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

- **Version 6** (Aug 2011, Document History, see page 37)

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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 foods of plant origin such as fruits (including dried fruits), vegetables, cereals and processed products thereof as well as honey.

Residues are extracted from the test portion following water adjustment and the addition of acidified methanol. The mixture is centrifuged, filtered and directly analyzed by LC-MS/MS. Various options for the simultaneous LC-MS/MS 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 (IL-ISTDs). So far available, these IL-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 the sample preparation as well as matrix-effects during measurement.

2. Apparatus and Consumables

2.1. Powerful sample processing equipment,

e.g. Stephan UM 5 or Retsch Grindomix GM 300.

2.2. 50 mL centrifuge tubes with screw caps,

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

2.3. Automatic pipettes,

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

2.4. 10 mL solvent-dispenser,

for the acidified methanol (**3.5**).

2.5. Centrifuge,

suitable for the centrifuge tubes employed in the procedure (**2.2**) and capable of achieving > 2500 g.

2.6. Syringe filters,

e.g. Polyester filters 0.45 µm pore size.

2.7. Syringes

e.g. 2 or 5 mL disposable polypropylene syringes suitable for **2.6.**

2.8. Autosampler vials,

suitable for LC auto-sampler, use plastic vials when Paraquat, Diquat, Streptomycin and Glyphosate are within the scope.

2.9. Volumetric flask with stoppers,

for the preparation of stock and working solutions. E.g. 20 mL; 25 mL; 50 mL, 100 mL glass flasks. Use plastic flasks / stoppers for solutions containing Paraquat, Diquat, Streptomycin and Glyphosate.

2.10. LC-MS/MS instrumentation,

equipped with ESI source and appropriate columns, see details in chapters **5.6.1** till **5.6.6** .

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. Acidified methanol,

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

3.6. Citric acid monohydrate (p.a.)

3.7. Dimethylamine,

e.g. 40 % by Fluka (article-no. 38940).

3.8. Ammonium formate (p.a.)

3.9. Pesticide Standards,

of known purity.

3.10. Dry ice,

technical grade can be used, but it should be periodically demonstrated not to contain any pesticides at relevant levels.

3.11. Pesticide Standards,

of known purity.

3.12. Pesticide stock solutions,

e.g. 1 mg/mL solutions of pesticide standards (**3.9**) in a water miscible solvent (e.g. water (**3.1**), methanol (**3.2**), acetonitrile (**3.3**) or mixtures thereof). See solvent-suggestions in Table 12. Use plastic vessels in the case of Paraquat, Diquat, Streptomycin and Glyphosate.

3.13. Pesticide working solutions / mixtures,

prepared at appropriate concentrations by diluting pesticide stock solutions (**3.12**) of one or more pesticides with water-miscible solvents as required for the spiking of samples, in recovery experiments (**5.4**), or for the preparation of calibration standards (**5.5**). See suggestions in **Table 12** in the Annex. Use plastic vessels in the case of Paraquat, Diquat, Streptomycin and Glyphosate.

3.14. Internal Standards (ISTDs),

see details in **Table 13**.

3.15. ISTD Stock solutions,

e.g. 1 mg/mL solutions of ISTDs (**3.14**) in a water miscible solvent (e.g. methanol, acetonitrile, water or mixtures thereof). For solvent-suggestions see **Table 12**. Use plastic vessels in the case of Paraquat-D6, Diquat-D4, Dihydrostreptomycin and Glyphosate (1,2-$^{13}C_2$ ^{15}N).

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

prepared at appropriate concentrations by diluting ISTD stock solutions (**3.15**) of one or more ISTDs with water-miscible solvents. Suggestions for solvents and concentrations are shown in **Table 12** and **Table 14**. Use plastic vessels in the case of Paraquat-D6, Diquat-D4, Dihydrostreptomycin and Glyphosate (1,2-$^{13}C_2$ ^{15}N).

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

prepared at appropriate concentrations by diluting ISTD working solution I (**3.16**) with water-miscible solvents. Suggestions for solvents and concentrations are shown in **Table 12** and **Table 14**. Use plastic vessels in the case of Paraquat-D6, Diquat-D4, Dihydrostreptomycin and Glyphosate (1,2-$^{13}C_2$ ^{15}N), (see also subnote 3 in **Table 1**).

3.18. LC-MS/MS mobile phases,

see details in chapters **5.6.1** till **5.6.6**.

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. For fruits and vegetables cryogenic milling (e.g. using dry ice) is to be preferred to minimize degradations and improve homogeneity.

5.1.1. For dried fruits and similar commodities (< 30 % water content) the following procedure is proposed: Add 850 g of cold water to 500 g frozen dried fruits and homogenize the mixture using a strong mixer (**2.1**), if possible with addition of dry ice (**3.10**). 13.5 g of this homogenate will correspond to 5 g sample.

5.2. Extraction / Centrifugation / Filtration

5.2.1. Weigh a representative portion (m_a) of the sample homogenate (**5.1**) into a 50 mL centrifuge tube (**2.2**). In case of fresh fruits and vegetables as well as juices take 10 g ± 0.1 g of the homogenized sample. In case of dried fruits, dried vegetables, dried mushrooms take 5 g ± 0.05 g or 13.5 g ± 0.1 g of the re-hydrated and homogenized material according to **5.1.1** (corresponding to 5 g sample). In case of cereals, dried pulses and honey also take 5 g ± 0.05 g.

Notes:

- *Smaller sample portions may have to be used for extract-rich commodities, such as spices or fermented products, or commodities with very high water absorbing capacity not allowing proper extraction.*

5.2.2. Add water (**3.1**) to a content of ca. 10 g in total according to the indications in **Table 15**

Notes:

- *No water adjustment is needed where re-hydrated commodities (**5.1.1**) are employed.*
- *Where no ISTDs are used or where they are added after extract aliquotation, water adjustment is essential. Where the appropriate ISTDs are employed before any aliquotation has taken place water adjustment is less critical and can be skipped for commodities containing ≥80% water (see **Table 15**)*

5.2.3. Add 10 mL acidified methanol (**3.5**) and 50 µL of the ISTD-WS I (**3.16**) containing isotopically labeled analogues of one or more of the analytes of interest (added ISTD mass = m_{ISTD}^{sample}).

Notes:

- *The resulting extract volume, taking into account the natural water content of the sample and the water added in **5.2.2**, should be ca. 20 mL (corresponds to ca. 0.5 g sample per mL extract if 10 g sample is employed for extraction).*
- *For screening purposes the ISTD can be alternatively added to the (e.g. 1 mL) sample extract aliquot that is transferred to the auto-sampler vial (see **5.2.8**), assuming that 1 mL extract corresponds to exactly 0.5 g sample equivalents. This way the added amount of ISTD per sample can be drastically reduced (20-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**. Keep in mind that the final volume of the extract will deviate from 20 mL if water is not adjusted and additionally due to the ca. 2.5% volume contraction oc-*

*curring when methanol is mixed with water. The latter effect was considered in the water volumes listed in **Table 15**.*

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

Notes:

- *In case of dry products the 1 minute shaking is to be followed by a soaking period of 10 minutes and a subsequent second 1 minute vigorous shaking*
- *Alternatively mechanical shaking at prolonged shaking times (e.g.5-20 minutes) may be employed for all sample types (no soaking period for dry commodities is necessary in this case)*

5.2.5. For **Paraquat and Diquat** the 1 minute shaking is followed by a thermal treatment of 15 minutes at 80 °C in a water bath. Then shake again for 1 minute and wait for the sample to cool down to room temperature before centrifuging.

5.2.6. Centrifuge (e.g. for 5 min at >2500 g).

5.2.7. Using a syringe (**2.7**) filter an aliquot of the extract (e.g. 3 mL) through a syringe filter (**2.6**) into a sealable storage vessel.

Notes:

- *In case of Paraquat, Diquat, Streptomycin or Glyphosate use plastic storage vessel or transfer an aliquot (e.g. 1 mL) directly into a plastic auto-sampler vial.*

5.2.8. Transfer, as required, one or more aliquots (e.g. 1 mL each) of the filtered extract into autosampler vials (**2.8**)

5.3. Blank extracts

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

5.4. Recovery experiments

Weigh an appropriate portion (see **5.2.1)** of a blank commodity homogenate into a 50 mL centrifuge tube (**2.2**) and spike it with a suitable pesticide working solution (**3.13** and **Table 12**). Spike directly to the matrix, prior to any water or solvent addition. Use small volumes of pesticide working solutions (e.g. 50-300 µL), to avoid too strong dilution. Conduct sample preparation exactly as described in **5.2**.

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 IL-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[4]			without ISTD[5]			using ISTD[4]		
Calibration levels in µg pesticide /mL OR in µg pesticide/ "ISTD-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)		-	-	-	900 µL	900 µL	900 µL	850 µL	850 µL	850 µL
1:1 (v/v) mix of water (3.1) and acidified MeOH (3.5)		900 µL	850 µL	900 µL	50 µL	-	50 µL	50 µL	-	50 µL
Pesticide working solutions (3.13) [2]	**1 µg/mL**	50 µL	100 µL	-	50 µL	100 µL	-	50 µL	100 µL	-
	5 µg/mL	-	-	50 µL	-	-	50 µL	-	-	50 µL
ISTD-WS II (3.17)[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 ISTD portion would correspond to the ISTD mass contained in 50 µL ISTD-WS II (the volume 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 recommended to prepare the ISTD-WS II **(3.17)** by diluting 20-fold the ISTD-WS I **(3.16)**. The same volume and pipette as in **5.2.3** can then be used for the preparation of the calibration standards.*

*[4] When employing IL-ISTDs matrix-matching and volume adjustments are of less importance as the ISTD compensates for any matrix-related signal suppressions / enhancements. Solvent-based calibrations can be used here.. Important in this case is 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 calibra-*

*tion standard(s) (**5.5.1** and **5.5.2**). For convenience this mass ratio should be kept constant throughout all calibration levels (e.g. at 20:1 when preparing calibration standards of 1 mL).*

*[4] Where IL-ISTDs are not 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 total volume of the sample extracts is assumed to be exactly 20mL.*

[6] The calibration level 0.05 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 the relation between pesticide concentrations and the corresponding detection responses is linear throughout the relevant concentration range.

Several aliquots of the final sample extract are fortified with increasing volumes of an appropriate pesticide working solution (**3.13**) as shown in **Table 2**. This procedure requires knowledge of the approximate residue level in the sample ($w_{R(approx)}$). This info is derived from a preliminary analysis.

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 0.5 g sample equivalents per mL and an estimated residue level ($w_{R(approx)}$)of 0,5 mg/kg

Additions	Vial 1	Vial 2	Vial 3	Vial 4
Volume of sample extract	500 µl (= 0.25 g sample)	500 µl (= 0.25 g sample)	500 µl (= 0.25 g sample)	500 µl (= 0.25 g sample)
ISTD	none	none	none	none
Added volume of pesticide working solution containing 5 µg/ml (**3.13**)	-	50 µl	100 µl	150 µl
Resulting mass ($m_{pest}^{std\ add}$) of pesticide added to each vial		0.25 µg	0.5 µg	0.75 µg
Volume of solvent	150 µl	100 µl	50 µl	-
Final volume	650 µl	650 µl	650 µl	650 µl

5.6. LC-MS/MS Measurement Conditions

Any suitable LC and MS/MS conditions may be used. Below you will find some exemplary instrument measurement conditions.

5.6.1. Method 1 (for "Glyphosate & Co.")

Table 3: Proposed LC-MS/MS conditions for Ethephon, HEPA (ethephon metabolite), Glyphosat, AMPA (glyphosate metabolite), Glufosinate, MPPA (glufosinate metabolite), N-Acetylglufosinate (glufosinate metabolite)

Instrument parameters	**Conditions**		
Ionization mode	ESI neg		
Column/temperature **(see also notes below)**	Dionex IonPac AS 11 2 x 250 mm (P/N 44077); 40°C		
Pre-column	Dionex IonPac AG11 2 x 50 mm (P/N 44079)		
Pre-filters	e.g. Supelco column saver 2.0 µm Filter (optional)		
Eluent A	Water (**3.1**)		
Eluent B	1 mM citric acid in water adjusted to pH 11 with dimethylamine (DMA) **Note:** You will need ca. **0.5 mL DMA*** solution for 500 mL 1 mM citric acid in water **Make sure your eluent filters can handle alkaline solvents (see notes)!!**		
Gradient	**%A**	**Flow [mL/min]**	**Time [min]**
	100	0.3	0
	50	0.3	8
	50	0.3	15
	100	0.3	15.1
	100	0.3	23
Injection volume	10-20 µL (Note: in case of analyzing only ethephon 5 µL may be enough -depending on the instrument)		
Calibration standards and levels	e.g. 0.05 or 0.1 µg/ISTD-portion** + one level at the reporting limit		
Acquired mass transitions (m/z)	**Compound**	**Mass Transitions (m/z)**	
	Glyphosate Glyphosate 13C215N (ISTD)	168/63, 168/124, 168/150, 168/81 171/63	
	AMPA AMPA 13C15N (ISTD)	110/63, 110/79, 110/81 112/63	
	Ethephon Ethephon D4 (ISTD)	143/107, 143/79, 145/107 147/111	
	HEPA HEPA D4 (ISTD)	125/79, 125/95, 125/63 129/79	
	Glufosinate Glufosinate D3 (ISTD)	180/63, 180/136, 180/85, 180/95 183/63	
	N-AG N-AG D3 (ISTD)	222/63, 222/59, 222/136 225/63	
	MPPA MPPA D3 (ISTD)	151/63, 151/107, 151/133 154/63	

AMPA: Aminomethylphosphonic acid; MPPA: 3-Methylphosphinicopropionic acid; HEPA: 2-Hydroxyethylphosphonic acid (= ethephon-hydroxy), N-AG: N-Acetylglufosinate

** The value of 1 mL DMA solution given in Version 4 of this document was an error (it should have referred to 1 L)*

*** One ISTD portion is the absolute ISTD-mass contained in the prepared calibration standard solution (see also* **Table 1***).*

Notes on LC-operation:

- *Reconditioning: The column should be flushed from time to time (e.g. after injection of 50-100 extracts) 30 mM NaOH (e.g. for 1 hour at a flow rate of e.g. 0.3 mL/min = ca 18 mL in total).* ***The NaOH solution has to go directly into a waste bottle and SHOULDN'T reach the MS ion source!.*** *After conditioning the column should be flushed with 3-10 mL of water to remove NaOH. Before starting analyzing samples, run the system 3-4 times with the full method for the column to re-equilibrate.*

- *Pre-filters: If pre-filters are used these should be regularly checked. In case pressure significantly increases they should be exchanged. For practical and convenience reasons pre-filters are exchanged when performing other maintenance operations such as reconditioning or pre-column exchange. Losses of Glyphosate, that could be clearly linked to interactions with a dirty pre-filter, have been once observed.*

- *Pre-column (guard column): The pre-column should be exchanged as soon as a clear deterioration of the separation performance (worsening of peak-shape) is noticed. If after pre-filter exchange (see above) the pressure does not come back to normal levels the frit of the pre-column should be exchanged.*

- *Column storage: Following normal operation, as described above, the columns can be stored directly after any normal sequence/run (full gradient). For columns that were stored for longer periods (e.g. >2 weeks) reconditioning, as descried above, is recommended. Columns flushed with NaOH should be flushed again with water (3-10 mL) before putting them aside for storage.*

- *LC-system specifications: As the pH of the mobile phase is quite high, it is recommendable to* ***use alkali-compatible components****, e.g. metal frits instead of silica frits in the Eluent B reservoir; borosilicate 3.3 bottles instead of glass bottles for eluent B; rotor-seals from alkali-persistent materials, such as PEEK (polyetherketone) or Tefzel, rather than Vespel.*

Figure 1: Typical chromatograms of Glyphosate, AMPA, Glufosinate, MPPA and Ethephon

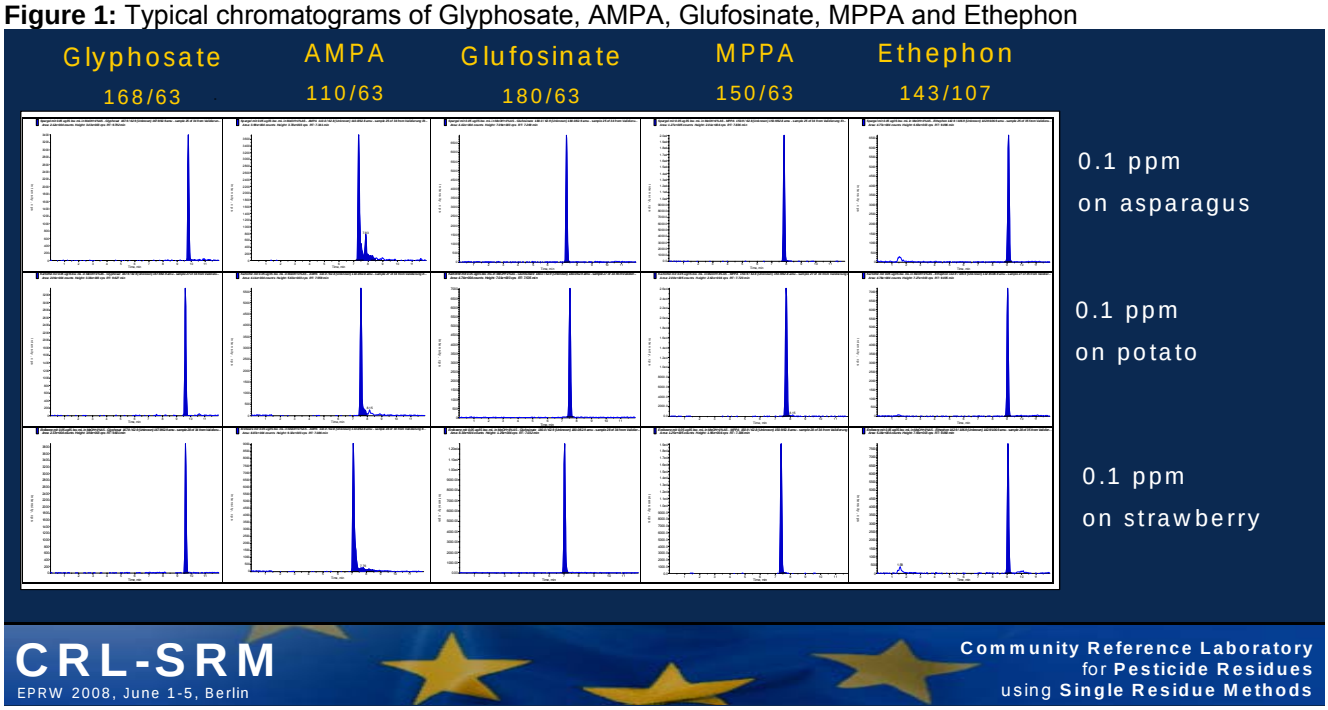
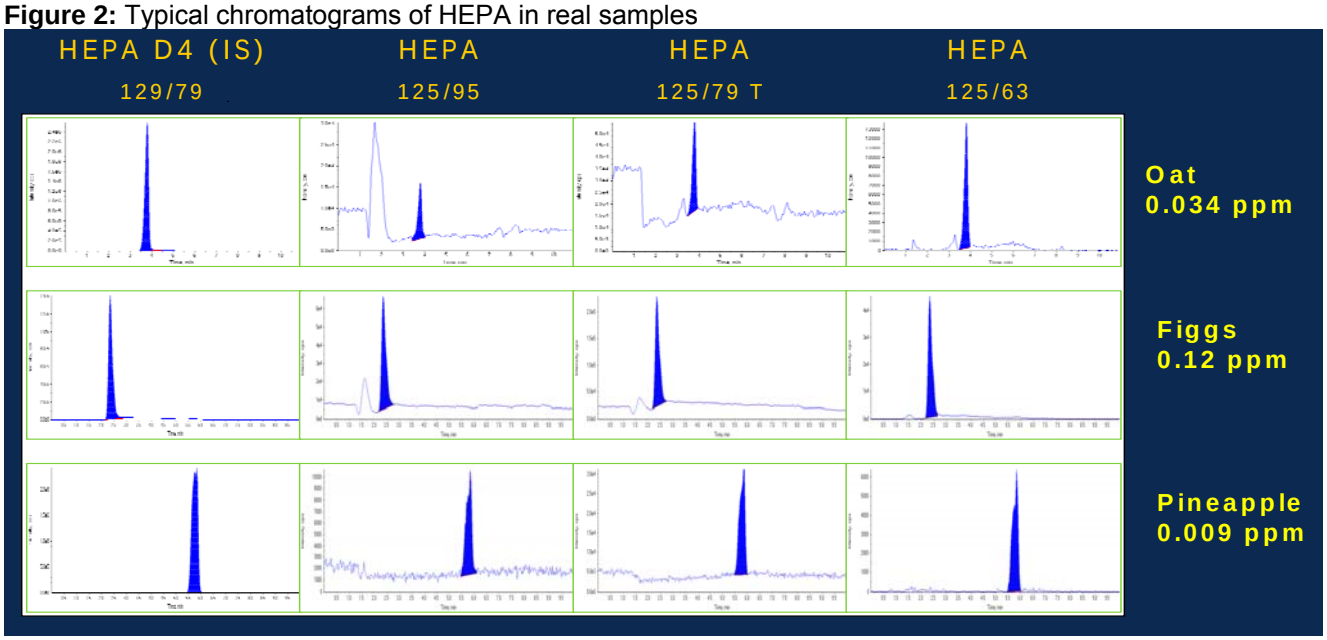


Figure 2: Typical chromatograms of HEPA in real samples



5.6.2. Method 2 (for Fosetyl and Maleic Hydrazide):

Table 4: Proposed LC-MS/MS conditions for Fosetyl-Al, Maleic Hydrazide and Perchlorate

Instrument parameters	
Ionization mode	ESI neg
Column/temperature	Obelisc R 2.1 x 150 mm 5 µm 100 Å; (SIELC; OR-21.150.0510)
Pre-filters	e.g. Supelco column saver 2.0 µm Filter
Pre-column	Obelisc R 2.1 x 10mm 5 µm (SIELC; OR-21.G.0510)
Eluent A	50 mmol NH_4-formate in water + 0.1 % formic acid use brown glass bottles
Eluent B	Acetonitrile
Gradient	**%A** / **Flow [mL/min]** / **Time [min]** 3 / 0.3 / 0 10 / 0.3 / 6 70 / 0.5 / 15 70 / 0.5 / 18 3 / 0.5 / 18.1 3 / 0.5 / 28
Injection volume	5 µL
Calibration standards and levels	e.g. 0.05 or 0.1 µg/ISTD portion*, + one level at the reporting limit For maleic hydrazide (MH) an additional level at 1 or 2 µg/ml may be useful as well, due to high residue levels; consider that MH is typically only relevant for potatoes and crops of the leek family (onions etc.)
Acquired mass transitions	**Compound** / **Mass Transitions (m/z)**
	Fosetyl-Al (detected as foseyl) Fosetyl-Al D15 (ISTD) / 109/81, 109/63 114/82 (D5-fosetyl)
	Maleic hydrazide Maleic hydrazide D2 (ISTD) / 111/82, 111/42, 111/55, 111/83 113/42
	Perchlorate Perchlorate $^{18}O_4$ (ISTD) / 99/83, 101/85 107/89

** One ISTD portion is the absolute ISTD-mass contained in the prepared calibration standard solution (see also* **Table 1***).*

Note: *It should be kept in mind that standards of isotopically labeled pesticides may contain small amounts of native (unlabelled) compounds as impurities. Typically these impurities are at low levels, so that the added amounts of native-pesticides resulting from the addition of ISTDs are insignificant. In the case of maleic hydrazide (MH), however the amount of ISTD added is comparably high due to the low detection sensitivity achieved for this compound. Assuming native MH being contained as impurity in D2-MH at 0.25 % the resulting concentration of native MH following the addition of 20 µg D2-MH to 10 g sample will be at 0.005 mg /kg sample. This aspect is to be considered in the selection of Reporting Limits as well as when judging residue levels in samples having low MRLs (e.g. baby food) or organic food.*

Figure 3: Typical chromatograms of Fosetyl-Al

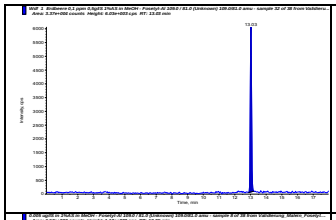
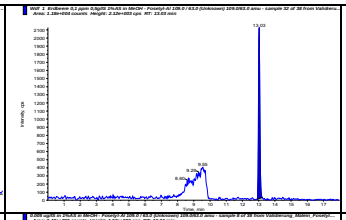
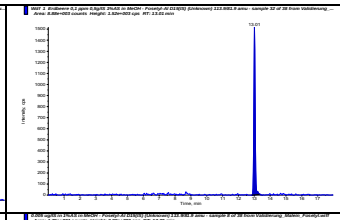
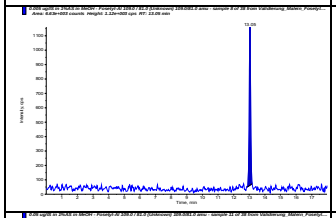
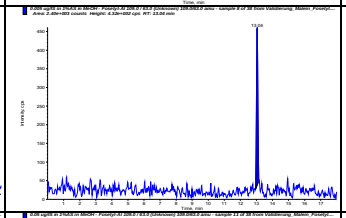
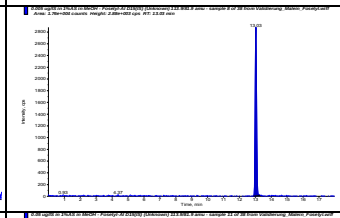
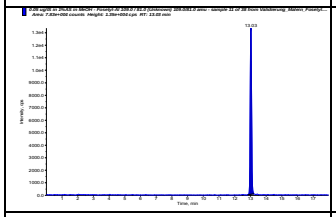
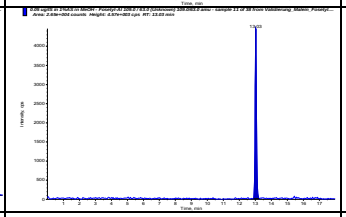
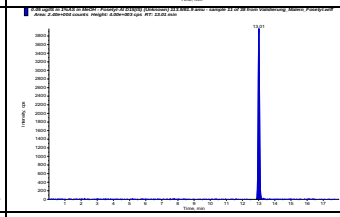
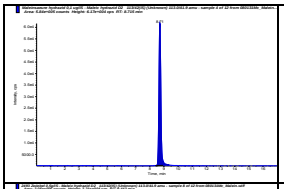
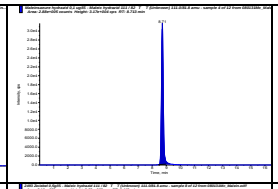
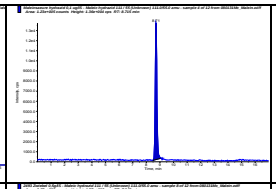
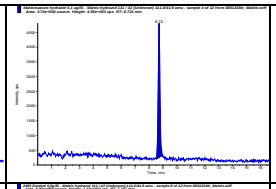
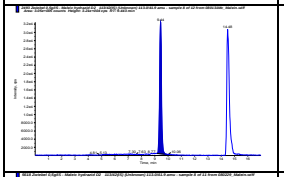
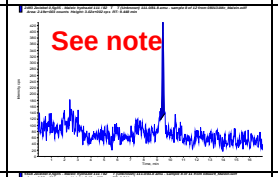
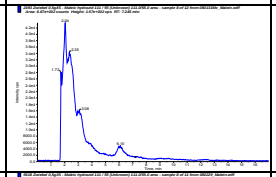
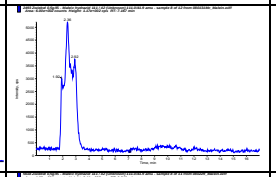
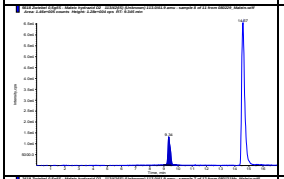
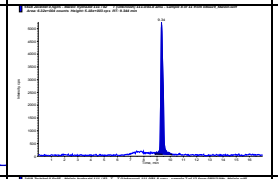
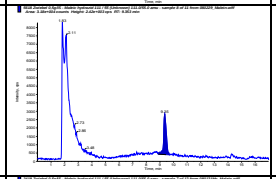
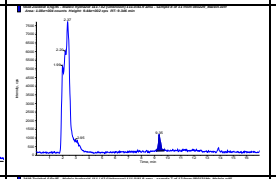
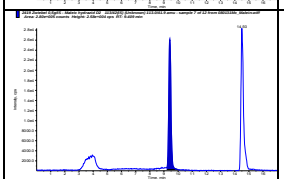
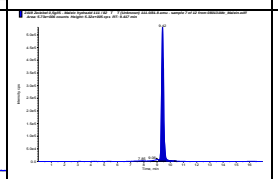
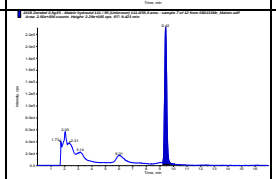
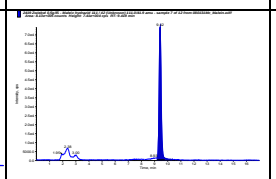
			**Recovery test on strawberry** **0.1 mg/kg** **= 0.05 µg/mL**
			**Fosetyl-Al solvent calib.** **0.005 µg/mL** **= 0.01 mg/kg**
			**Fosetyl-Al solvent calib.** **0.05 µg/mL** **= 0.1 mg/kg**
Fosetyl-Al 109 / 81	**Fosetyl-Al 109 / 63**	**Fosetyl-Al D15 114 / 82 (ISTD)**	

Figure 4: Typical chromatograms of Maleic Hydrazide

				**Maleic hydrazide solvent calib.** **= 0.05 µg/ml**
				**Onion sample 0.5 g/ml** **n.d.**
				**Onion sample 0.5 g/ml** **~ 0.1 mg/kg Maleic hydrazide**
				**Onion sample 0.5 g/IS** **~ 4 mg/kg Maleic hydrazide**
Maleic hydrazide D2 113/42 (ISTD)	**Maleic hydrazide 111 / 82 (target ion)**	**Maleic hydrazide 111 / 55**	**Maleic hydrazide 111 / 42**	

5.6.3. Method 3 (for Amitrole & Co)

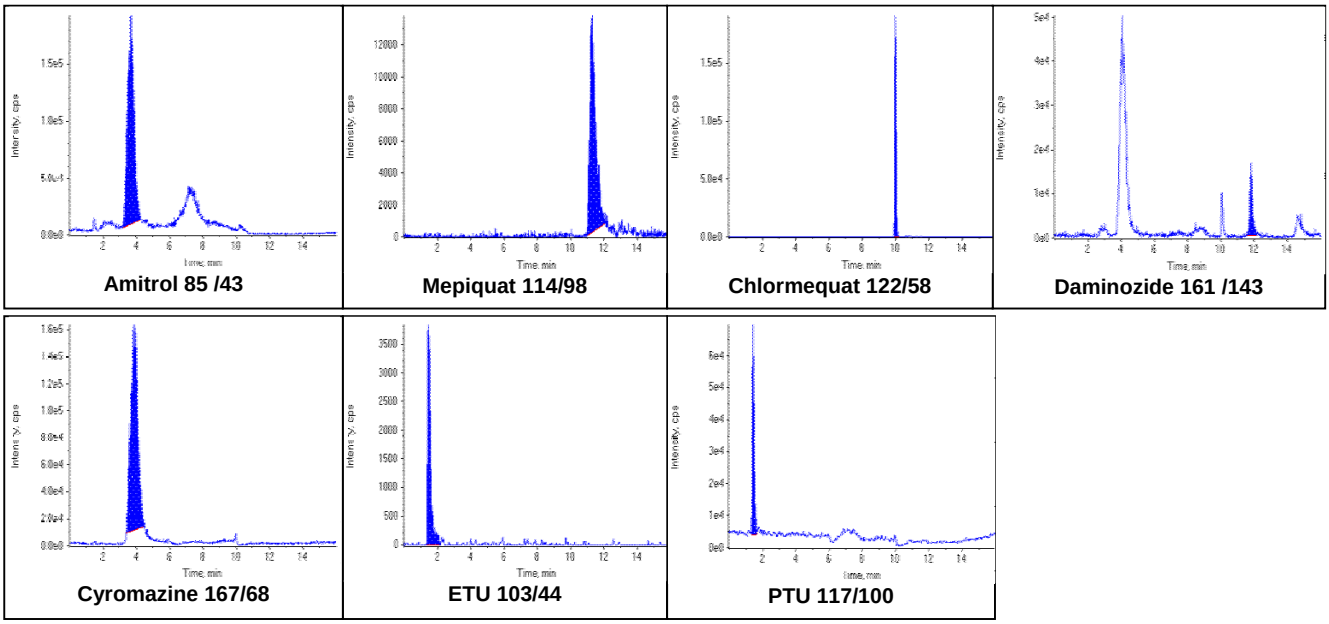
Table 5: Proposed LC-MS/MS conditions for Amitrole, Chlormequat, Mepiquat, Daminozide, ETU, PTU, Trimesium, Cyromazine

Instrument parameters	**Conditions**	
Ionisation mode	ESI pos	
Column/temperature	Obelisc R 2.1 x 150 mm 5 µm 100 Å (SIELC; OR-21.150.0510); 40°C	
Pre-column	Obelisc R 2.1 x 10 mm 5 µm (SIELC; OR-21.G.0510)	
Pre-filters	e.g. Supelco column saver 2.0 µm Filter	
Eluent A	5 mmol NH_4-formate in water Use brown glass bottles	
Eluent B	5 mmol NH_4-formate Acetonitrile/Water 95 :5 (v/v)	
Gradient	**%A** / **Flow [mL/min]** / **Time [min]** 2 / 0.4 / 0 2 / 0.4 / 2.5 80 / 0.4 / 5 80 / 0.4 / 11 2 / 0.4 / 11.1 2 / 0.4 / 18	
Injection volume	5 µL	
Calibration standards and levels	e.g. 0.05 or 0.1 µg/ISTD portion* + one level at the reporting limit	
Acquired mass transitions	**Compound**	**Mass Transitions (m/z)**
	Amitrole Amitrole ^{15}N (ISTD) Amitrole ^{15}N / ^{13}C (ISTD)	85/57, 85/43 86/43 87/xx
	Chlormequat Chlormequat D4 (ISTD)	122/58, 122/63, 124/58 126/58
	Mepiquat Mepiquat D3 (ISTD):	114/98, 114/58 117/101
	Daminozide Daminozide D6 (ISTD):	161/143, 161/101, 161/61, 161/115 167/149
	Cyromazine Cyromazine D4 (ISTD):	167/85, 167/125, 167/68 171/86
	ETU ETU D4 (ISTD):	103/44, 103/60, 103/86 107/48
	PTU PTU D6 (ISTD)	117/100, 117/58, 117/60 123/64
	Trimesium No ISTD currently available	77/62, 77/47 -

** One ISTD portion is the absolute ISTD-mass contained in the prepared calibration standard solution (see also* **Table 1***).*
ETU: Ethylenethiourea; PTU: Propylenethiourea ; Trimesium=Trimethylsulfonium-cation

***Note:** For Paraquat, Diquat and N,N-Dimethylhydrazine better run Method 4 (**5.6.4**)*

Figure 6: Typical chromatograms of Amitrole, Chlormequat, Mepiquat, Daminozide, ETU, PTU and Cyromazine in Apple at 0.01 mg/kg



5.6.4.Method 4 (for "Quats & Co")

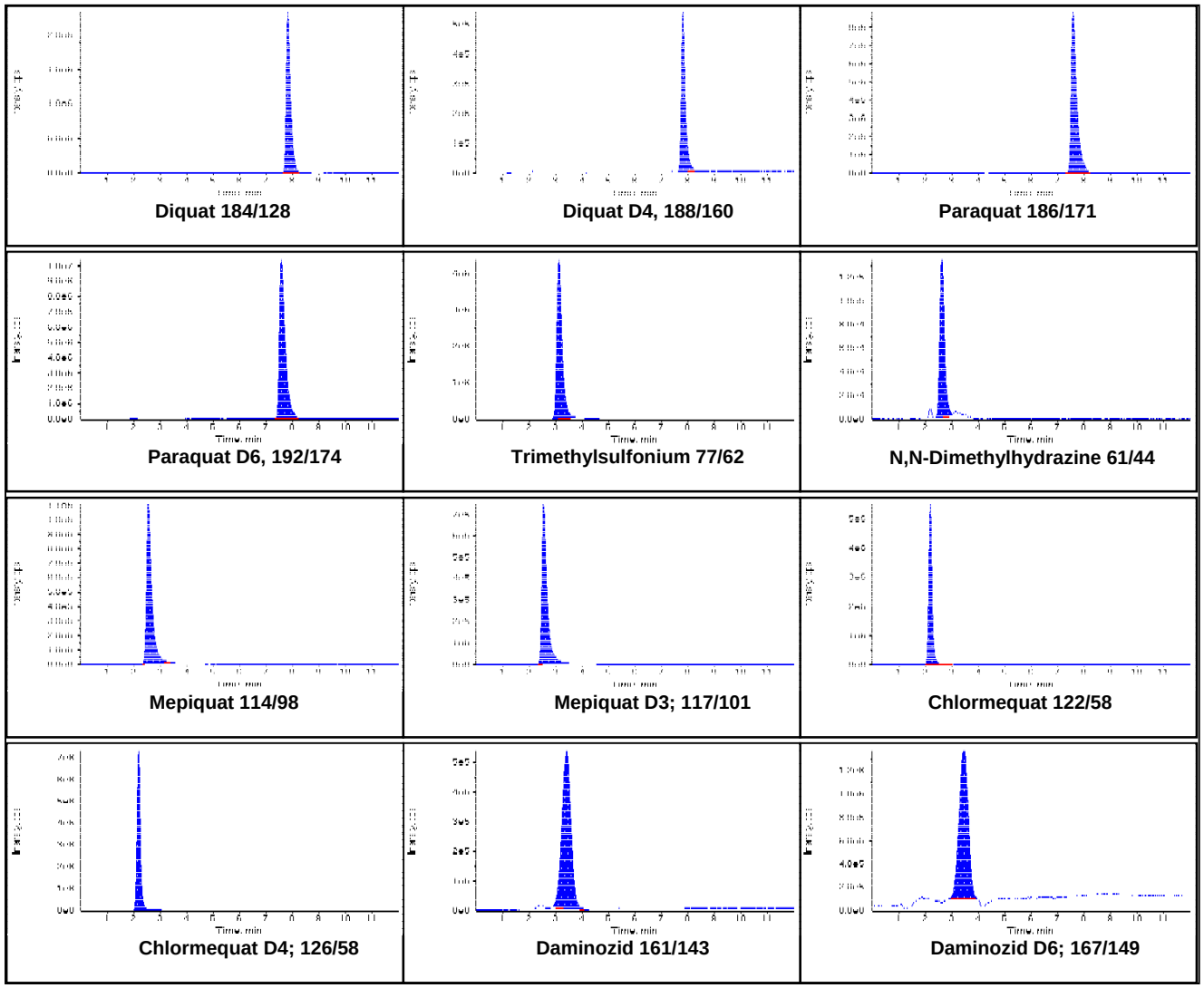
Table 6: Proposed LC-MS/MS conditions Diquat, Paraquat, Chlormequat, Mepiquat, Daminozide N,N-Dimethylhydrazine, Cyromazine, Trimesium (counterion of Glyphosate) and Nereistoxin.

Instrument parameters	**Conditions**	
Ionisation mode	ESI pos	
Column/temperature	Obelisc R 2.1 x 150 mm 5 µm 100 Å (SIELC; OR-21.150.0510); 40°C	
Pre-filters	e.g. Supelco column saver 2.0 µm Filter	
Pre-column	Obelisc R 2.1 x 10 mm 5 µm (SIELC; OR-21.G.0510)	
Eluent A	20 mmol NH_4-formate in water (adjust to pH 3 with formic acid), for this mix 1.8 mL formic acid (**3.4**) with 500 mL 20 mmol NH_4-formate in water **Use brown glass bottles!**	
Eluent B	Acetonitrile	
Gradient	**%A** / **Flow [mL/min]** / **Time [min]** 20 / 0.4 / 0 80 / 0.4 / 4 80 / 0.4 / 12 20 / 0.4 / 12.1 20 / 0.4 / 20	
Injection volume	10 µL	
Calibration standards and levels	e.g. 0.05 or 0.1 µg/ISTD portion* + one level at the reporting limit (**use plastic vials if Paraquat and Diquat are within your scope**!)	
Acquired mass transitions	**Compound**	**Mass Transitions (m/z)**
	Diquat** Diquat D4 (ISTD):	184/128, 183/157, 184/156 188/160
	Paraquat** Paraquat D6 (ISTD):	186/171, 171/77, 171/155 192/174
	Chlormequat Chlormequat D4 (ISTD):	122/58, 122/63, 124/58 126/58
	Mepiquat Mepiquat D3 (ISTD):	114/98, 114/58 117/101
	Daminozide Daminozide D6 (ISTD):	161/143, 161/101, 161/61, 161/115 167/149
	N,N-Dimethylhydrazine No ISTD currently available	61/44, 61/45 -
	Cyromazine Cyromazine D4 (ISTD):	167/85, 167/125, 167/68 171/86
	Trimesium No ISTD currently available	77/62, 77/47 -
	Nereistoxin Nereistoxin D6	150/105, 150/61, 150/71 156/105

** One ISTD portion is the absolute ISTD-mass contained in the prepared calibration standard solution (see also* **Table 1***).*
*** **Diquat and Paraquat require special extraction conditions** (see **5.2.5**)*

***Note:** For ETU, PTU and Amitrole better run Method 3 (**5.6.3**) or Method 5 (**5.6.5**), for Morpholin, Diethanolamine (DEA) and Triethanolamine (TEA) better run Method 7 (**5.6.7**). As DEA converts to Morpholine in the ion source, chromatographic separation of these two is paramount. With Method 4 these two peaks do not sufficiently separate.*

Figure 5: Typical chromatograms of Diquat, Paraquat, Chlormequat, Mepiquat, Daminozide, N,N-Dimethylhydrazine and Trimethylsulfonium-Cation (Trimesium) in Apple at 0.01 mg/kg



5.6.5. Method 5 (alternative method for Chlormequat and Mepiquat)

Table 7: Proposed alternative LC-MS/MS conditions for Chlormequat and Mepiquat

Instrument parameters	**Conditions**		
Ionisation mode	ESI pos		
Column/temperature	MonoChrom MS 100x2 mm; 5 µm (Varian); at 40°C		
Eluent A	5 mmol/L NH_4-acetate + 0.1% acetic acid		
Eluent B	Acetonitrile		
Gradient	**%A**	**Flow [mL/min]**	**Time [min]**
	5	0.4	0
	95	0.4	2
	95	0.4	5
	5	0.4	5.1
	5	0.4	15
Injection volume	5 µL		
Calibration standards and levels	e.g. 0.05 or 0.1 µg/ISTD portion*+ one level at the reporting limit		
Acquired mass transitions	**Compound**	**Mass Transitions (m/z)**	
	Chlormequat Chlormequat D4 (ISTD):	122/58, 122/63, 124/58 126/58	
	Mepiquat Mepiquat D3 (ISTD):	114/98, 114/58 117/101	
	ETU ETU D4 (ISTD):	103/44, 103/60, 103/86 107/48	
	PTU PTU D6 (ISTD)	117/100, 117/58, 117/60 123/64	

** One ISTD portion is the absolute ISTD-mass contained in the prepared calibration standard solution (see also* **Table 1***).*

For more information on method 5 please refer to the following document within the EURL homepage:
http://www.crl-pesticides.eu/library/docs/srm/meth_ChlormequatMepiquat_CrlSrm.pdf

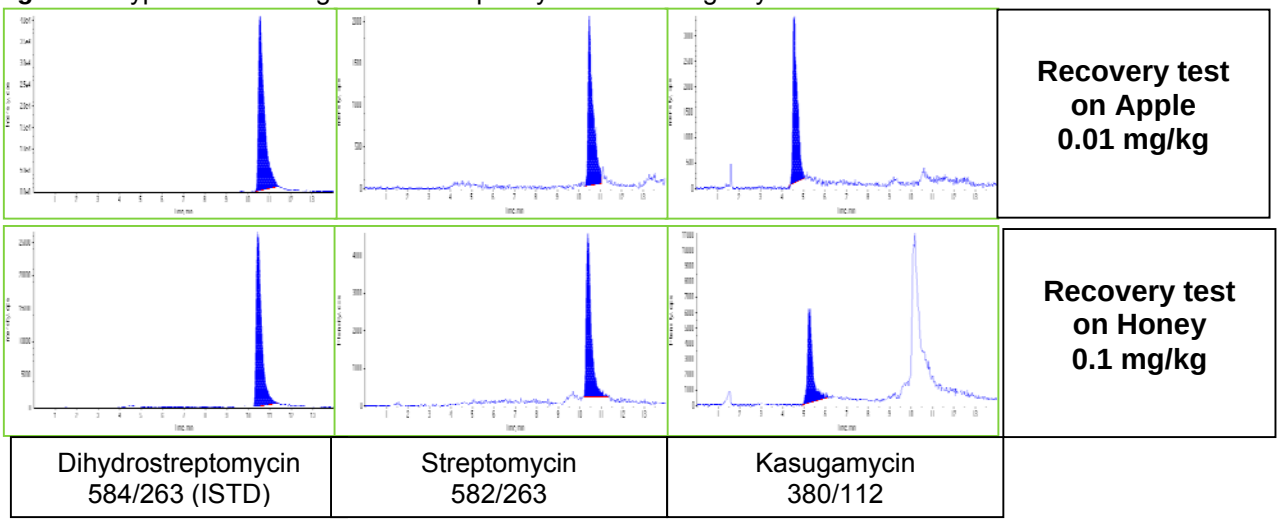
5.6.6. Method 6 (for Streptomycin and Kasugamycin)

Table 8: Proposed LC-MS/MS conditions Streptomycin and Kasugamycin

Instrument parameters	**Conditions**
Ionisation mode	ESI pos
Column	Obelisc R 2.1 x 150 mm 5µm 100 Å (SIELC; OR-21.150.0510); 40°C
Pre-filters	e.g. Supelco column saver 2.0 µm Filter
Pre-column	Obelisc R 2.1 x 10 mm 5 µm (SIELC; OR-21.G.0510)
Eluent A	0.1% formic acid in water
Eluent B	0.1% formic acid in acetonitrile
Gradient	**%A** / **Flow [mL/min]** / **Time [min]** 20 / 0.3 / 0 20 / 0.3 / 5 80 / 0.5 / 10 80 / 0.5 / 14 20 / 0.3 / 14.1 20 / 0.3 / 22
Injection volume	50 µL
Calibration standards and levels	e.g. 0.05 or 0.1 µg/ISTD portion+ one level at the reporting limit (use plastic vials if streptomycin is within your scope)
Acquired mass transitions	**Compound** / **Mass Transitions (m/z)** Streptomycin Dihydrostreptomycin (ISTD) / 582/263, 582/246, 582/ 221 584/263 Kasugamycin No ISTD currently available / 380/112, 380/200 -

** One ISTD portion is the absolute ISTD-mass contained in the prepared calibration standard solution (see also* **Table 1***).*

Figure 7: Typical chromatograms of Streptomycin and Kasugamycin



5.6.7.Method 7 (for Morpholine, Diethanolamine and Triethanolamine)

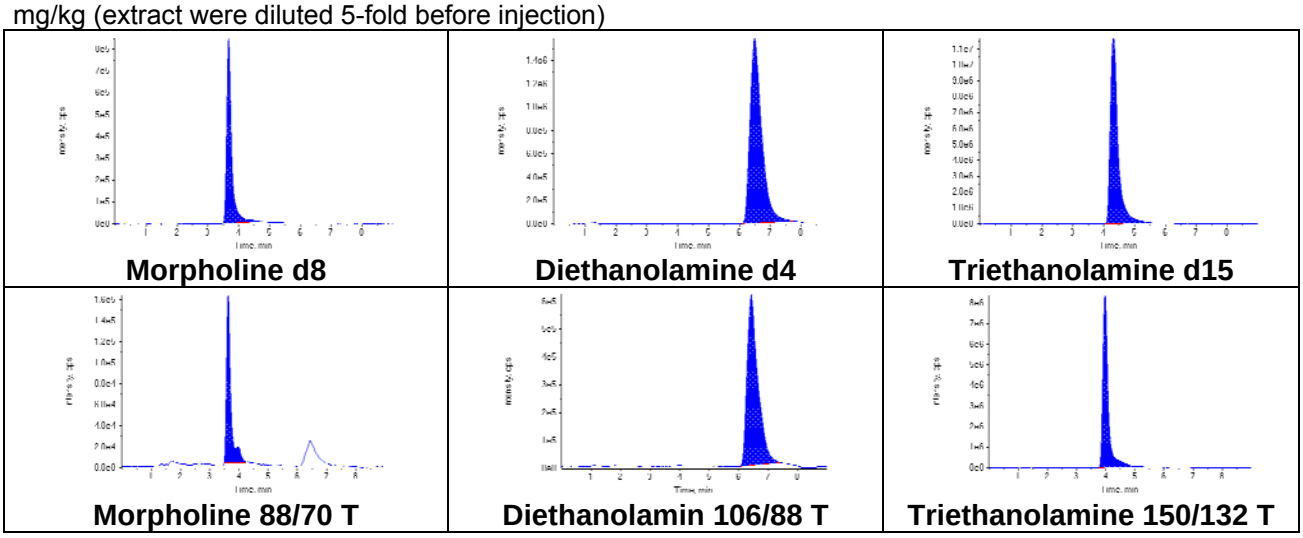
Table 9: Proposed LC-MS/MS conditions Morpholine, Diethanolamine and Triethanolamine

Instrument parameters	**Conditions**		
Ionisation mode	ESI pos		
Column	Dionex Acclaim Trinity P1 2.1 x 100 mm (3 µm) (P/N 071389); 40°C		
Pre-filters	e.g. Supelco column saver 2.0 µm Filter		
Pre-column	Dionex Acclaim Trinity P1 2.1 x 10 mm (3 µm) (P/N 071391)		
Eluent A	20 mmol NH_4-formate in water (adjust to pH 4 with formic acid) **Use brown glass bottles!**		
Eluent B	Acetonitrile		
Gradient	**%A**	**Flow [mL/min]**	**Time [min]**
	10	0.4	0
	10	0.4	10
Injection volume	5 µL		
Calibration standards and levels	e.g. 0.05 or 0.1 µg/ISTD portion+ one level at the reporting limit (use plastic vials if streptomycin is within your scope)		
Acquired mass transitions	**Compound**	**Mass Transitions (m/z)**	
	Morpholine Morpholine D8 (ISTD)	88/70, 88/45, 88/44 96/78	
	Diethanolamine (DEA) Diethanolamine D4 (ISTD)	106/88, 106/70, 106/45 110/92	
	Triethanolamine (TEA) Triethanolamine D12 (ISTD)	150/132, 150/87, 150/70 162/144	

** One ISTD portion is the absolute ISTD-mass contained in the prepared calibration standard solution (see also* **Table 1***).*

Morpholin, DEA and TEA are not pesticides, they are additive of waxes used to coat crops (citrus, apples and mangoes etc). They are included in this method for the sake of convenience and synergy. As these three compounds can be analyzed very sensitively 5-10-fold dilution of the extracts before injection is recommendable where possible, especially in absence of an ISTD requiring standard additions approach (**5.5.3**)

Figure 8: Typical chromatograms of Morpholine, Diethanolamine and Triethanolamine in Apple extracts at 0.05 mg/kg (extract were diluted 5-fold before injection)



5.7. Calibration and Calculations

5.7.1. Using ISTD

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

The following calculation approach requires that the ratio of the ISTD masses added to the test portions (5.2.3) and to the calibration standard(s) (5.5) (m_{ISTD}^{sample} / $m_{ISTD}^{cal\ mix}$) is known and constant. By keeping the ISTD constant throughout the calibration levels the peak ratio $PR^{cal\ mix}$ ($A_{pest}^{cal\ mix}$/ $A_{ISTD}^{cal\ mix}$) of each calibration level can be plotted against the absolute mass of the pesticide $m_{pest}^{cal\ mix}$ rather than the ratio $m_{pest}^{cal\ mix}$ / $m_{ISTD}^{cal\ mix}$(the $m_{ISTD}^{cal\ mix}$ is set as 1).

The calibration graph (to be plotted for each pesticide separately) is described by the following formula:

$$PR^{cal\ mix} = a_{cal} \times m_{pest}^{cal\ mix} + b_{cal} \quad (1)$$

The mass fraction (w_R) of a given pesticide in a given sample can be calculated as follows using the respective peak ratio of pesticide and internal standard obtained from the sample extract (PR^{sample} = A_{pest}^{Sample} / A_{ISTD}^{Sample}), the correction factor (m_{ISTD}^{sample} / $m_{ISTD}^{cal\ mix}$) as well as the weight of the test portion (m_a).

$$w_R = \frac{(PR^{Sample} - b_{cal})}{a_{cal}} \times \frac{1}{m_a} \times \frac{m_{ISTD}^{Sample}}{m_{ISTD}^{cal\ mix}} \left(\frac{\text{mg}}{\text{kg}}\right) \quad (2)$$

Reasonably (but not necessarily) the calibration standards should be prepared in such a way that the ratio m_{ISTD}^{sample} / $m_{ISTD}^{cal\ mix}$ equals 20 (the assumed volume ratio of sample extract versus calibration standard). The absolute masses of the ISTD-WS I and II do not need to be necessarily known (see also the notes of **Table 1**.

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

When adding the ISTD to an aliquot of the extract (e.g. 1 mL) theknowledge of the exact total volume of the sample extract becomes important. Water adjustment is thus essential and if it is done as described in 5.2.2 and **Table 15,** the total volume can be asumed to be exactly 20 mL. In this case 1 mL sample extract will correspond to 1/20th 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. Reasonably (but not

necessarily) $m_{ISTD}^{aliquot}$ should be derived using the following formula $m_{ISTD}^{aliquot} = m_{ISTD}^{sample} \times V_{aliquot}/20$, with m_{ISTD}^{sample} being the ISTD mass that would have been added to the entire sample portion according to 5.2.2 and **Table 15**.

Following the above, the mass fraction (w_R) of a given pesticide in a given sample can be calculated as follows using the respective peak ratio of pesticide and internal standard obtained from the sample extract ($PR^{sample} = A_{pest}^{sample} / A_{ISTD}^{sample}$), the correction factor ($m_{ISTD}^{aliquot} / m_{ISTD}^{cal\ mix}$) as well as the weight of the sample equivalents in the aliquot ($m_{aliquot} = m_a \times V_{aliquot}/20$).

$$w_R = \frac{(PR^{sample} - b_{cal})}{a_{cal}} \times \frac{1}{m_{aliquot}} \times \frac{m_{ISTD}^{aliquot}}{m_{ISTD}^{cal\ mix}} \left(\frac{\text{mg}}{\text{kg}}\right) \quad (3)$$

Variables used

Mass of pesticide in calibration mixture	$m_{pest}^{cal\ mix}$	µg
Mass of pesticide in final extract	m_{pest}^{sample}	µg
Mass of internal standard in calibration mixture	$m_{ISTD}^{cal\ mix}$	µg
Mass of internal standard added to test portion (sample)	m_{ISTD}^{sample}	µg
Mass of internal standard added to aliquot of sample extract	$m_{ISTD}^{aliquot}$	µg
Volume of sample extract aliquot used (**5.7.1.2** and **5.5.3**) to spike the ISTD or for standard additions	$V^{aliquot}$	ml
Mass of test portion	m_a	g
Mass of test portion represented in an aliquot	$m_{aliquot}$	g
Mass fraction of pesticide in the sample	w_R	mg/kg
Peak area of pesticide obtained from calibration standard (mixture)	$A_{pest}^{cal\ mix}$	(counts)
Peak area of ISTD obtained from calibration standard (mixture)	$A_{ISTD}^{cal\ mix}$	(counts)
Peak area of pesticide obtained from the injected extract	A_{pest}^{sample}	(counts)
Peak area of ISTD obtained from the injected extract	A_{ISTD}^{sample}	(counts)
Peak ratio of pesticide vs. ISTD obtained from calibration mixture	$PR^{cal\ mix}$	(dimensionless)
Peak ratio of pesticide vs. ISTD obtained from injected extract	PR^{sample}	(dimensionless)
Slope of calibration graph	a_{cal}	(dimensionless)
Bias of calibration graph (intercept)	b_{cal}	(dimensionless)

5.7.2. Not using ISTD

If no appropriate ISTDs are used it is of high importance to 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 sample extract is exactly 20 mL. 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

The calibration graph (to be plotted for each pesticide separately) is described by the following formula:

$$A_{pest}^{cal\ mix} = a_{cal} \times C_{pest}^{cal\ mix} + b_{cal} \quad (1)$$

The mass fraction (w_R) of a given pesticide in a given sample can be calculated as follows using the respective peak area of the pesticide obtained from the sample extract (A_{pest}^{sample}) and a correction factor (V) as well as the weight of the test portion (m_a).

$$w_R = \frac{(A_{pest}^{Sample} - b_{cal})}{a_{cal}} \times \frac{1}{m_a} \times V_{end} \left(\frac{\text{mg}}{\text{kg}}\right) \quad (2)$$

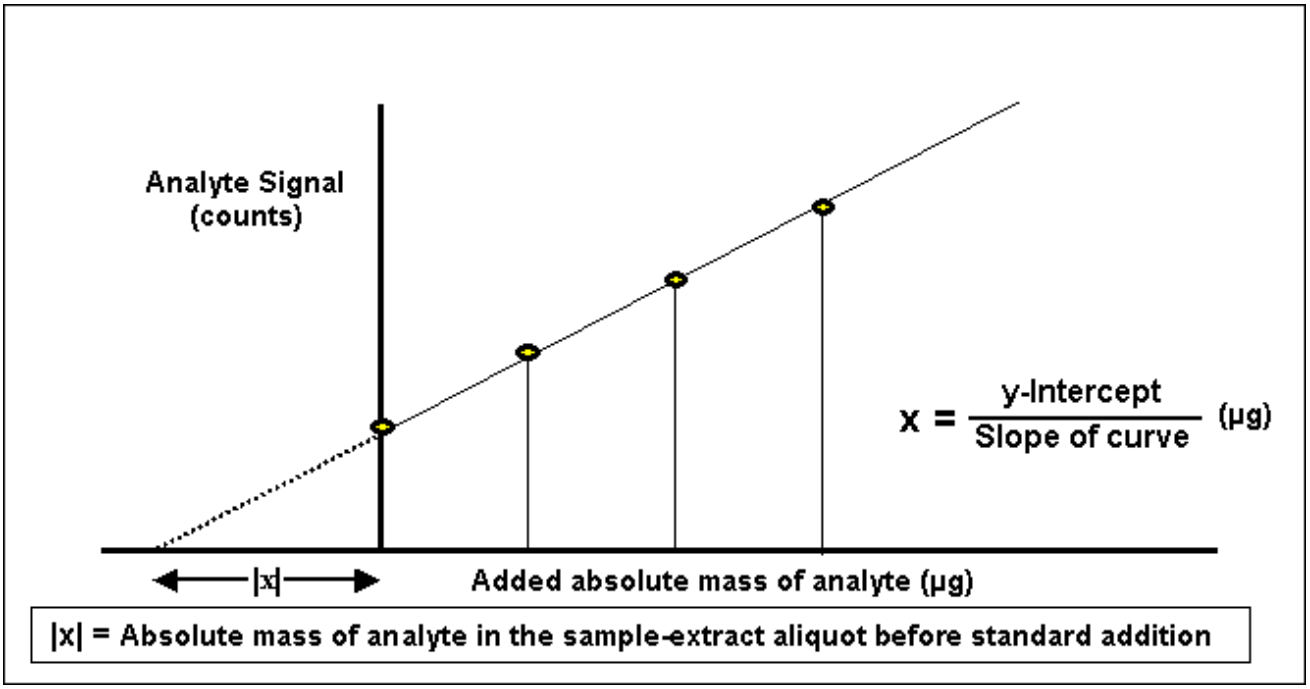
where V_{end} is the total volume of the sample extract (20 mL).

All other variables are listed in 5.7.1.2**.**

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 the matrix-matched calibrations (**5.5.2**). The mass fraction of the pesticide in the sample (w_R) is calculated via linear regression using a graphical presentation as shown in Figure 1. The Y-intercept of the calibration graph will indicate the pesticide mass contained in the non-fortified aliquot of the sample extract.

Figure 1 — Internal calibration using the procedure of standard additions, schematically



Key:

Y Peak area of analyte

X Added absolute mass of analyte $m_{pest}^{std\ add}$ in µg

|x| absolute amount of analyte in the sample extract (in µg) before standard addition (y = 0)

With x = $\frac{y - \text{intercept } (b)}{\text{slope of the curve } (a)}$ (µg)

The calculation is performed as follows using the regression graph shown in Figure 1

$$w_R = \frac{b}{a} \times \frac{V}{V_{al} \times m_a} \left(\frac{\text{mg}}{\text{kg}} \right)$$

where:

b Y-intercept of the calibration graph of the analyte in question;

a Slope of the calibration graph of the analyte in question (1/µg);

V_{end} Volume of sample extract (mL) (should be 20 mL)

V_{al} Volume of aliquots used for the standard additions approach (mL)

m_a Weight of initial sample portion (g)

6. Performance Data

Exemplary results of recovery experiments (n=5) using matrix matched calibrations (for more information see method validation database at **www.crl-pesticides-datapool**).

Table 10: Overview of exemplary Validation Data

Pesticide	Target Transition (m/z)	Matrix	Spiking Level [mg/kg]	ISTD	Mean Recovery [%]	RSD [%]
Amitrol	85/57	Cucumber	0.01	None, but matrix-matched	107	4.6
AMPA	110/63	Wheat Flour	0.1	AMPA $^{13}C^{15}N$	119	5.7
Chlormequat	122/58	Cherry	0.05	Chlormequat D4	97	4.6
Cyromazine	167/85	Pineapple	0.05	Cyromazine D4	100	4.1
Daminozide	161/143	Grape	0.05	Daminozide D6	98	2.8
Diethanolamine	106/88	Apple Apple	0.5 1	Diethanolamine D4	87 93	
Diquat	184/128	Potato	0.01	Diquat D4	101	2,4
Ethephon	143/107	Cherry	0.05	Ethephon D4	92	5.9
ETU	103/44	Carrot	0.1	ETU D4	79	2.8
Fosetyl-Al	109/81	Pineapple	0.05	Fosetyl-Al D15	100	4.1
Glufosinate	180/63	Cherry	0.05	Glufosinate D3	94	4.6
Glyphosate	168/63	Cherry	0.05	Glyphosate $^{13}C_2{}^{15}N$	98	7.6
HEPA	125/79	Cherry	0.05	HEPA D4	93	3.5
Kasugamycin	380/112	Apple Honey	0.01 0.1	None, but matrix-matched calibration	89 88	1.2 3.0
Maleic Hydrazide	111/82	Pineapple	0.05	Maleic Hydrazide D2	89	3.7
Mepiquat	114/98	Cherry	0.05	Mepiquat D3	102	4.9
Morpholin	88/70	Lime	1	Morpholin D8	106	
MPPA	151/63	Cherry	0.05	MPPA D3	92	1.3
N-AG	222/63	Cherry	0.05	N-AG D3	104	3.5
Nereistoxin	150/105	Cuccumber	0.1	Nereistoxin D6	93	
Paraquat	186/171	Barley	0.01	Paraquat D6	92	7,0
PTU	117/100	Carrot	0.1	None, but matrix-matched	94	3.2
Streptomycin	582/263	Apple	0.01	Dihydrostreptomycin	115	5.8
Triethanolamine	150/132	Apple Apple	0.5 1	Triethanolamine D12	104 106	
Trimesium	77/62	Wheat Flour	0.1	None, but matrix-matched calibration	104	1.9

Table 11: Overview of approximate limits of quantification (LOQs)*

Method	Pesticide	Most fruits and Vegetables (tested on Tomato, Cucumber, Apples) [mg/kg]	Citrus (tested on Orange) [mg/kg]	Cereals (tested on Barley) [mg/kg]
M1	Ethephon	0.01	0.01	0.02
M1	HEPA	0.01	0.01	0.02
M1	Glyphosate	0.01	0.02	0.02
M1	AMPA	0.01	0.02	0.02
M1	Glufosinate	0.01	0.02	0.02
M1	MPPA	0.01	0.02	0.02
M1	N-AG	0.02	0.02	0.02
M1 / M2	Fosetyl	0.1 / 0.005	n.a. / 0.005	n.a. / 0.005
M2	Maleic Hydrazide	0.01	0.01	0.02
M3	Amitrol	0.01	0.01	0.02
M3 / M5	ETU	0.01 / 0.01	0.02 / n.a.	0.02 / n.a.
M3 / M5	PTU	0.01 / 0.01	0.02 / n.a.	0.02 / n.a.
M3 / M4 / M5	Chlormequat	0.005 / 0.005	0.005 / 0.005	0.01 / 0.01
M3 / M4 / M5	Mepiquat	0.005 / 0.01	0.005 / 0.01	0.001 / 0.02
M3 / M4	Cyromazine	0.01 / 0.01	0.01 / 0.01	0.02 / 0.02
M3 / M4	Daminozide	0.01 / 0.02	0.01 / 0.02	0.02 / 0.04
M3 / M4	Trimethylsulfonium-Cation	0.01 / 0.005	0.01 / 0.005	0.02 / 0.01
M3 / M4	Nereistoxin	0.01 / 0.01	n.a. / n.a.	n.a. / n.a.
M4	N,N-Dimethylhydrazine	0.005	0.005	0.01
M4	Diquat	0.005	0.005	0.005
M4	Paraquat	0.005	0.005	0.005
M6	Streptomycin	0.01	n.a.	n.a.
M6	Kasugamycin	0.01	n.a.	n.a.
M7	Morpholine	0.01**	0.01**	n.a.
M7	Diethanolamine	0.01**	0.01**	n.a.
M7	Triethanolamine	0.01**	0.01**	n.a.

* using API 5500 instrument
** value derived from 10-fold diluted extract (0,05 g sample equivalents/mL)

7. 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

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

ANNEX

Table 12: Exemplary concentrations of pesticide stock and working solutions:

Compound	Method	Stock Solution (exemplary)		Working Solutions (exemplary)	
		Solvent used to prepare	[mg/mL]	Solvent used to prepare	[µg/mL]
Ethephon	M1	Methanol + 1% Formic acid	1	Methanol + 1% Formic acid	5 / 1 / 0,1
HEPA	M1	Methanol	1	Methanol + 1% Formic acid	5 / 1 / 0,1
Glyphosate	M1	Water / Methanol (3:1)	0,2	Methanol + 1% Formic acid	5 / 1 / 0,1
AMPA	M1	Water *	0,01	Methanol + 1% Formic acid	5 / 1 / 0,1
Glufosinate	M1	Water / Methanol (2:1)	1	Methanol + 1% Formic acid	5 / 1 / 0,1
MPPA	M1	Acetonitril*	0,01	Methanol + 1% Formic acid	5 / 1 / 0,1
N-Acetyl-Glufosinate	M1	Methanol	1	Methanol + 1% Formic acid	5 / 1 / 0,1
Fosetyl-Aluminium	M2	Water / Methanol (3:1)	0,1	Methanol	5 / 1 / 0,1
Maleic Hydrazide	M2	Methanol	1	Methanol	5 / 1 / 0,1
Amitrol	M3	Methanol	1	Methanol	5 / 1 / 0,1
ETU	M3	Methanol	1	Methanol	5 / 1 / 0,1
PTU	M3	Methanol	1	Methanol	5 / 1 / 0,1
Trimethylsulfonium-Cation (trimesium)	M3,4	Methanol	1	Methanol	5 / 1 / 0,1
Cyromazine	M3,4	Methanol	1	Methanol	5 / 1 / 0,1
Daminozide	M3,4	Methanol	1	Methanol	5 / 1 / 0,1
Chlormequat	M3,4,5	Methanol	1	Methanol	5 / 1 / 0,1
Mepiquat	M3,4,5	Methanol	1	Methanol	5 / 1 / 0,1
Diquat**	M4	Methanol + 1% Formic acid	1	Methanol + 1% Formic acid	5 / 1 / 0,1
Paraquat**	M4	Methanol + 1% Formic acid	1	Methanol + 1% Formic acid	5 / 1 / 0,1
Streptomycin**	M6	Water / Methanol (1:1)	1	Methanol	5 / 1 / 0,1
Kasugamycin	M6	Methanol	1	Methanol	5 / 1 / 0,1

* solutions as provided by the provider
** use plastic vessels and stoppers for Paraquat (M4), Diquat (M4), streptomycin (M6) and Glyphosate (M1)

Table 13: Providers of isotopically labeled internal standards (exemplary)

Name		Source	Article-No.	Conc. [µg/mL]	Amount per unit	Prices		
						1 unit	2 µg*	0.1 µg**
Amitrole	(^{15}N)	1	XA10240100ME	100	1.1 mL	179 €	325 c	16 c
	(^{15}N / ^{13}C)	1	XA10240110AL	100	1.1 mL	360 €	655 c	33 c
		7	A633382		10 mg	1138€	23 c	1.1 c
AMPA (^{13}C ^{15}N)		1	XA10205100WA	100	1.1 mL	330 €	600 c	30 c
		8	CIL-CDNLM-6786-1.2	100	1.2 mL	532 €	887 c	44 c
		5	CDNLM-6786-1.2	100	1.2 mL	338 €	563 c	28 c
Chlormequat chloride (1,1,2,2-D4)		1	X 11340100DO	100	10 mL	310 €	62 c	3 c
		1	XA11340100DO	100	1.1 mL	79 €	144 c	7.2 c
		6	D3386		5 mg	605 €	24 c	1.2 c
		1	CA11340100		5 mg	380 €	15.2 c	0.8 c
Chlormequat-chloride D9		3	673151		5 mg	310 €	12.4 c	0.6 c
Cyromazine D4		1	XA11920010EA	100	1.1 mL	128 €	232 c	11.6 c
		7	C989302		10 mg	797 €	15.9 c	0.8 c
Daminozide D6		1	XA11960100AL	100	1.1 mL	94 €	171 c	8.5 c
Diethanolamine D4		4	D-5307/0.1		0.1 g	432 €	0.86 c	0.04 c
Dihydrostreptomycin sesquisulfate hydrate		1	C 12635300		100 mg	27 €	0.05 c	0.003 c
Dihydrostreptomycin sulphate		8	EPD1954000		25 mg	120 €	0.96	0.048
Diquat dibromide D4 monohydrate		1	XA12960010DO	100	1.1 mL	89 €	162 c	8.1 c
		7	D492902		5 mg	87 €	3.5 c	0.17 c
		6	D17071		50 mg	534 €	2.1 c	0.1 c
Ethephon D4 (2-Chloroethyl-1,1,2,2-D4)		1	XA13230100AC	100	1.1 mL	138 €	251 c	12.5 c
		6	D8328		5 mg	1387 €	55.5 c	2.8 c
		7	C366177		10 mg	854 €	17.1 c	0.85 c
Ethylene thiourea (ETU) D4		1	C 13330100		50 mg	310 €	1.2 c	0.06 c
			XA13330100AC	100	1.1 mL	138 €	251 c	12.5 c
		6	D1965		100 mg	391 €	0.78 c	0.04 c
		7	I367002		10 mg	75 €	1.5 c	0.08 c
Fosetyl-aluminium D15		1	CA13940010		10 mg	380 €	8 c	0.4 c
Glufosinate D3		2	-	friendly donation				
		7	G596952		10 mg	1423 €	29 c	1.4 c
Glyphosate (1,2-$^{13}C_2$ ^{15}N)		1	XA14050100WA	100	1.1 mL	330 €	600 c	30 c
		5	CNLM-4666-1.2	100	1.2 mL	267 €	445 c	22 c
		5	CNLM-4666-10	100	10 mL	890 €	178 c	8.9 c
		8	CIL-CNLM-4666-1.2	100	1.2 mL	394 €	657 c	33 c
		6	CN10570		5 mg	1510 €	60.4 c	3.0 c
		7	G765002		10 mg	800 €	16 c	0.8 c
		10	608629-SPEC		10 mg	247 €	5 c	0.2 c
Hydroxy-Ethephon (HEPA) D4		1	CA13230200		10 mg	240 €	5 c	0.2 c
		2	-	friendly donation				
Maleic hydrazide (MH) D2		1	C 14730100		10 mg	230 €	23 c (10µg)	1.2 c (0.5 µg)
		3	673799		10 mg	199 €	20 c (10µg)	1 c (0.5 µg)
Mepiquat-d16 Chloride		6	D14539		50 mg	1352 €	5.4 c	0.3 c
Mepiquat iodide D3 (methyl D3)		1	X 14880100DO	100	10 mL	410 €	82 c	4 c
		1	XA14880100DO	100	1.1 mL	73 €	133 c	6.6 c
Morpholin D8		4	D-1895/0.5		500 mg	468 €	0.94 c (10µg	0.05 c (0.5µg)

Single Residue Methods

Name	Source	Article-No.	Conc. [µg/mL]	Amount per unit	Prices		
					1 unit	2 µg*	0.1 µg**
N-Acetyl-Glufosinate (N-AG) D3	2	-	friendly donation				
Nereistoxin oxalate D6	1	C 15502010		10 mg	240 €	5 c	0.0x c
MPPA D3	2	-	friendly donation				
	7	M326162		10 mg	1423 €	28 c	1.4 c
Perchlorate $^{18}O_4$	5	OLM-7310-1.2	100	1.2 mL	249 €	415 c	21 c
Paraquat diiodide D6	1	C 15870200		50 mg	250 €	1 c	0.05 c
	1	XA15870200DO	100	1.2 mL	58 €	97 c	4.8 c
Propylene thiourea (PTU)D6	1	D-5959/0.1		100 mg	297 €	0.6 c	0.03 c
	6	D535		100 mg	1067 €	2.1 c	0.1 c
Triethanolamine-d15	1	D-5459/0.1		100 mg	315 €	0.63 c	0.03 c

Providers of compounds (**Disclaimer**: The use of trade names is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the EURL of any product to the exclusion of others. Market prices may be subject to changes, shipping costs are not included in the pricing):

1: Dr. Ehrenstorfer

2: Bayer Crop Science

3: HPC (High Purity Compounds)

4: CDN Isotopes (via Dr. Ehrenstorfer)

5: Cambridge Isotope Lab. Inc

6: Medical isotopes

7: Toronto Research Chemicals

8: LGC Standards

9: Crescent Chemical Co., Inc.

10: Sigma-Aldrich

* 2 µg ISTD are typically employed to samples (typically 10 g) at the beginning of the procedure
** 0.1 µg are typically added to 1 mL aliquots of sample extracts (typically corresponding to 0.5 g sample)

Table 14: Exemplary concentrations of ISTD working solutions (**3.16**)

Method	ISTD*	ISTD -Addition to samples (5.2.3)		ISTD -Addition to calibration standard(s) (5.5)		Expected approx. ISTD-concentration in sample extracts (~20 mL) and calibration standards (~1 mL)
		Suggested concentration of ISTD-WS I (3.16)	Absolute mass of ISTD spiked to sample (50 µL ISTD-WS I) (m_{ISTD}^{sample})	Suggested concentration of ISTD- WS II (3.17) **	Absolute mass of ISTD spiked to calibration standard (50 µL ISTD-WS II) ($m_{ISTD}^{cal\ mix}$)	
		µg/mL	µg	µg/mL	µg	µg/mL
M1	Ethephon D4	40	2	2	0,1	0,1
M1	HEPA D4	40	2	2	0,1	0,1
M1	Glyphosat $^{13}C_2$ ^{15}N	40	2	2	0,1	0,1
M1	AMPA ^{13}C ^{15}N	40	2	2	0,1	0,1
M1	Glufosinat D3	40	2	2	0,1	0,1
M1	MPPA D3	40	2	2	0,1	0,1
M1	N-Acetyl-Glufosinate D3	40	2	2	0,1	0,1
M1,2	Fosetyl D5 (from fosetyl-aluminium D15)	40	2	2	0,1	0,1
M2	Maleic hydrazide D2	200	10	10	0,5	0,5
M2	Perchlorat $^{18}O_4$	40	2	2	0,1	0,1
M3	ETU D4	40	2	2	0,1	0,1
M3	PTU D6	40	2	2	0,1	0,1
M3,4	Cyromazin D4	40	2	2	0,1	0,1
M3,4	Daminozid D6	40	2	2	0,1	0,1
M3,4	Nereistoxin D4	40	2	2	0,1	0,1
M3,4,5	Chlormequat D4	40	2	2	0,1	0,1
M3,4,5	Mepiquat D3	40	2	2	0,1	0,1
M4	Diquat D4	40	2	2	0,1	0,1
M4	Paraquat D6	40	2	2	0,1	0,1
M6	Dihydrostreptomycin****	40	2	2	0,1	0,1
M7	Morpholin D8	40	2	2	0,1	0,1
M7	Diethanolamine D6	40	2	2	0,1	0,1
M7	Triethanolamine D12	40	2	2	0,1	0,1

* The concentration of the ISTD should be high enough to ensure good detection with little influence of the noise on the signal (e.g. S/N>20 is fine). It should be kept in mind, however, that isotopically labeled ISTDs (IL-ISTDs) typically contain small amounts of the non-labeled analogues. To minimize the risk of false positives the amount of IL-ISTD added to the samples should thus not be higher than necessary. Quantification of the parent is typically not affected to a great extend as the cross-contamination is typically at low levels and as similar concentrations of the native pesticide originating from the IL-ISTD will also be present in the calibration standards and thus subtracted via the intercept. In the case of maleic hydrazide, where the IL-ISTD is added at higher concentrations to the samples special attention is necessary (see also comments under **5.6.2**).

** a 20-fold dilution of the ISTD working solution used to spike samples in step **5.2.3** .

*** Dihydrostreptomycin is not isotopically labeled but still suitable for compensation of matrix effects on streptomycin if LC conditions are adjusted in such a way to ensure exact co-elution and thus equivalent matrix-effects

NOTE: *If detections of a compound are rather seldom and the ISTD expensive it is advisable to add the ISTD to the 1 mL aliquot transferred to the auto-sampler vial (see **5.2.7**). Alternatively, it can be even skipped entirely in the first screening analysis and only added in a second analysis in case the first one was positive. The first approach is to be preferred especially where the retention times of a compound tends to shift. By comparing the retention time between the ISTD and the suspected peak as well as the peak shape the certainty of identification significantly improves.*

Table 15: Water content of selected foods and water amount to be added to test portions prior to extraction (**5.2.2**) depending on the analytical approach

Commodity group	Commodity	Typical water content g/100 g	mL of water to be added to 10 g test portions [g] (where water-addition refers to different sample weights this is specified)		Remarks
			When quantifying with ISTD that was added at the beginning of the procedure (5.2.3)	When no ISTD is used or when ISTD is added after aliquotation (5.7.1.2)	
Fruits					
Citrus fruit	citrus juices	90	-	1	
	grapefruit	90	-	1	
	lemon/lime	85	-	1.5	
	orange	85	-	1.5	
	tangerine	90	-	1	
Pome fruit	apple	85	-	1.5	
	apple (dried)	30	8.5 to 5 g sample (see **5.1.1**)	8.5 to 5 g sample (see **5.1.1**)	Weigh 13.5 g rehydratized homogenate
	apple sauce	80	-	2	
	apple juice	90	-	1	
	pear	85	-	1.5	
	quince	85	-	1.5	
Stone fruit	apricot	85	-	1.5	
	apricot (dried)	30	8.5 to 5 g sample (see **5.1.1**)	8.5 to 5 g sample (see **5.1.1**)	Weigh 13.5 g rehydratized homogenate
	apricot nectar	85	-	1.5	
	cherry	85	-	1.5	
	mirabelle	80	-	2	
	nectarine	85	-	1.5	
	peach	90	-	1	
	peach (dried)	20	8.5 to 5 g sample (see **5.1.1**)	8.5 to 5 g sample (see **5.1.1**)	Weigh 13.5 g rehydratized homogenate
	plum	85	-	1.5	
	plum (dried)	20	8.5 to 5 g sample (see **5.1.1**)	8.5 to 5 g sample (see **5.1.1**)	Weigh 13.5 g rehydratized homogenate

Commodity group	Commodity	Typical water content g/100 g	mL of water to be added to 10 g test portions [g] (where water-addition refers to different sample weights this is specified)		Remarks
			When quantifying with ISTD that was added at the beginning of the procedure (5.2.3)	When no ISTD is used or when ISTD is added after aliquotation (5.7.1.2)	
Soft and small fruit	blackberry	85	-	1.5	
	blueberry	85	-	1.5	
	currant	85	-	1.5	
	elderberry	80	-	2	
	gooseberry	90	-	1	
	grapes	80	-	2	
	raspberry	85	-	1.5	
	raisins	20	8.5 to 5 g sample (see **5.1.1**)	8.5 to 5 g sample (see **5.1.1**)	Weigh 13.5 g rehydratized homogenate
	strawberry	90	-	1	
	pineapple	85	-	1.5	
Other fruits	banana	75	2.5	2.5	
	fig	80	-	2	
	fig (dired)	20	8.5 to 5 g sample (see **5.1.1**)	8.5 to 5 g sample (see **5.1.1**)	Weigh 13.5 g rehydratized homogenate
	kiwi	85	-	1.5	
	mango	80	-	2	
	papaya	90	-	1	
Vegetables					
Root and tuber vegetables	beetroot	90	-	1	
	carrot	90	-	1	
	celeriac	90	-	1	
	horseradish	75	2.5	2.5	
	parsley root	90	-	1	
	radish	95	-	0.5	
	black salsify	80	-	2	
	potato	80	-	2	
	garlic	60	7 to 5 g sample	7 to 5 g sample	

Commodity group	Commodity	Typical water content g/100 g	mL of water to be added to 10 g test portions [g] (where water-addition refers to different sample weights this is specified)		Remarks
			When quantifying with ISTD that was added at the beginning of the procedure (5.2.3)	When no ISTD is used or when ISTD is added after aliquotation (5.7.1.2)	
Leek plants	onion	90	-	1	
	leek	85	-	1.5	
	shallot	80	-	2	
	chive	85	-	1.5	
Fruiting vegetables	aubergine	90	-	1	
	cucumber	95	-	0.5	
	melon	90	-	1	
	Pepper, sweet	90	-	1	
	pumpkin	95	-	0.5	
	tomato	95	-	0.5	
	zucchini	95	-	0.5	
	broccoli	90	-	1	
Cabbage	brussel sprouts	85	-	1.5	
	cauliflower	90	-	1	
	chinese cabbage	95	-	0.5	
	kale	90	-	1	
	kohlrabi	90	-	1	
	red cabbage	90	-	1	
	savoy cabbage	90	-	1	
	white cabbage	90	-	1	
	lettuce varieties	95	-	0.5	
	endive	95	-	0.5	
Leafy vegetables and herbs	cress	90	-	1	
	lamb's lettuce	85	-	1.5	
	parsley	80	-	2	
	rucola	85	-	1.5	
	spinach	90	-	1	

Commodity group	Commodity	Typical water content g/100 g	mL of water to be added to 10 g test portions [g] (where water-addition refers to different sample weights this is specified)		Remarks
			When quantifying with ISTD that was added at the beginning of the procedure (5.2.3)	When no ISTD is used or when ISTD is added after aliquotation (5.7.1.2)	
Stem vegetables	asparagus	95	-	0.5	
	celery	95	-	0.5	
	leek	85	-	1.5	
	rhubarb	95	-	0.5	
	artichokes	85	-	1.5	
Legumes	beans, peas, lentils (dried)	<10	10 to 5 g sample	10 to 5 g sample	
	beans, peas	75	2.5	2.5	
Miscellaneous					
Cereals	grain, flour. etc.	10	10 to 5 g sample	10 to 5 g sample	Different sample amounts may be used depending on water-absorbing properties of material
High Extract commodities	coffee beans	<10	10 to 2 g sample	10 to 2 g sample	Different sample amounts may be used depending on extract-richness
	tea	<10	10 to 2 g sample	10 to 2 g sample	
	dry herbs and spices	<10	10 to 2 g sample	10 to 2 g sample	
Other	mushrooms	90	-	1	
	wine	90	-	1	
	Honey	20	9 to 5 g sample	9 to 5 g sample	

Table 16: Exemplary LC-MS/MS parameters for ABI 5500

	Method 1	**Method 2**	**Method 3+4+5**	**Method 6**
Ion source/Mode	Turbo Ion Spray (ESI)/negative	Turbo Ion Spray (ESI)/negative	Turbo Ion Spray (ESI)/positive	Turbo Ion Spray (ESI)/positive
Curtain gas	Nitrogen 30 psi (2,07 bar)	Nitrogen 30 psi (2,07 bar)	Nitrogen 40 psi (2,76 bar)	Nitrogen 40 psi (2,76 bar)
Collision gas	med	med	med	med
Ion spray voltage	-4500	-4500	1500	5500
Gas 1	Nitrogen 50 psi (3,45 bar)			
Gas 2	Nitrogen 50 psi (4,14 bar)			
Temperatur Gas 2	600°C	500°C	500°C	550°C
Resolution MS 1	unit (ca. 0.7 amu FWHM*)			
Resolution MS 2	unit (ca. 0.7 amu FWHM)			
Dwell time	20	50	20	50

*FWHM = full width at half maximum

Table 17: Document History

Action	When?	Version
Development of Method by the CRL-SRM	2006-2008	-
Presentation of method at the EPRW in Berlin (oral presentation plus poster)	Jun. 2008	
Drafting of V1	Nov.-Dec. 2008	V1
Placing of V1 in CRL-Website	Jan. 2009	
Update of Table 1, Expected concentrations of ISTDs were calculated with a wrong dilution factor in previous version. Arithmetical errors were corrected.	Aug. 2009	V2
Introduction of measurement conditions for HEPA within the “Glyphosate & Co.” method		
Introduction of measurement conditions for the screening of diquat and paraquat within the “Quats & Co. method”	Nov 2009	V3
Introduction of measurement conditions for Amitrole, chlormequat, mepiquat and daminozide “Amitrol & Co.” method		
Extensive text revisions		
Introduction of measurement conditions for Streptomycin Kasugamycin	May 2010	V4
Introduction of measurement conditions for the screening of Perchlorate ion		
Extensive text revisions		
Extensive text revisions and restructuring of document	Nov 2010	V5
Introduction of measurement conditions for ETU, ETU D4, PTU, PTU D6, Cyro-mazine, Cyromazine D4, N-Acetyl-Glufosinate (NAG), NAG-D3, Glufosinate D3, MPPA D3, Morpholin, Morpholin D8		
Introduction of an acronym for the method (QuPPe)	Jul 2011	V6
Advice to use plastic vessels and stoppers for Glyphosate		
Minor modification and additional instructions in Method 1		
Modification of mobile phase of Method 3 to improve analysis of ETU and PTU		
Introduction of measurement conditions for Amitrole (^{15}N / ^{13}C) and Amitrole (^{15}N) in Method 3		
Introduction of measurement conditions for Nereistoxin and Nereistoxin D6 in Method 4		
New method for the analysis of Morpholin/Morpholin D8; Di-ethanonamine/diethanolanmineD6; Triethanolamine/Triethanolamine D12 (Method 7), removal of Morpholin from Method 4 as it does not separate from the interfering diethanolamine		
Introduction of ETU and PTU and their corresponding IL_ISTDs in Method 5		
Correction of dimension of stock solutions concentration in Table 12 (from µg/mL to mg/mL)		
Text and Table revisions		
Extensive actualization of table concerning possible sources of purchase of ISTDs		