mainly consist of the water naturally contained in the sample, the water added during the procedure (including that of the EDTA solution), the extraction solvent added, the IS solution added as well as the extra volume of formic acid. Volume contraction is also taking place to a certain degree and it is partly complemented by the addition of IS and formic acid. Further alternatives to avoid errors due to volumetric deviations are calibrations that compensate for recovery, such as the approach of standard additions to sample portions and the procedural calibrations approach using a suitable blank matrix.
For screening purposes the IS can be alternatively added to an aliquot of the sample extract (e.g. the 1 mL aliquot transferred to the autosampler vial, see below), assuming that 1 mL extract entails exactly 0.25 g sample equivalents. This way the added amount of IS per sample can be drastically reduced (e.g. 40-fold if added to 1 mL extract). The IS added at this step will compensate for matrix effects including retention-time shifts but not for recovery and volume deviations. The quantitative result should therefore be considered tentative. For more accuracy samples should be re-extracted with the IS being added to the analytical portion before aliquotation.

b) Animal fat (isolated fat or adipose tissue homogenate):

Add 10 mL acidified methanol with 30% water (3.6) and an appropriate small volume (e.g. 100 µl) of the IS-WSln-1 (**3.19**) containing isotopically labeled analogues of the analytes of interest (added IS mass = m_{IS}^{sample}). Close the tube shake well for a few seconds and place it in a water bath of 80°C for 3-4 minutes until the fat has completely melted. While still hot, shake intensively for 1 minute by hand or for 2-15 min by an automatic shaker, to ensure distribution of the polar pesticides into the aqueous phase.

Notes: Due to the poor miscibility of the aqueous methanol with the fat, the final extract volume can be considered as being 10 mL, which corresponds to 0.5 g sample per mL. For screening purposes the IS can be alternatively added to an aliquot of the sample extract (e.g. the 1 mL aliquot transferred to the autosampler vial, see below), assuming that 1 mL extract entails exactly 0.5 g sample equivalents. This way the added amount of IS per sample can be drastically reduced (e.g. 10-fold if added to 1 mL extract). See further commends under **5.2.3**. Although melting points of animal fat usually are between 30 and 50 °C it is more suitable to heat up the sample to at least 60 °C to ensure that the fat melts quickly and stays liquid during shaking.

5.2.4. **Freeze-Out and Centrifugation**

Depending on the available centrifugation equipment there is various options, e.g.:

(1) **Centrifugation following freeze-out:** Place the tubes with the extracts from **5.2.3** into a freezer (e.g. at ca, -80 °C for 30 min or for > 90 min at ca. -20 °C) and centrifuge them while still cold for 5 min at ≥3,000 g. Higher centrifugation forces (e.g. ≥10,000 g) are preferable.

(2) **Refrigerated high-speed centrifugation:** Centrifuge the extracts **5.2.3** for ≥20 min at high centrifugation speed (e.g. >10,000 g) and low temperatures (e.g. lower than -5 °C). Centrifugation time may be reduced to 5 min if the extract is pre-frozen.

Notes: Low temperatures reduce the solubility of interfering matrix components resulting in increased precipitation, which considerably facilitates the filtration step as well as the subsequent LC-MS/MS analysis by reducing matrix effects and increasing the lifespan of columns. It is recommended to proceed immediately with the next steps to avoid redissolvation of matrix components. Otherwise transfer an aliquot of the cold supernatant into a sealable container for later use.

5.2.5. dSPE and dilution with ACN for removal of lipids and protein precipitation

a) Liver and Milk:

Transfer a 2 mL aliquot of the supernatant from **5.2.4** into a 10 mL centrifuge tube with screw cap (**2.4**), which already contains 2 mL of acetonitrile (**3.3**) and 100 mg of C18-sorbent (**3.8**) and shake for 1 min. Then centrifuge for 5 minutes at >3,000 g (see **2.7**).

b) Animal fat:

Where the supernatant was isolated while still very cold, this step may be skipped. Otherwise, transfer a 4 mL aliquot of the supernatant from **5.2.4** into a 10 mL centrifuge tube with screw cap (**2.4**), which already contains 200 mg of C18-sorbent (**3.8**) and shake for 1 min. Then centrifuge for 5 minutes at >3,000 g (see **2.7**).

5.2.6. Filtration

a) Liver and Milk:

Transfer a 3 mL aliquot of the supernatant from **5.2.5-a)** into an ultrafiltration unit (**2.10**) and centrifuge at 3,000 g until enough filtrate is accumulated in the reservoir (5 min are typically enough). Transfer an aliquot of the filtrate into an autosampler vial.

b) Animal fat:

Withdraw an aliquot (e.g. 2-3 **m**L) of the supernatant from **5.2.4** or **5.2.5** using a syringe (**2.7**) and filter it through a syringe filter (**2.8**) either directly into an auto-sampler vial (2.9) or into a sealable storage vessel.

Notes: The cleaned-up extract will contain ca. **0.5 g sample equivalents per mL extract** in the case of animal fat and for all other commodities 0.25 g sample equivalents per mL where 10 g sample (e.g. milk, liver) are employed.
Instead of adding the IS 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 IS per sample can be drastically reduced (e.g. 40-fold[2] if added to 1 mL extract). The IS 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 IS being added in step Fehler! Verweisquelle konnte nicht gefunden werden.

[2] 10-fold in the case of animal fat

QuPPe-AO-Method at a glance – Liver and Milk

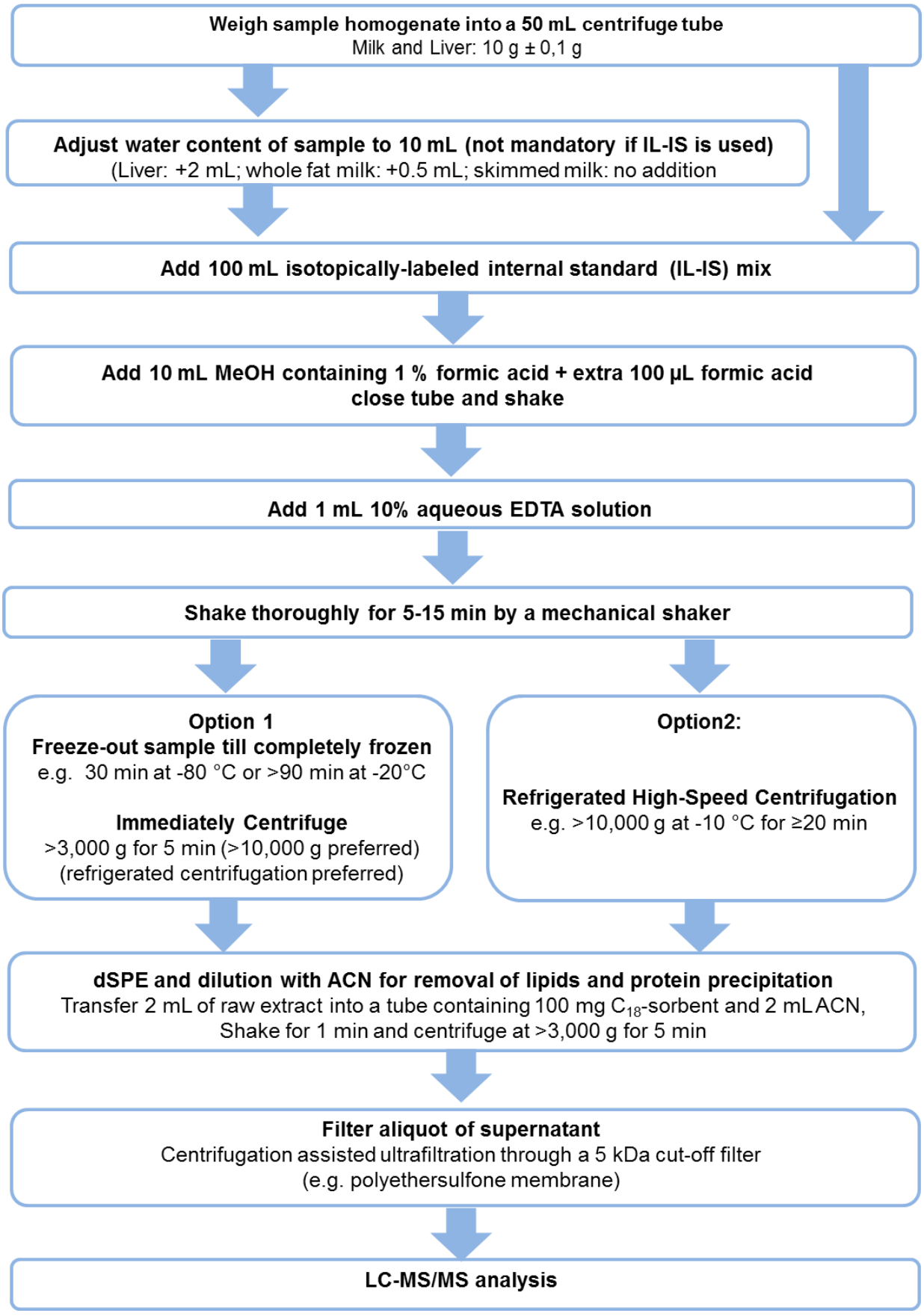

Figure 1: *Method at a glance liver and milk*

QuPPe-AO-Method at a glance – Animal Fat

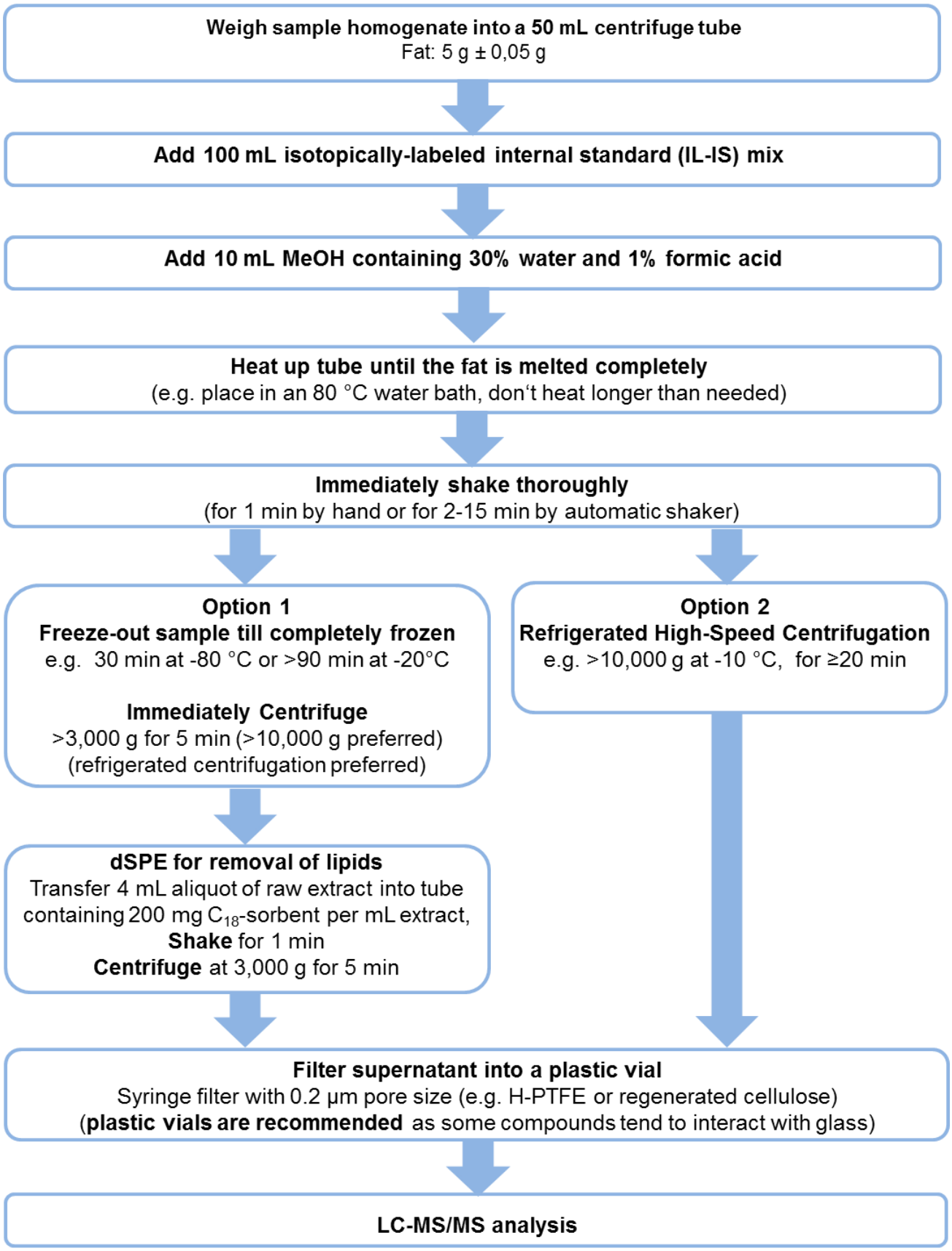

Figure 2: *Method at a glance fat*

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. In the case of fat samples incurred residues will be better simulated if the fat portions to be analyzed are first melted (water bath), then cooled down and spiked while the fat is still liquid (e.g. at 45 °C). Following fat gentle stirring to distribute the residues the spiked fat portions are placed in the fridge or freezer to cool down and solidify before being extracted as shown above.

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 2**. 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 2.
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 2: Exemplary pipetting scheme for the preparation of calibration standards

		Calibration standards								
		Solvent based (5.5.1)			Matrix-matched (5.5.2)					
		using IS[4]			without IS[5]			using IS[4]		
Calibr. levels in µg pesticide /mL OR in µg pesticide/ "IS-portion"[1]		0.05[6]	0.1	0.25	0.05	0.1	0.25	0.05	0.1	0.25
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 methanol (3.6)		925 µL	900 µL	825 µL	100 µL	75 µL	-	100 µL	75 µL	-
Pesticide working solutions (3.16) [2]	0.5 µg/mL	25 µL	50 µL	125 µL	25 µL	50 µl	125 µL	25 µL	50 µL	125 µL
IS-WSln-2 (0)[1,3]		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] One IS portion would correspond to the IS mass contained in 50 µL IS-WSln-2 (which in the particular example is added to each calibration standard).
[2] 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.
[3] For calibration standards of 1 mL it is highly recommended to prepare the IS-WSln-2 **(3.20)** by diluting IS-WSln-1 **(3.19)** 40-fold. The same volume and pipette as in **5.2.3** can be used for preparing the calibration standards.
[4] When employing IL-ISs matrix-matching and volume adjustments are of less importance as the IS 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 IS in the respective calibration standards and b) the ratio between the IS mass added to the sample (**5.2.3**) and the IS 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 calibr. standards of 1 mL).

[4] Where ILISs are not available/employed, matrix-matched standards **Table 2**) 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).

[6] The calibration level of 0.05 µg/mL corresponds to 0.1 mg pesticide /kg sample, when using 10 g test portions, or to 0.2 mg/kg sample when using 5 g test portions.

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 (**3.16**) 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.

An exemplary pipetting scheme according to Example A in shown in **Table 3**. The calculation of the mass fraction of the pesticide in the sample w_R is shown in **5.7.2.2**.

Table 3: 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 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)
Internal Standard (IS)	none	none	none	none
Added volume of pesticide working solution containing 1 µg/mL (3.16)	-	50 µL	100 µL	150 µL
Mass of pesticide added to each vial ($m_{pest}^{std\ add}$)	-	0.25 µg	0.5 µg	0.75 µg
Volume of solvent (for volume equalization)	150 µL	100 µL	50 µL	-
Final volume	1150 µL	1150 µL	1150 µL	1150 µL

5.5.4. Procedural calibration standards

Procedural calibration is most useful where numerous samples of the same commodity type are analyzed within the same badge and can help to largely compensate for recovery losses and matrix effects. An ideal precondition is the availability of a blank matrix of exactly the same type as the samples to be analyzed. For this prepare 4 analytical portions of a suitable blank sample and spike three of them with increasing amounts of the pesticides of interest (as done in recovery experiments, see also **5.4**). The aim should be to bracket the expected concentration range of the analytes in the samples. These spiked samples are extracted as described above and the obtained extracts are used in the same way as any other matrix-matched standards.

5.6. LC-MS/MS Measurement Conditions

For measurement conditions please refer to the latest version of the QuPPe-PO-Method. Any suitable LC and MS/MS conditions may be used. For food of animal origin only methods M 1.3, M 1.4, M 1.6 and M 4.2. have been tested so far. Exemplary chromatograms obtained by the various methods are shown below.

5.6.1. **Exemplary LC-MS/MS chromatograms (method M 1.3)**

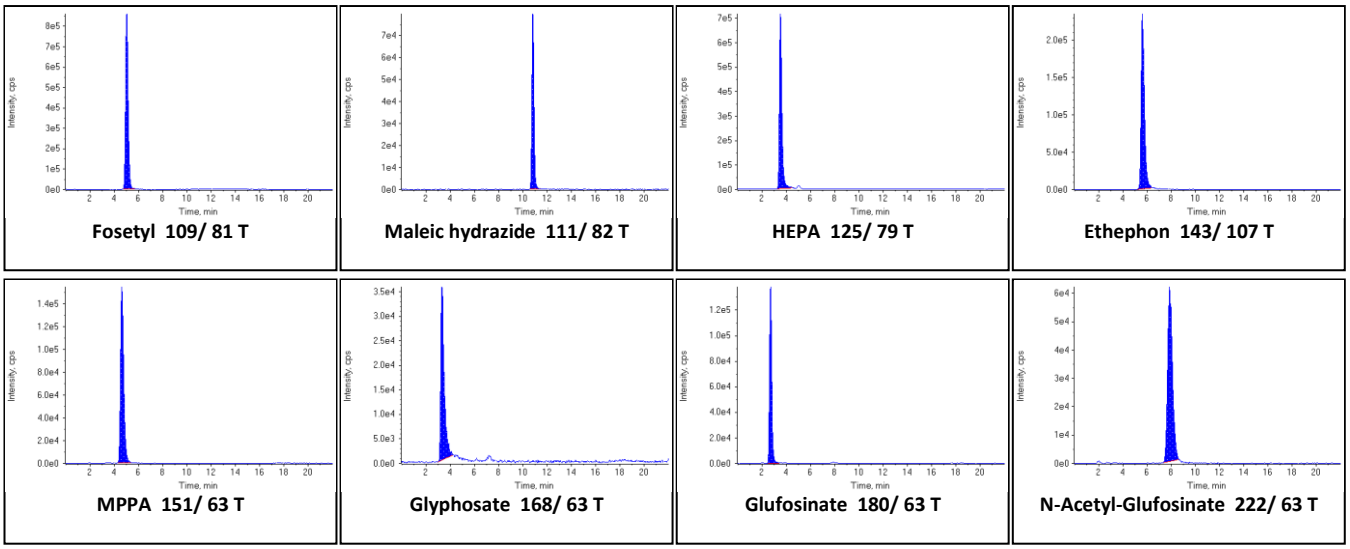

Figure 3: *Chromatograms of Fosetyl, Maleic Hydrazide, HEPA, Ethephon, MPPA, Glyphosate, Glufosinate, N-Acetyl-Glufosinate, at 0.1 µg/mL in MeOH (with 1% formic acid).*

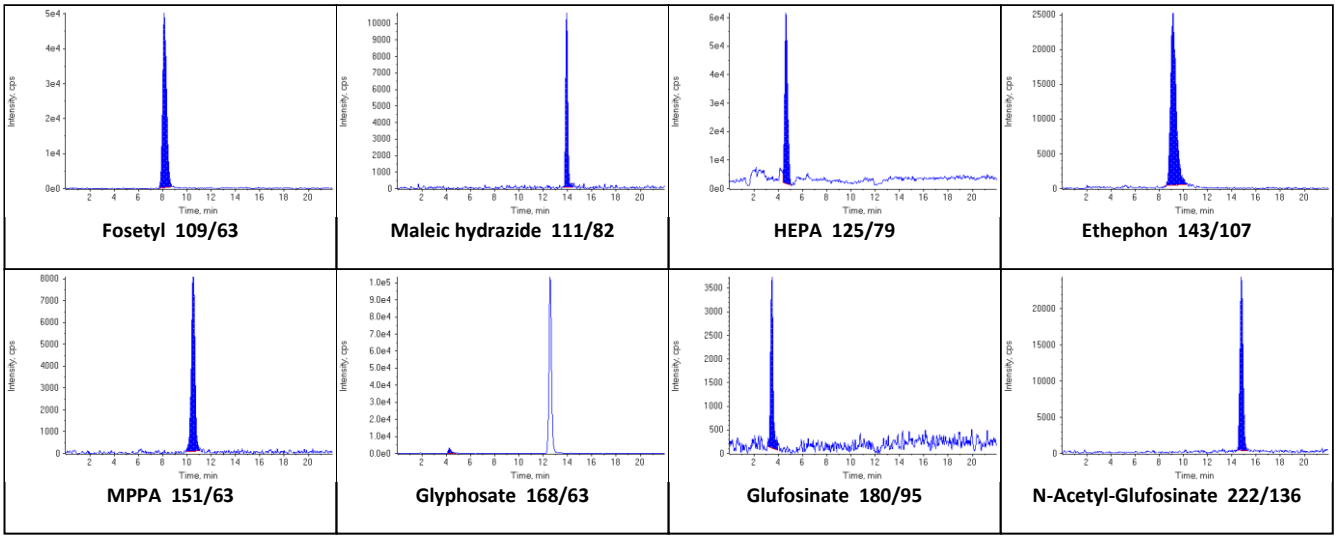

Figure 4: *Chromatograms of Fosetyl, Maleic Hydrazide, HEPA, Ethephon, MPPA, Glyphosate, Glufosinate, N-Acetyl-Glufosinate, at 0.0125 µg/mL respectively 0.05 mg/kg in whole cow's milk extract. The extract was prepared without use of EDTA solution, see former Version of QuPPe AO (V2).*

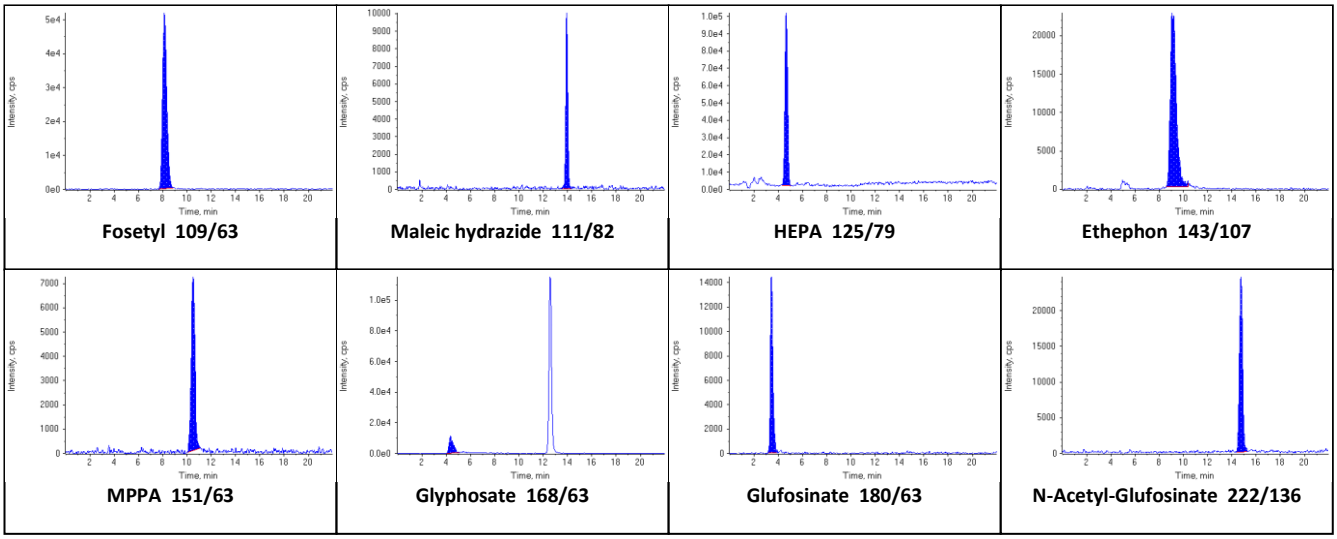

Figure 5: *Chromatograms of Fosetyl-Al, Maleic Hydrazide, HEPA, Ethephon, MPPA, Glyphosate, Glufosinate, N-Acetyl-Glufosinate, at 0.0125 µg/mL respectively 0.05 mg/kg in chicken eggs extract. The extract was prepared without use of EDTA solution, see former Version of QuPPe AO (V2).*

5.6.2. Exemplary LC-MS/MS chromatograms (method M 1.6)

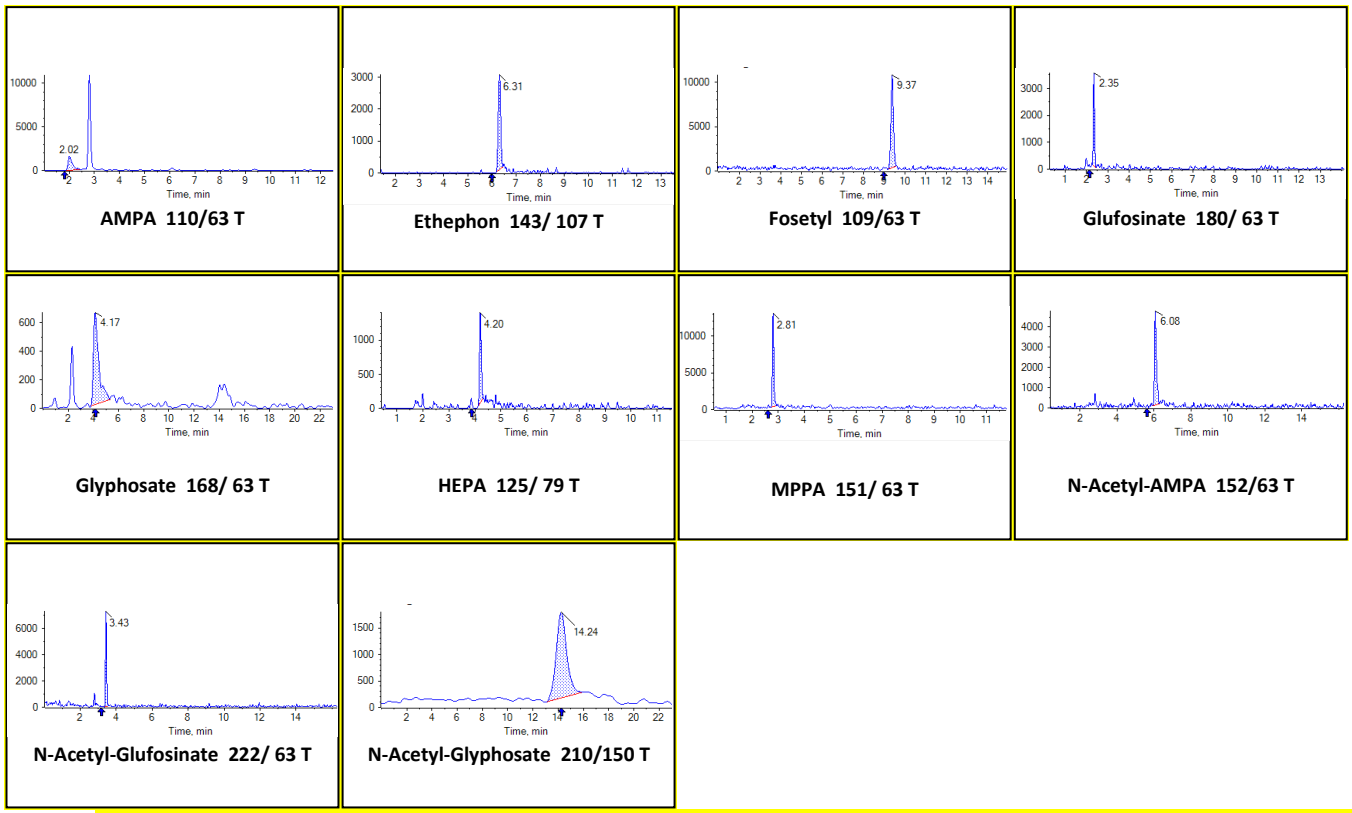

Figure 6 *: Chromatograms of AMPA, Ethephon, Fosetyl, Glufosinat, Glyphosate, Glufosinate, HEPA, MPPA, N-Acetyl-AMPA, N-Acetyl-Glufosinate at 0.0015 µg/mL in MeOH (with 1% formic acid) and N-Acetyl-Glyphosate at 0,003 µg/mL in MeOH (with 1% formic acid). The extract was prepared with use of EDTA solution; current Version of QuPPe AO (V3).*

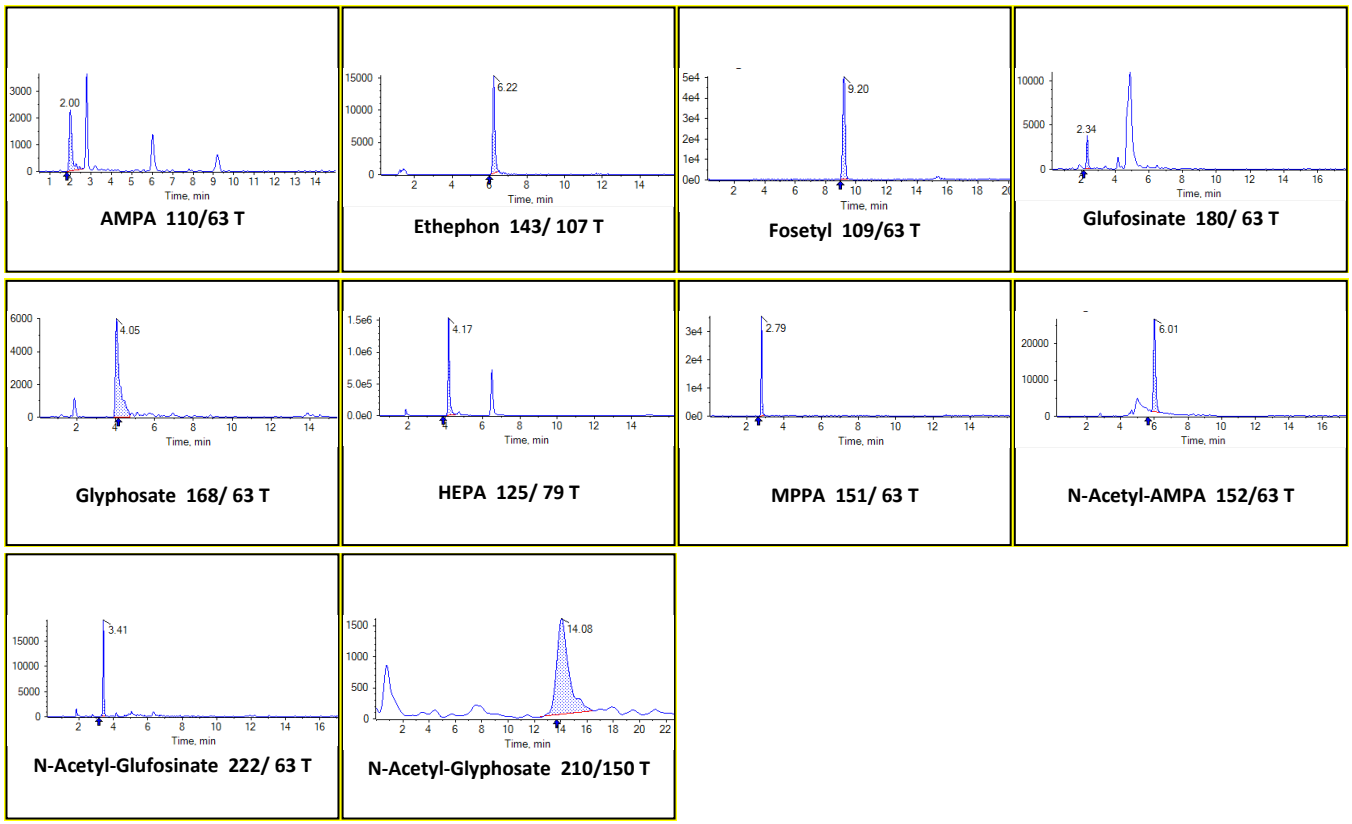

***Figure 7**: Chromatograms of AMPA, Ethephon, Fosetyl, Glufosinat, Glyphosate, Glufosinate, HEPA, MPPA, N-Acetyl-AMPA, N-Acetyl-Glufosinate and N-Acetyl-Glyphosate at 0,0125 µg/mL respectively 0,05 mg/kg in bovine liver extract. The extract was prepared with use of EDTA solution; current Version of QuPPe AO (V3).*

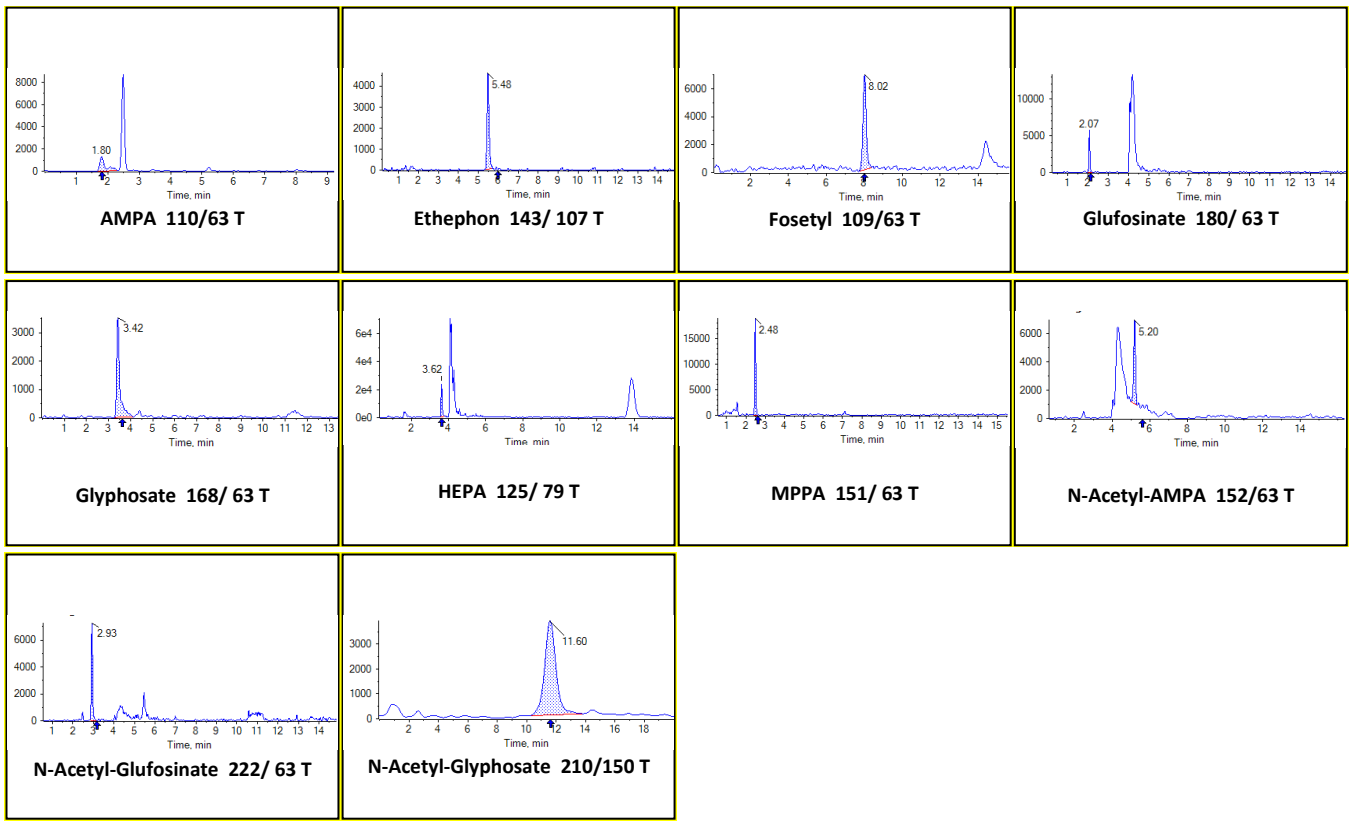

***Figure 8**: Chromatograms of AMPA, Ethephon, Fosetyl, Glufosinat, Glyphosate, Glufosinate, HEPA, MPPA, N-Acetyl-AMPA, N-Acetyl-Glufosinate at 0,0025 µg/mL respectively 0,01 mg/kg in whole cow's milk extract and N-Acetyl-Glyphosate at 0,0125 µg/mL respectively 0,05 mg/kg in whole cow's milk extract. The extract was prepared with use of EDTA solution; current Version of QuPPe AO (V3).*

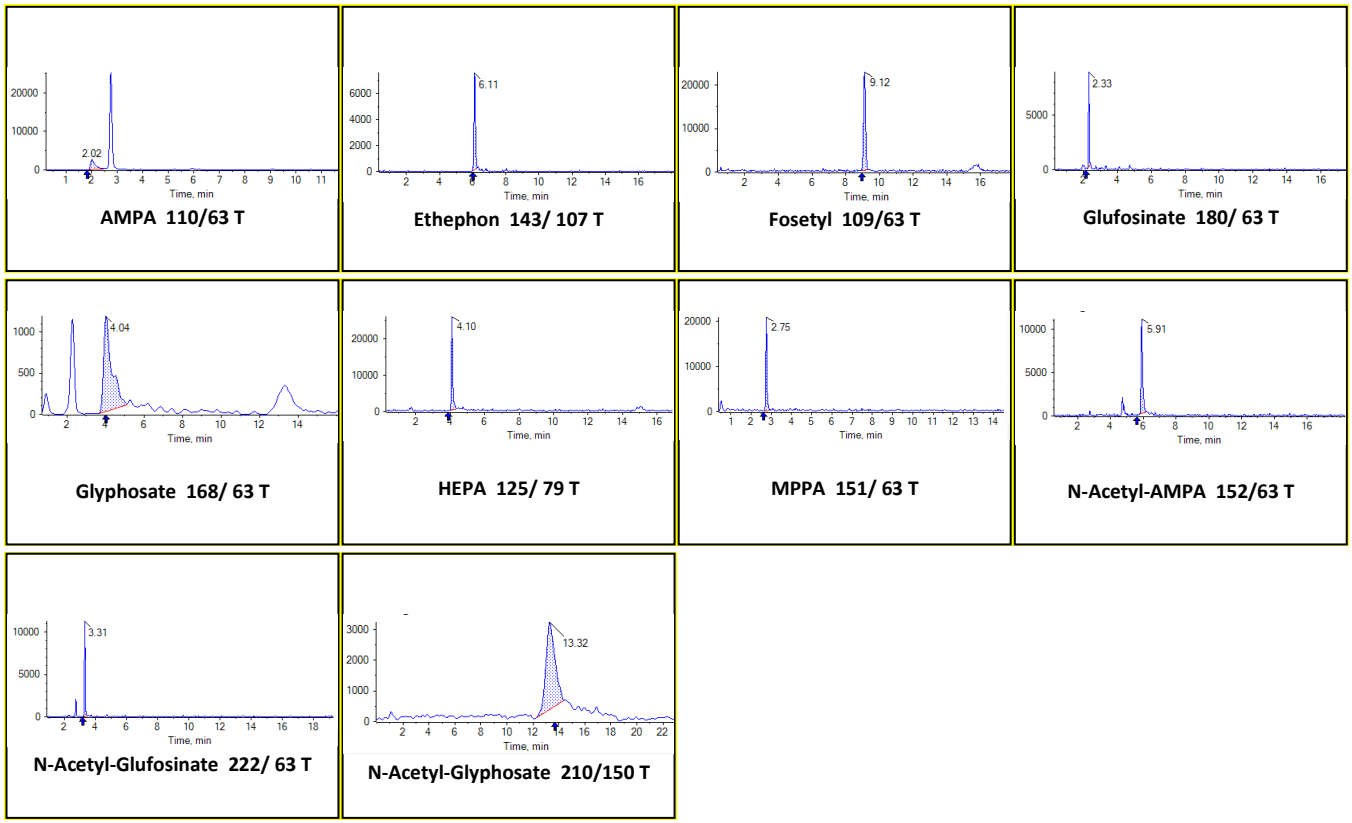

***Figure 9**: Chromatograms of AMPA, Ethephon, Fosetyl, Glufosinat, Glyphosate, Glufosinate, HEPA, MPPA, N-Acetyl-AMPA, N-Acetyl-Glufosinate at 0,0025 µg/mL respectively 0,005 mg/kg in butter oil extract and N-Acetyl-Glyphosate at 0,01 µg/mL respectively 0,02 mg/kg in butter oil extract. The extract was prepared according to the current Version of QuPPe AO (V3).*

5.6.3. **Exemplary LC-MS/MS chromatograms (method M 4.2)**

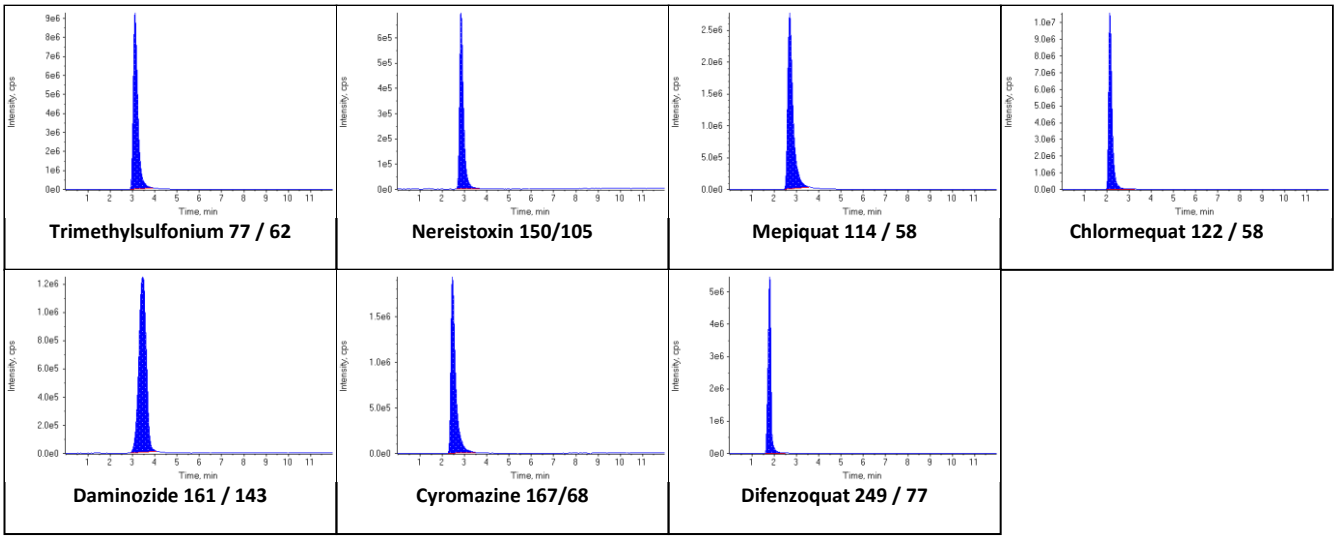

Figure 10: *Chromatograms of Trimethylsulfonium cation, Nereistoxin, Mepiquat, Chlormequat, Daminozide, Cyromazine, Difenzoquat at 0.1 µg/mL in MeOH (with 1% formic acid). The extract was prepared without use of EDTA solution, see former Version of QuPPe AO (V2).*

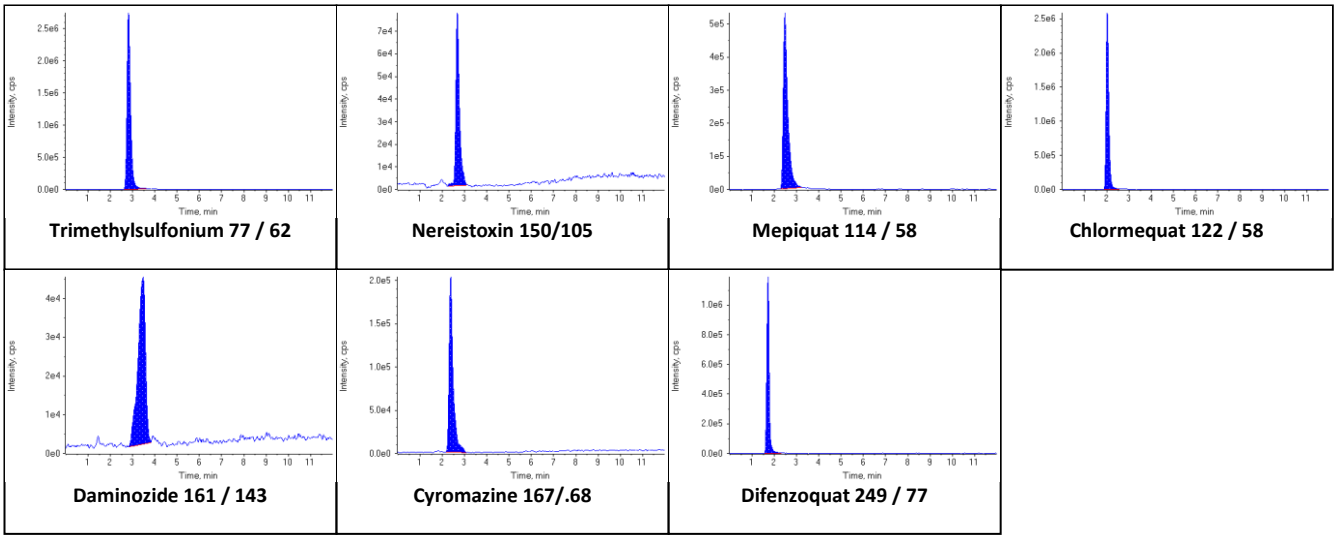

Figure 11: *Chromatograms of Trimethylsulfonium cation, Nereistoxin, Mepiquat, Chlormequat, Daminozide, Cyromazine, Difenzoquat at 0.0125 µg/mL respectively 0.05 mg/kg in whole cow's milk extract. The extract was prepared without use of EDTA solution, see former Version of QuPPe AO (V2).*

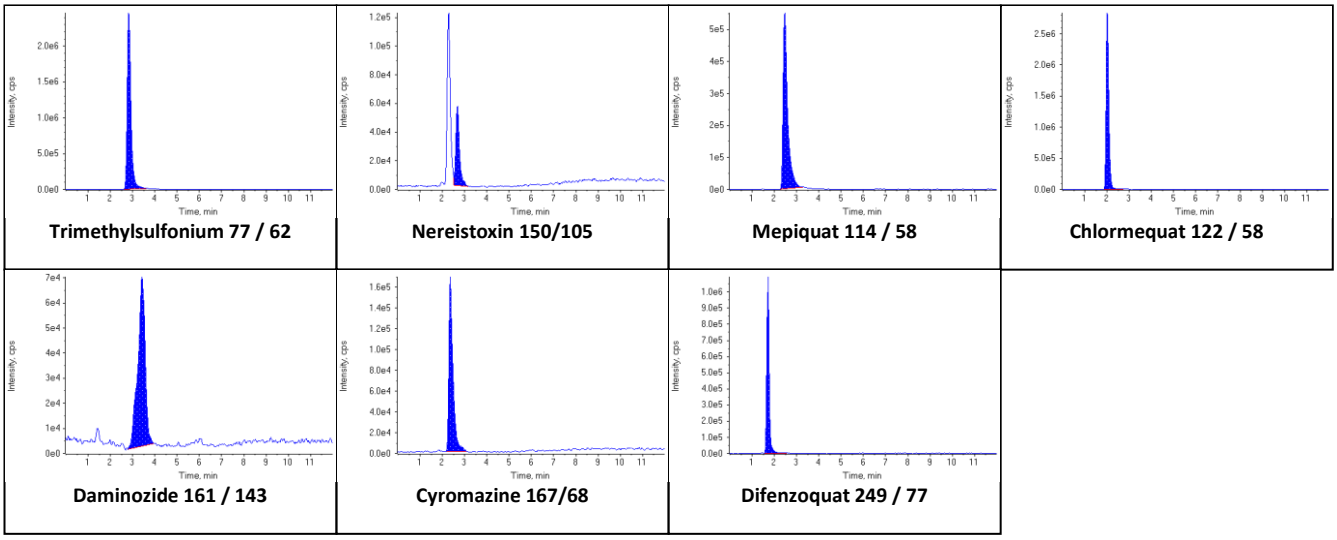

***Figure 12**: Chromatograms of Trimethylsulfonium cation, Nereistoxin, Mepiquat, Chlormequat, Daminozide, Cyromazine, Difenzoquat at 0.0125 µg/mL respectively 0.05 mg/kg in chicken eggs extract. The extract was prepared without use of EDTA solution, see former Version of QuPPe AO (V2).*

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 clean-up). 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

Table 4 : Overview of lowest successfully validated levels per matrix. The extract was prepared according to the current Version of QuPPe AO (V3). *Further commodities will be added soon.*

Method	Analyte	Matrix	Spiking Level (mg/kg)	n	Mean Recovery (%)	RSD
M 1.3	AMPA	Butter fat	0.02	5	102	2
	Ethephon	Butter fat	0.005	5	91	1
	Fosetyl	Whole cow´s milk	0.05	5	103	0.1
	Fosetyl	Butter fat	0.005	5	97	1
	Glufosinate	Butter fat	0.005	5	95	2
	Glyphosate	Butter fat	0.005	5	97	2
	HEPA	Butter fat	0.005	5	107	8
	MPPA	Butter fat	0.005	5	99	3
	N-Acetyl-AMPA	Butter fat	0.02	5	104	3
	N-Acetyl-Glufosinate	Butter fat	0.005	5	97	2
	N-Acetyl-Glyphosate	Butter fat	0.005	5	106	2
M 1.4	Phosphonic acid*	Whole cow´s milk	0.2	5	96	4
	Phosphonic acid*	Bovine liver	0.2	5	107	3
	Phosphonic acid*	Butter fat	0.2	5	97	1
	Chlorate	Whole cow´s milk	0.01	5	94	15
	Chlorate	Bovine liver	0.01	5	102	1
	Chlorate	Butter fat	0.01	5	100	1
	Perchlorate	Whole cow´s milk	0.01	5	108	1
	Perchlorate	Bovine liver	0.01	5	107	1
	Perchlorate	Butter fat	0.01	5	94	1
M 1.6	AMPA	Whole cow´s milk	0.01	5	107	8
	AMPA	Bovine liver	0.05	5	105	14
	AMPA	Butter fat	0.005	5	97	5
	Ethephon	Whole cow´s milk	0.01	5	92	2
	Ethephon	Bovine liver	0.01	5	103	8
	Ethephon	Butter fat	0.005	5	100	4
	Fosetyl	Whole cow´s milk	0.01	5	103	4
	Fosetyl	Bovine liver	0.01	5	98	2
	Fosetyl	Butter fat	0.005	5	100	2
	Glufosinate	Whole cow´s milk	0.01	5	104	11
	Glufosinate	Bovine liver	0.05	5	88	6
	Glufosinate	Butter fat	0.005	5	94	12
	Glyphosate	Whole cow´s milk	0.05	5	106	1
	Glyphosate	Bovine liver	0.05	5	113	6
	Glyphosate	Butter fat	0.005	5	102	7
	HEPA	Whole cow´s milk	0.01	5	104	2
	HEPA	Butter fat	0.005	5	89	9

Method	Analyte	Matrix	Spiking Level (mg/kg)	n	Mean Recovery (%]	RSD
	MPPA	Whole cow´s milk	0.01	5	95	9
	MPPA	Bovine liver	0.01	5	102	10
	MPPA	Butter fat	0.005	5	103	11
	N-Acetyl-AMPA	Whole cow´s milk	0.01	5	92	6
	N-Acetyl-AMPA	Bovine liver	0.01	5	76	18
	N-Acetyl-AMPA	Butter fat	0.005	5	99	2
	N-Acetyl-Glufosinate	Whole cow´s milk	0.01	5	101	6
	N-Acetyl-Glufosinate	Bovine liver	0.01	5	102	18
	N-Acetyl-Glufosinate	Butter fat	0.005	5	94	5
	N-Acetyl-Glyphosate	Whole cow´s milk	0.05	5	110	3
	N-Acetyl-Glyphosate	Bovine liver	0.05	5	107	6
	N-Acetyl-Glyphosate	Butter fat	0.02	5	106	6
M 4.2	Aminocyclopyrachlor	Whole cow´s milk	0.01	5	97	2
	Aminocyclopyrachlor	Butter fat	0.005	5	116	12
	Amitrole	Whole cow´s milk	0.01	5	93	12
	Amitrole	Bovine liver	0.05	5	106	6
	Amitrole	Butter fat	0.005	5	98	3
	Chlormequat	Whole cow´s milk	0.01	5	103	2
	Chlormequat	Bovine liver	0.01	5	103	2
	Chlormequat	Butter fat	0.005	5	93	6
	Chloridazon-desphenyl	Whole cow´s milk	0.01	5	86	11
	Chloridazon-desphenyl	Butter fat	0.005	5	104	2
	Cyromazine	Whole cow´s milk	0.01	5	103	2
	Cyromazine	Bovine liver	0.05	5	105	3
	Cyromazine	Butter fat	0.005	5	93	1
	Mepiquat	Whole cow´s milk	0.01	5	98	1
	Mepiquat	Bovine liver	0.01	5	107	8
	Mepiquat	Butter fat	0.005	5	95	4
	Morpholine	Whole cow´s milk	0.05	5	99	10
	Morpholine	Butter fat	0.02	5	99	4
	Nereistoxin	Whole cow´s milk	0.01	5	94	4
	Nereistoxin	Bovine liver	0.05	5	111	4
	Nereistoxin	Butter fat	0.005	5	87	4
	Trimethylsulfonium	Whole cow´s milk	0.01	5	105	1
	Trimethylsulfonium	Bovine liver	0.01	5	104	1
	Trimethylsulfonium	Butter fat	0.005	5	91	4
	Propamocarb	Whole cow´s milk	0.01	5	103	1
	Propamocarb	Bovine liver	0.01	5	101	1
	Propamocarb	Butter fat	0.005	5	97	0
	Melamine	Whole cow´s milk	0.01	5	100	2
	Melamine	Bovine liver	0.05	5	109	7
	Melamine	Butter fat	0.005	5	94	3

*Please take note that the results only refer to target transitions 81/79 which is unique to Phosphonic acid. When analyzing Phosphonic acid the interference of Phosphonic acid by Phosphoric acid has to be considered, especially in matrixes of animal origin (see also latest Version of QuPPe-PO-Method).

Table 5: *Overview of lowest successfully validated levels per matrix. The extract was prepared without use of EDTA solution, see former Version of QuPPe AO (V2).*

Method	Analyte	Matrix	Spiking Level (mg/kg)	n	Mean Recovery (%]	RSD
M 1.3	Ethephon	Whole cow´s milk	0.1	5	99	4
	Ethephon	Chicken Egg	0.1	5	114	4
	Fosetyl	Whole cow´s milk	0.1	5	99	2
	Fosetyl	Chicken Egg	0.1	5	104	2
	Glufosinate	Whole cow´s milk	0.1	5	94	9
	Glufosinate	Chicken Egg	0.1	5	100	4
	Glyphosate	Chicken Egg	0.1	5	117	1
	HEPA	Whole cow´s milk	0.1	5	105	1
	HEPA	Chicken Egg	0.1	5	102	3
	Maleic hydrazide	Whole cow´s milk	0.1	5	106	4
	Maleic hydrazide	Chicken Egg	0.1	5	107	5
	MPPA	Whole cow´s milk	0.1	5	103	5
	MPPA	Chicken Egg	0.1	5	100	8
	N-Acetyl-Glufosinate	Whole cow´s milk	0.1	5	103	2
	N-Acetyl-Glufosinate	Chicken Egg	0.1	5	104	3
M 1.4	Phosphonic acid*	Whole cow´s milk	0.02	5	103	4
	Phosphonic acid*	Chicken Egg	0.05	5	99	3
	Chlorate	Whole cow´s milk	0.02	5	102	4
	Chlorate	Chicken Egg	0.05	5	97	3
	Perchlorate	Whole cow´s milk	0.02	5	104	3
	Perchlorate	Chicken Egg	0.05	5	97	2
M 4.1	Diquat	Infant formula	0.05	5	103	3
M 4.2	Amitrole	Infant formula	0.03	5	103	5
	Nicotine	Infant formula	0.02	5	91	6
	Trimethylsulfonium	Whole cow´s milk	0.1	5	99	1
	Trimethylsulfonium	Chicken Egg	0.1	5	90	1
	Nereistoxin	Whole cow´s milk	0.1	5	98	2
	Nereistoxin	Chicken Egg	0.1	5	98	3
	Mepiquat	Whole cow´s milk	0.1	5	98	2
	Mepiquat	Chicken Egg	0.1	5	100	1
	Chlormequat	Whole cow´s milk	0.1	5	102	2
	Chlormequat	Chicken Egg	0.1	5	98	2
	Daminozide	Whole cow´s milk	0.1	5	90	4
	Daminozide	Chicken Egg	0.1	5	91	7
	Cyromazine	Whole cow´s milk	0.1	5	100	1
	Cyromazine	Chicken Egg	0.1	5	104	2
	Difenzoquat	Whole cow´s milk	0.1	5	92	2
	Difenzoquat	Chicken Egg	0.1	5	78	2
M 8	1,2,4-Triazole (TRZ)	Whole cow´s milk	0.2	5	87	7
	Triazole acetic acid (TAA)	Whole cow´s milk	0.02	5	89	6
	Triazole alanine (TA)	Whole cow´s milk	0.02	5	85	21
	Triazole lactic acid (TLA)	Whole cow´s milk	0.02	5	97	4

*****Please take note that the results only refer to target transitions 81/79 which is unique to Phosphonic acid.

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**
A.1.1.1 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
General revision of document	Dec. 2018	V3
Elaboration of the chapter concerning homogenization		
Optimized Extraction Method for milk, liver involving addition of EDTA;		
New extraction method for fat		
New LC-Method M 1.6 applied (chromatograms)		
Addition of Validation Data		