Protocols of Interlaboratory Validation Studies Round 1 to Round 3

Content

1. Overview

For evaluating the performance and transferability of the QuPPe method into various laboratories, three rounds of inter-laboratory validation studies were conducted. The participating laboratories were free to decide in which extent they wanted to participate in each round. In each validation round one or two (for two different matrix groups) protocols were prepared describing thoroughly the extraction procedure and giving instructions for measurement (e.g. measurement sequence). The laboratories were free to narrow down the scope of their validation experiments as they wished, e.g. skip certain compounds, matrices and spiking levels. The matrices had to be bought by the laboratories themselves, whereas all analytical standards required (native analytes and ILISs) were provided by the organizers (EU Reference Laboratory for Pesticides requiring single residue methods – EURL-SRM).

For each validation round, the organizers prepared special Excel sheets to allow convenient data tracking by the participants. In these Excel sheets the participating laboratories, could enter their raw data (i.e. the peak areas) for the analytes and their internal standards. Within the evaluation sheets, validation criteria according to the SANTE guidelines were verified, such as the average recovery rate (between 70 and 120%), the relative standard deviation (RSD; <20%), the drift and the residuals.

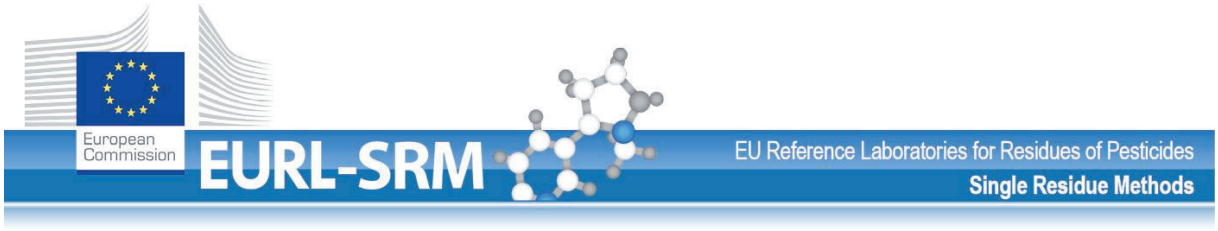
The 1st round (see **2** and **3**) covered 9 compounds (Cyromazine, Daminozide, Chlormequat, Mepiquat, Trimethylsulfonium, Nereistoxin, Paraquat, Diquat and Melamine), five matrices (potatoes, grapes, rye flour, avocados and milk) and three spiking levels (0.01, 0.05 and 0.2 mg/kg). Overall, 14 laboratories took part in this round, which was conducted in 2014 and 2015.

The 2nd round (see **4**) covered 4 compounds (Bromide, Chlorate, Perchlorate and Phosphonic acid), five matrices (carrots, lemons, rye flour, avocados and milk) and three spiking levels per compound. The levels differed between compounds and were chosen considering background levels, MRLs and analyte sensitivity. Overall, 17 laboratories took part in this round, which was conducted in 2017 and 2018.

The 3rd round (see **5**) covered 12 compounds (Ethephon, Fosetyl, HEPA, Maleic Hydrazide, Cyanuric acid, Glufosinate, MPPA, N-Acetyl-Glufosinate, Glyphosate, AMPA and N-Acetyl-Glyphosate), seven matrices (cucumbers, strawberries, rice, soybeans, milk, liver and kidney) and three spiking levels per compound. The spiking levels differed between compounds and were chosen mainly based on analyte sensitivity. Overall, 15 laboratories took part in this round, which was conducted in 2019 and 2021.

The completed Excel sheets entailing the validation data, as well as data concerning the periphery (e.g. instrument type, column type) had to be sent to the organizers for further evaluation and plausibility checks. Prior to uploading certain data had to be discarded. This included data strongly deviating from the general trend (e.g. RSD>50%) or because of strong background levels, or because of a clear deviation from the prescribed procedure or because of an obvious lack of measurement sensitivity. Finally, the data was uploaded to the EURL Datapool, where an outlier test was applied.

2. Protocol Round 1 – Plant Origin



Protocol Round 1 (PO)

for the Interlaboratory Validation of the QuPPe-PO Method (draft version 3)

Introduction:

This document describes the procedures to be followed by laboratories participating in the interlaboratory validation study of the QuPPe method organized by the EURL-SRM[1] in collaboration with EPRA[2]. The study foresees recovery experiments of selected pesticides from selected commodities at 3 spiking levels. 5 replicate experiments are to be conducted for each pesticide mixture/commodity/spiking level -combination. **Table 1** gives an overview of the scope of the study.

Table 1: Overview of the study

Mix	Compounds	IL-IS	Spiking Levels	Matrices	LC-Method*
I	Cyromazine	Cyromazine D4	LOW: 0.01 mg/kg MED: 0.05 mg/kg HIGH: 0.2 mg/kg	Potatoes Grapes Rye flour Avocados	M4
	Daminozide	Daminozide 13C4			
	Chlormequat	Chlormequat D4			
	Mepiquat	Mepiquat D3			
	Trimethylsulfonium	Trimethylsulfonium D9			
	Nereistoxin	Nereistoxin D6			
	Paraquat	Paraquat D6			
	Diquat	Diquat D4			
	Melamine	Melamine triamine 15N3			

* based on the nomenclature appearing in the QuPPe method protocol published in the EURL-SRM-website.

All required **standard solutions will be delivered to the participants by the Organizers** of this study. Participating laboratories do not have to perform the full range of experiments foreseen in Table 1, i.e. they may choose to process less commodities, less levels and/or less repetitions. **All other technical aspects in this protocol must, however, be followed as closely as possible to avoid results from having to be excluded from the final evaluation. Any relevant deviations from the protocol should be reported!**

[1] EURL-SRM = EU-Reference Laboratory for pesticides requiring Single Residue Methods
[2] EPRA = Expertengruppe für PSM Rückstandsanalytik (German Pesticide Residue Analysis Expert Group)

Test Materials (Matrices):

The required **blank commodities** are to be acquired by the participating labs. The test materials employed should be tested to not contain any of the pesticides included in this study at levels >0.002 mg/kg. The use of **organically grown crops is thus recommended**.

The commodities can be homogenized the way they are typically homogenized in each lab. The use of dry ice is recommended in all cases but not mandatory. Rye flour is already homogeneous and can be employed as such.

Apparatus and Consumables:

See latest version of QuPPe-PO method published in the EURL-SRM-website.

Chemicals for Sample Preparation:

See latest version of QuPPe-PO method published in the EURL-SRM-website.

Standard Solutions:

All standard solutions necessary for this validation study will be provided by the Organizers. Native pesticides and IL-ISs will be delivered in separate mixtures as listed in **Table 2** and **3**. The volumes of pesticide and IL-ISs mixtures to be used for the spiking and the preparation of calibration solutions are shown in the pipetting schemes in **Table 3** and **4** respectively.

Table 2: Standard solutions for spiking sample portions

Solution Names	Pesticide / IL-IS conc. [µg/mL]	Solvent	Corresponding Pesticide Spiking Levels [mg/kg] on…		Expected approximate concentrations of Pesticides / IL-ISs in final extract [µg/mL]
			Rye	Grapes, Potato and Avocado	
SPIKE LOW	1	MeOH +1% FA	0.02	0.01	0.005
SPIKE MED	5	MeOH +1% FA	0.1	0.05	0.025
SPIKE HIGH	20	MeOH +1% FA	0.4	0.2	0.1
IL-IS for SPIKE	20	MeOH +1% FA	See above		0.1

Table 3: Standard solutions to prepare calibration standards

Solution Names	Pesticide / IL-IS conc. [µg/mL]	Solvent	Corresponding Calibration Levels of Pesticides* [mg/kg]		Approx. concentrations of Pesticides / IL-ISs in calibration solutions [µg/mL]
			Rye	Grapes, Potato and Avocado	
CAL LOW	0.05	MeOH +1% FA	0.012; 0.024	0.006; 0.012	0.003 / 0.006
CAL MED	0.25	MeOH +1% FA	0.06; 0.12	0.03; 0.06	0.015 / 0.03
CAL HIGH	1	MeOH +1% FA	0.24; 0.48	0.12; 0.24	0.06 / 0.12
IL-IS for CAL	1	MeOH +1% FA	-	-	0.1**

* These calibration levels correspond to 60 and 120% of the expected level at full recovery.

** Obtained by adding 100 µL of "**IL-IS for CAL"** to each calibration solution.

SAMPLE PREPARATION

1) WEIGHING OF TEST MATERIAL INTO PLASTIC EXTRACTION VESSELS:

- **Grape, Potato, Avocado: 10 g ± 0.1 g,**
- **Rye Flour: 5 g ± 0.05 g.**

Note: If the study will be performed at 3 spiking levels in quintuplicate it is necessary to prepare 16 extraction vessels for each test material (1 blank and 3x5 spiked).

Close the vessels and put them in the **freezer** (except rye flour). Remove the set of samples to be analyzed early enough from the freezer so that the material is half thawed when spiking takes place.

Note: In practice real samples are processed in frozen condition, but spiking of thawed material allows the residues to better distribute within the sample.

2) SPIKING (5 x per level and commodity):

- **0.01 mg/kg Levels*:** add 100 µL of „SPIKE LOW“ and 100 µL of „IL-IS SPIKE “
- **0.05 mg/kg Levels*:** add 100 µL of „SPIKE MED“ and 100 µL of „IL-IS SPIKE “
- **0.2 mg/kg Levels*:** add 100 µL of „SPIKE HIGH“ and 100 µL of „IL-IS SPIKE “
- **Blank:** no spiking, but add the same volume of methanol + 1% FA as it was added to the spiked samples (e.g. 200 µL).

Note: for rye flour the levels are to be considered as twice as high.

Shake the liquid samples gently to distribute the pesticides (a vortex mixer can be used here). Let the samples stand for 5 min before you continue.

Table 4: Pipetting-Scheme for spiking at 3 levels:

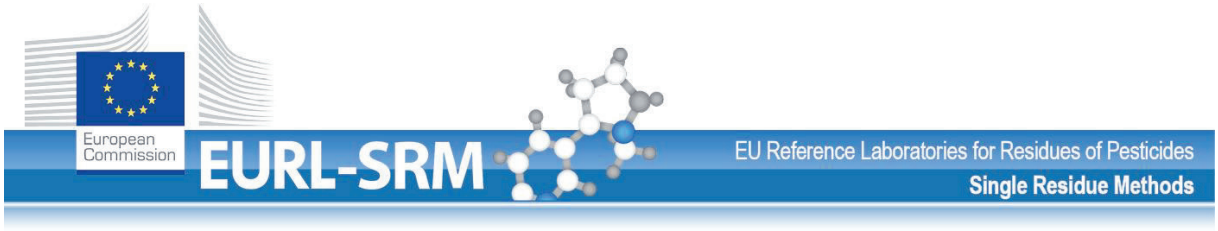
Suggested Labelling of Extraction Vessels	Spiking of 10g Sample Portions with...			Volume of “IL-IS for SPIKE” to be added	Volume adjustment MeOH (addition to samples)*
	SPIKE LOW	SPIKE MED	SPIKE HIGH		
[Matrix Code] LOW (1~5)	100 µL	-	-	100 µL	-
[Matrix Code] MED (1~5)	-	100 µL	-	100 µL	-
[Matrix Code] HIGH (1~5)	-	-	100 µL	100 µL	-
BLANK EXTRAKT	-	-	-	-	200 µL

* equal to the volume of spiking standards added to the equivalent spiked samples

3) ADJUSTMENT OF WATER CONTENT TO 10 mL

- **Grape: add 2 mL of water**
- **Potato: add 2 mL of water**
- **Avocado: add 3 mL of water**
- **Rye Flour: add 10 mL of water**

Note: In this study IL-ISs are used which can correct both volumetric errors and matrix effects. Volume adjustment is thus not essential. Nevertheless, we additionally intent to evaluate the effectiveness of matrix-matched calibration and this is the reason why we ask for volumetric adjustment.



4) ADDITION OF EXTRACTION SOLVENT:

Add **10 mL of acidified methanol (methanol with 1% formic acid v/v)** to all samples (e.g. using a solvent dispenser)

Let the Rye-Flour sample soak for 10 min

5) EXTRACTION:

Tightly close the extraction vessels and **shake vigorously for 1 min. (by hand or with a powerful mechanic shaker)**

6) CENTRIFUGATION:

Centrifuge **for 5 min.** (at ca. 4000 rpm).

7) C18-CLEANUP (ONLY FOR AVOCADO):

Transfer a 4 mL aliquot into a 10 mL plastic centrifuge tube, which already contains 200 mg of C18 (ODS) sorbent. Close the tubes and shake 1 min by hand and centrifuge for 5 min (at ca. 4000 rpm).

Note: use same C18 sorbent as used in QuPPe-AO procedure.

8) FILTRATION:

Filter at least 1.5-3 mL of centrifuged extracts into plastic tubes, for example the QuPPe extraction tubes, using polyester filters with 0.45 µm pore size.

9) PREPARATION OF SOLUTIONS FOR MEASUREMENT:

see pipetting scheme in **Table 5**

Use plastic vials, because some compounds tend to interact with the glass surface.

Table 5: Pipetting-Scheme for calibration points:

Solution to be used	Transfer volume to HPLC-Vials	Spiking of Extracts with...				Volume adjustment w. MeOH (addition to extracts)	Suggested Labelling of Vials	Final-volume
		CAL LOW	CAL MED	CAL HIGH	„IL-IS for Cal“			
BLANK EXTRACT	1 mL	60 µL	-	-	100 µL	60 µL	[Matrix Code] -CAL- 0.006	1.22 mL
	1 mL	120 µL	-	-	100 µL	-	[Matrix Code] -CAL- 0.012	1.22 mL
	1 mL	-	60 µL	-	100 µL	60 µL	[Matrix Code] -CAL- 0.03	1.22 mL
	1 mL	-	120 µL	-	100 µL	-	[Matrix Code] -CAL- 0.06	1.22 mL
	1 mL	-	-	60 µL	100 µL	60 µL	[Matrix Code] -CAL- 0.12	1.22 mL
	1 mL	-	-	120 µL	100 µL	-	[Matrix Code] -CAL- 0.24	1.22 mL
	1 mL	-	-	-	100 µL	120 µL	[Matrix Code] -BLANK	1.22 mL
MeOH with 1% FA	1 mL	60 µL	-	-	100 µL	60 µL	S-CAL-0.006	1.22 mL
	1 mL	120 µL	-	-	100 µL	-	S-CAL-0.012	1.22 mL
	1 mL	-	60 µL	-	100 µL	60 µL	S-CAL-0.03	1.22 mL
	1 mL	-	120 µL	-	100 µL	-	S-CAL-0.06	1.22 mL
	1 mL	-	-	60 µL	100 µL	60 µL	S-CAL-0.12	1.22 mL
	1 mL	-	-	120 µL	100 µL	-	S-CAL-0.24	1.22 mL
	1 mL	-	-	-	100 µL	120 µL	S-BLANK	1.22 mL
Extracts of spiked samples (LOW, MED and HIGH)	1 mL	-	-	-	-	220 µL	[Matrix Code]-LOW (1~5) [Matrix Code]-MED (1~5) [Matrix Code]-HIGH (1~5)	1.22 mL

Determinative Analysis and Data Processing

10) MEASUREMENT:

Measurement should be conducted by LC-MS/MS following the conditions indicated in the QuPPe-PO method protocol. The following **injection-sequence** is recommended**:**

- **Low Level**

1) S-BLANK1 (check for interferences and report them if relevant)
2) [Matrix Code]-BLANK (check for interferences and report them if relevant)
3) S-CAL-0.006
4) S-CAL-0.012
5) [Matrix Code]-CAL-0.006
6) [Matrix Code]-CAL-0.012
7) [Matrix Code]-LOW1
8) [Matrix Code]-LOW2
9) [Matrix Code]-LOW3
10) [Matrix Code]-LOW4
11) [Matrix Code]-LOW5
12) S-CAL-0.006
13) S-CAL-0.012
14) [Matrix Code]-CAL-0.006
15) [Matrix Code]-CAL-0.012
16) S-BLANK2 (check for carry-over and report if relevant, should be <2%).

- **Med Level**

1) S-BLANK1 (check for interferences and report them if relevant)
2) [Matrix Code]-BLANK (check for interferences and report them if relevant)
3) S-CAL-0.03
4) S-CAL-0.06
5) [Matrix Code]-CAL-0.03
6) [Matrix Code]-CAL-0.06
7) [Matrix Code]-MED1
8) [Matrix Code]-MED2
9) [Matrix Code]-MED3
10) [Matrix Code]-MED4
11) [Matrix Code]-MED5
12) S-CAL-0.03

13) S-CAL-0.06
14) [Matrix Code]-CAL-0.03
15) [Matrix Code]-CAL-0.06
16) S-BLANK2 (check for carry-over and report if relevant, should be <2%).

- **High Level**

1) S-BLANK1 (check for interferences and report them if relevant)
2) [Matrix Code]-BLANK (check for interferences and report them if relevant)
3) S-CAL-0.12
4) S-CAL-0.24
5) [Matrix Code]-CAL-0.12
6) [Matrix Code]-CAL-0.24
7) [Matrix Code]-HIGH1
8) [Matrix Code]-HIGH2
9) [Matrix Code]-HIGH3
10) [Matrix Code]-HIGH4
11) [Matrix Code]-HIGH5
12) S-CAL-0.12
13) S-CAL-0.24
14) [Matrix Code]-CAL-0.12
15) [Matrix Code]-CAL-0.24
16) S-BLANK2 (check for carry-over and report if relevant, should be <2%).

11) DOCUMENTATION AND FURTHER DATA PROCESSING:

The measured areas of the pesticides and IL-ISs should be tracked into an **Excel sheet specially prepared by the Organizer for this study and distributed to the participants**. All recovery calculations are done automatically.

The recovery figures obtained by the participants will be entered in the Method Validation Database within the EURL-Datapool (www.eurl-pesticides-datapool.eu).

3. Protocol Round 1 – Animal Origin

European Commission
EURL-SRM
EU Reference Laboratories for Residues of Pesticides
Single Residue Methods

Protocol Round 1 (AO)
for the Interlaboratory Validation of the QuPPe-AO Method
(draft version 3)

Introduction:

This document describes the procedures to be followed by laboratories participating in the interlaboratory validation study of the **QuPPe-AO** method organized by the EURL-SRM[1] in collaboration with EPRA[2]. The study foresees recovery experiments of selected pesticides from **Milk** at 2 spiking levels. 5 replicate experiments are to be conducted. **Table 1** gives an overview of the scope of the study.

NOTE: Before conducting the experiment at the low level (0.01 mg/kg) check the sensitivity of your instrument by injecting the lowest level of calibration [S-CAL-0.006] or [Milk-CAL-0.006]. Instructions for preparation are given in the pipetting scheme in **Table 5**.

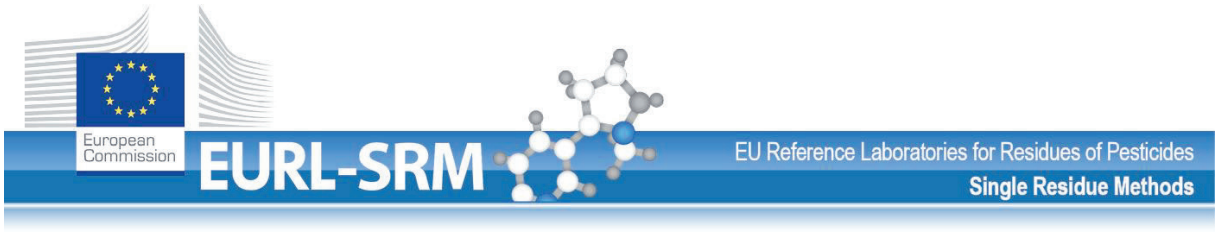
Table 1: Overview of the study

Mix	Compounds	IL-IS	Spiking Levels	Matrices	LC-Method*
I	Cyromazine	Cyromazine D4	LOW: 0.01 mg/kg MED: 0.05 mg/kg	Whole Milk (3.25-4 % fat)	M4
	Daminozide	Daminozide 13C4			
	Chlormequat	Chlormequat D4			
	Mepiquat	Mepiquat D3			
	Trimethylsulfonium	Trimethylsulfonium D9			
	Nereistoxin	Nereistoxin D6			
	Paraquat	Paraquat D6			
	Diquat	Diquat D4			
	Melamine	Melamine tramine 15N3			

* nomenclature appearing in the QuPPe-PO method protocol published in the EURL-SRM-website.

All required **standard solutions will be delivered to the participants by the Organizers** of this study. Participating laboratories do not have to perform the full range of experiments foreseen in Table 1, i.e. they may choose to process less levels and/or les repetitions. **All technical aspects in this protocol must be, however, followed as closely as possible to avoid results from having to be excluded from the final evaluation. Any relevant deviations from the protocol should be reported!**

[1] EURL-SRM = EU-Reference Laboratory for pesticides requiring Single Residue Methods
[2] EPRA = Expertengruppe für PSM Rückstandsanalytik (German Pesticide Residue Analysis Expert Group)



Test Material (Matrix):

The required **blank milk** is to be acquired by the participating laboratories. It should be tested to not contain any of the pesticides included in this study at levels >0.002 mg/kg. The use of **organic milk is thus recommended**.

Apparatus and Consumables:

See latest version of QuPPe-AO method published in the EURL-SRM-website.

Chemicals for Sample Preparation:

See latest version of QuPPe-AO method published in the EURL-SRM-website.

Standard Solutions:

All standard solutions necessary for this validation study will be provided by the Organizers. Native pesticides and IL-ISs will be delivered in separate mixtures as listed in **Table 2** and **3**. The volumes of pesticide and IL-ISs mixtures to be used for the spiking and the preparation of calibration solutions are shown in the pipetting schemes in **Table 3** and **4** respectively.

Table 2: Standard solutions for spiking sample portions

Solution Names	Pesticide / IL-IS conc. [µg/mL]	Solvent	Corresponding Spiking Levels of Pesticides in Milk [mg/kg]	Expected approx. conc. of Pesticides / IL-ISs in final extract [µg/mL]
SPIKE LOW	1	MeOH +1% FA	0.01	0.0025
SPIKE MED	5	MeOH +1% FA	0.05	0.0125
IL-IS for SPIKE	20	MeOH +1% FA	-	0.05

Table 3: Standard solutions to prepare calibration standards

Solution Names	Pesticide / IL-IS conc. [µg/mL]	Solvent	Corresponding Calibration Levels of Pesticides* [mg/kg]	Approx. concentrations of Pesticides / IL-ISs in calibration solutions [µg/mL]
CAL LOW	0.05	MeOH +1% FA	0.006; 0.012	0.0015 / 0.003
CAL MED	0.25	MeOH +1% FA	0.03; 0.06	0.0075 / 0.015
IL-IS for CAL	1	MeOH +1% FA	-	0.05**

* These calibration levels correspond to 60 and 120% of the expected level at full recovery

** Obtained by adding 50 µL of "**IL-IS for CAL**" to each CAL solution.

SAMPLE PREPARATION

1) WEIGHING OF TEST MATERIAL INTO PLASTIC EXTRACTION VESSELS:

Milk: 10 g ± 0.05 g

Note: If the study will be performed at 2 spiking levels in quintuplicate it is necessary to prepare 11 extraction vessels (1 blank and 2x5 spiked).

2) SPIKING (5 x per level):

- **0.01 mg/kg Level:** add 100 µL of „SPIKE LOW“ and 100 µL of „IL-IS SPIKE “
- **0.05 mg/kg Level:** add 100 µL of „SPIKE MED“ and 100 µL of „IL-IS SPIKE “
- **Blank:** no spiking, but add the same volume of methanol + 1% FA as it was added to the spiked samples (e.g. 200 µL).

Shake gently to distribute the pesticides (a vortex mixer can be used here). Let the samples stand for 5 min before you continue.

Table 4: Pipetting-Scheme for spiking at 2 levels:

Suggested Labelling of Extraction Vessels	Spiking of 10 g Sample Portions with...		Volumeof “IL-IS for SPIKE” to be added	Vol.-adj. MeOH (addition to samples)*
	SPIKE LOW	SPIKE MED		
Milk-LOW-(1~5)	100 µL	-	100 µL	-
Milk MED-(1~5)	-	100 µL	100 µL	-
Milk-BLANK	-	-	-	200 µL

* equal to the volume of spiking standards added to the spiked samples.

3) ADJUST WATER TO 10 ml

- **Add 1.2 g of water.**

Note: In this study IL-ISs are used which can correct both volumetric errors and matrix effects. Volume adjustment is thus not essential. Nevertheless, we additionally intent to evaluate the effectiveness of matrix-matched calibration and this is the reason why we ask for volumetric adjustment.

4) ADDITION OF EXTRACTION SOLVENT:

Add **10 mL of acidified methanol (methanol with 1% formic acid v/v)** to all samples (e.g. using a solvent dispenser).

5) EXTRACTION:

Tightly close the extraction vessels and **shake vigorously for 1 min (by hand or with a powerful mechanic shaker)**.

6) CENTRIFUGATION:

Centrifuge **for 5 min.** (at ca. 4000 rpm).

7) C18-CLEANUP:

Transfer a 2 mL aliquot into a 10 mL plastic centrifuge tube, which already contains 2 mL of acetonitrile and 100 mg of C18 (ODS) sorbent. Close the tubes and shake for 1 min by hand.

8) CENTRIFUGATION:

Centrifuge **for 5 min** (at 4000 rpm).

9) FILTRATION:

Filter at least 1.5-2 mL of extract into plastic tubes, for example the QuPPe extraction tubes, using polyester filters with 0.45 µm pore size.

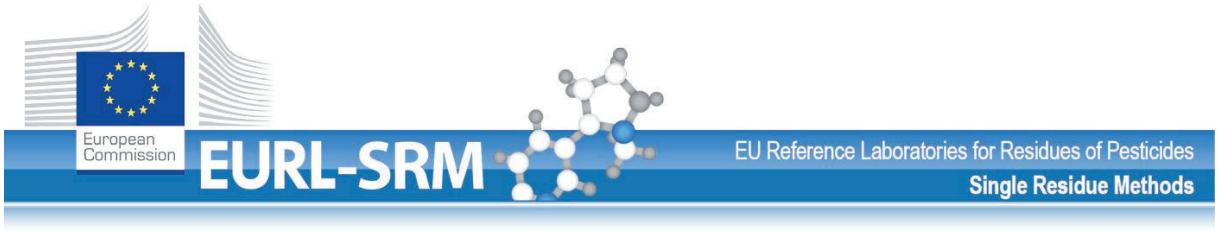
10) PREPARATION OF SOLUTIONS FOR MEASUREMENT:

See pipetting scheme in **Table 5**

Use plastic vials, because some compounds tend to interact with the glass surface.

Table 5: Pipetting-Scheme for calibration points:

Solution to be used	Transfer vol. to HPLC-Vials	Spiking of Extracts with...			Volume adjustment w. MeOH (addition to extracts)	Suggested Labelling of Vials	Final-volume
		CAL LOW	CAL MED	„IL-IS for Cal“			
BLANK EXTRACT	1 mL	30 µL		50 µL	30 µL	**Milk-CAL- 0.006**	1.10 mL
	1 mL	60 µL		50 µL	-	**Milk-CAL- 0.012**	1.10 mL
	1 mL		30 µL	50 µL	30 µL	**Milk-CAL- 0.03**	1.10 mL
	1 mL		60 µL	50 µL	-	**Milk-CAL- 0.06**	1.10 mL
	1 mL	-	-	50 µL	60 µL	**Milk-BLANK**	1.10 mL
MeOH with 1% FA	1 mL	30 µL		50 µL	30 µL	**S-CAL-0.006**	1.10 mL
	1 mL	60 µL		50 µL	-	**S-CAL-0.012**	1.10 mL
	1 mL		30 µL	50 µL	30 µL	**S-CAL-0.03**	1.10 mL
	1 mL		60 µL	50 µL	-	**S-CAL-0.06**	1.10 mL
	1 mL	-	-	50 µL	60 µL	**S-BLANK**	1.10 mL
Extracts of Spiked Samples (LOW and MED)	1 mL	-	-	-	110 µL	**Milk-LOW (1~5)** **Milk-MED (1~5)**	1.10 mL



Determinative Analysis and Data Processing

11) MEASUREMENT:

Measurement should be conducted by LC-MS/MS following the conditions indicated in the QuPPe-AO method protocol. The following **injection-sequence** is recommended**:**

- **LOW Level**

1) S-BLANK1 (check for interferences and report them if relevant)
2) Milk-BLANK (check for interferences and report them if relevant)
3) S-CAL-0.006
4) S-CAL-0.012
5) Milk-CAL- 0.006
6) Milk-CAL- 0.012
7) Milk-LOW1
8) Milk-LOW2
9) Milk-LOW3
10) Milk-LOW4
11) Milk-LOW5
12) S-CAL-0.006
13) S-CAL-0.012
14) Milk-CAL- 0.006
15) Milk-CAL- 0.012
16) S-BLANK2 (check for carry-over and report if relevant, should be <2%).

- **MED Level**

1) S-BLANK1 (check for interferences and report them if relevant)
2) Milk-BLANK (check for interferences and report them if relevant)
3) S-CAL-0.03
4) S-CAL-0.06
5) Milk-CAL-0.03
6) Milk-CAL-0.06
7) Milk-MED1
8) Milk-MED2
9) Milk-MED3
10) Milk-MED4
11) Milk-MED5
12) S-CAL-0.03

13) S-CAL-0.06
14) Milk-CAL-0.03
15) Milk-CAL-0.06
16) S-BLANK2 (check for carry-over and report if relevant, should be <2%).

12) DOCUMENTATION AND FURTHER DATA PROCESSING:

The measured areas of the pesticides and IL-ISs should be tracked into an **Excel sheet specially prepared by the Organizer for this study and distributed to the participants**. All recovery calculations are done automatically.

The recovery figures obtained by the participants will be entered in the Method Validation Database within the EURL-Datapool (www.eurl-pesticides-datapool.eu).

4. Protocol Round 2

EURL-SRM
EU Reference Laboratories for Residues of Pesticides
Single Residue Methods

Protocol Round 2

for the Interlaboratory Validation of the QuPPe-PO Method 1.4 "PerChloPhos"

(draft version 2, Note: Changes from Draft 1 to Draft 2 are highlighted in yellow**)**

Introduction:

This document describes the procedures to be followed by laboratories participating in the interlaboratory validation study of the QuPPe method organized by the EURL-SRM[1] in collaboration with EPRA[2]. The study foresees recovery experiments of selected pesticides from selected commodities at 3 spiking levels. 5 replicate experiments are to be conducted for each pesticide mixture/commodity/spiking level -combination. Table 1 gives an overview of the scope of the study.

Important Changes compared to the first draft:

- **Bromate was excluded** from the exercise.
- **Lemons are used instead of grapes**.
- **Phosphonic acid spiking levels were changed to:** 0.1 mg/kg, 0.2 mg/kg and 2 mg/kg

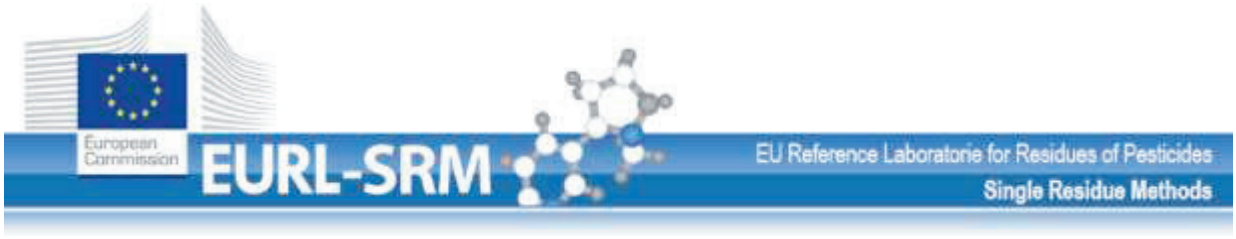
Table 1: Overview of the study

Mix	Compounds	IL-IS	Spiking Levels (refers to 10 g sample)*	Matrices	LC-Method**
II	**Bromide**	-	5 mg/kg 10 mg/kg 100 mg/kg	Carrot Lemon Rye flour Avocados Whole Fat Milk***	**M1.4**
	Chlorate	Chlorate $^{18}O_3$	0.01 mg/kg 0.02 mg/kg 0.2 mg/kg		
	Perchlorate	Perchlorate $^{18}O_4$			
	Phosphonic acid	Phosphonic acid $^{18}O_3$	0.1 mg/kg 0.2 mg/kg 2 mg/kg		

* where 5 g sample are used (rye) the spiking levels are double as high
** based on the nomenclature appearing in the QuPPe method protocol (Version 9.2), published on the EURL-SRM-website.
*** in the following also referred as "Milk"; based on the QuPPe-AO-Method document (Version 2) published on the EURL-SRM-website

All required **standard solutions will be delivered to the participants by the Organizers** of this study. Participating laboratories do not have to perform the full range of experiments foreseen in Table 1, i.e. they may choose to process less commodities and/or less levels. **All other technical aspects in this protocol must, however, be followed as closely as possible to avoid results from having to be excluded from the final evaluation. Any relevant deviations from the protocol should be reported!**

[1] EURL-SRM = EU-Reference Laboratory for pesticides requiring Single Residue Methods
[2] EPRA = Expertengruppe für PSM Rückstandsanalytik (German Pesticide Residue Analysis Expert Group)



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A. Test Materials (Matrices):

The required **blank commodities** are to be acquired by the participating labs. The test materials employed should be tested to not contain any of the compounds included in this study at levels >20% of the lowest validation level. The use of **organically grown crops is thus recommended** but it should be kept in mind that even organic samples may contain these compounds at certain levels. The commodities can be homogenized the way they are typically homogenized in each lab. The use of dry ice is recommended in all cases but not mandatory. If rye is obtained in form of flour, it can be employed as such.

B. Apparatus and Consumables:

See latest version of QuPPe-PO method published in the EURL-SRM-website.

C. Chemicals for Sample Preparation:

See latest version of QuPPe-PO method published in the EURL-SRM-website.

D. Standard Solutions:

All standard solutions necessary for this validation study will be provided by the Organizers. Native pesticides and IL-ISs will be delivered in separate mixtures as listed in Table 2 and

Table 3. The volumes of pesticide and IL-ISs mixtures to be used for the spiking and the preparation of calibration solutions are shown in the pipetting schemes in Table 6 and Table 7 respectively.

Handling of the standard solutions (including IL-ISs): Please keep the solutions in the refrigerator (approximately 4°C).

Table 2: Provided solutions for the Plant Origin (PO) and Animal Origin (AO)- part

Name of solution provided	Compounds contained in solutions provided and their concentrations	No. of vials and volume provided	Spiking Volume to Sample or to Calibration Solution	Comment
SPIKE LOW	Bromide **500 µg/mL** Chlorate/Perchlorate **1 µg/mL** Phosphonic acid **10 µg/mL**	1 á 4 mL	100 µL	Ready to use
SPIKE MED	Bromide **1000 µg/mL** Chlorate/Perchlorate **2 µg/mL** Phosphonic acid **20 µg/mL**	1 á 4 mL	100 µL	
SPIKE HIGH	Bromide **10000 µg/mL** Chlorate/Perchlorate **20 µg/mL** Phosphonic acid **200 µg/mL**	1 á 4 mL	100 µL	
CAL LOW	Bromide **25 µg/mL** Chlorate/Perchlorate **0.05 µg/mL** Phosphonic acid **0.5 µg/mL**	1 á 4 mL	60/120 µL	
CAL MED	Bromide **50 µg/mL** Chlorate/Perchlorate **0.1 µg/mL** Phosphonic acid **1 µg/mL**	1 á 4 mL	60/120 µL	
CAL HIGH	Bromide **500 µg/mL** Chlorate/Perchlorate **1 µg/mL**	1 á 4 mL	60/120 µL	

	Phosphonic acid **10 µg/mL**			

Table 3: IL-IS solutions provided for the Plant Origin (PO) and Animal Origin (AO)- part.

Name of provided IL-IS Stock solution	IL-ISs contained in provided solution	Conc. in provided solution	No. of Vials & Volume provided	Note
IL-IS Chlorate $^{18}O_3$ / Perchlorate $^{18}O_4$	Chlorate $^{18}O_3$ Perchlorate $^{18}O_4$	200 µg/mL	1 á 2 mL	**To be diluted before use! (see below)**
IL-IS Phosphonic acid $^{18}O_3$	Phosphonic acid $^{18}O_3$	200 µg/mL	1 á 2 mL	**To be diluted before use! (see below)**

Before the usage for the validation experiments the delivered IL-IS solutions need to be diluted.

In a first step the received IL-IS solutions need to be diluted 10-fold **to obtain the respective IL-IS SPIKE solutions** (see Table 4).

In a second step the IL-IS SPIKE solutions are mixed and diluted 20-fold to obtain the IL-IS CAL Mix (see Table **5**). **We recommend to dilute the IL-IS CAL solution freshly from the IL-IS SPIKE solution on every sample extraction day.**

Table 4: Information on the preparation of the IL-IS SPIKE solutions

Solution to be used for dilution	Provided IL-IS Conc.	Dissolved in …	Total Volume provided*	How to dilute to prepare respective IL-IS SPIKE	Name of Solution prepared
IL-IS Chlorate $^{18}O_3$/ Perchlorate $^{18}O_4$	200 µg/mL	H_2O	2 mL	10-fold with H_2O	**IL-IS SPIKE ChPe**
IL-IS Phosphonic acid $^{18}O_3$	200 µg/mL	ACN	2 mL	10-fold with ACN	**IL-IS SPIKE Ph**

* This volume is sufficient for 40 mL of the respective ILI-IS Spike. For this exercise, if all experiments are conducted once, max. 10.5 mL of each IL-IS SPIKE are needed.

Table 5: Information on the preparation of the IL-IS CAL Mix solution

Solution to be used for dilution	IL-IS Conc.	Dissolved in …	How to dilute to prepare IL-IS CAL Mix	Name of Solution prepared
IL-IS SPIKE ChPe	20 µg/mL	H_2O	20-fold with MeOH	**IL-IS CAL Mix**
IL-IS SPIKE Ph	20 µg/mL	ACN		

Additional information about the spinking and calibration solutions as well as the expected concentrations can be found in Table 6 and Table 7.

Table 6: Standard solutions for spiking sample portions

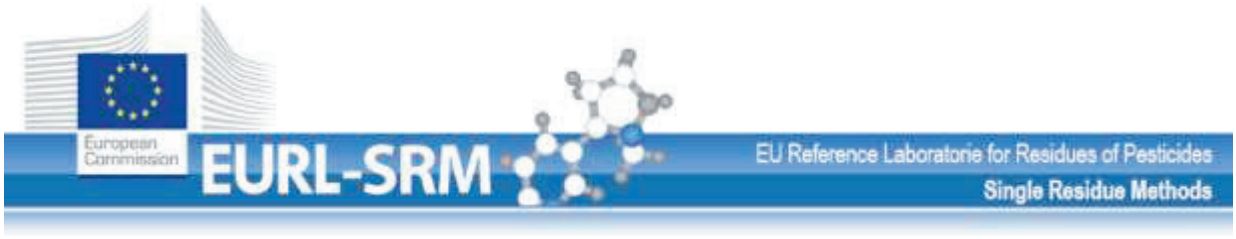
Compound	Solution Names	Pesticide / IL-IS conc. [µg/mL]	Dissolved in …	Corresponding Pesticide / IL-IS Spiking Levels [mg/kg] on…		Expected approximate concentrations of Pesticides / IL-ISs in final extract [µg/mL]	
				Rye	Carrot, Lemon, Avocado and Milk	Carrot, Lemon, Avocado and Rye	Milk
Bromide	**SPIKE LOW**	500	MeOH	10	5	2.5	1.25
Chlorate/Perchlorate		1		0.02	0.01	0.005	0.0025
Phosphonic acid		10		0.2	0.1	0.05	0.025
Bromide	**SPIKE MED**	1000	MeOH	20	10	5	2.5
Chlorate/Perchlorate		2		0.04	0.02	0.01	0.005
Phosphonic acid		20		0.4	0.2	0.1	0.05
Bromide	**SPIKE HGH**	10000	MeOH	200	100	50	25
Chlorate/Perchlorate		20		0.4	0.2	0.1	0.05
Phosphonic acid		200		4	2	1	0.5
Chlorate $^{18}O_3$ / Perchlorate $^{18}O_4$	**IL-IS SPIKE ChPe**	20	H_2O	0.4	0.2	0.1	0.05
Phosphonic acid $^{18}O_3$	**IL-IS SPIKE Ph**	20	ACN	0.4	0.2	0.1	0.05

Table 7: Standard solutions to prepare calibration standards

Compound	Solution Names	Pesticide / IL-IS conc. [µg/mL]	Dissolved in …	Corresponding Calibration Levels of Pesticides / IL-IS * [mg/kg]		Approx. Concentrations of Pesticides / IL-ISs in calibration solutions** [µg/mL]	
				Rye	Carrot, Lemon, Avocado and Milk	Carrot, Lemon, Avocado and Rye	Milk
Bromide	**CAL LOW**	25	MeOH	6 / 12	3 / 6	1.5 / 3	0.75 / 1.5
Chlorate/Perchlorate		0.05		0.012 / 0.024	0.006 / 0.012	0.003 / 0.006	0.0015 / 0.003
Phosphonic acid		0.5		0.12 / 0.24	0.06 / 0.12	0.03 / 0.06	0.015 / 0.03
Bromide	**CAL MED**	50	MeOH	12 / 24	6 / 12	3 / 6	1.5 / 3
Chlorate/Perchlorate		0.1		0.024 / 0.048	0.012 / 0.024	0.006 / 0.012	0.003 / 0.006
Phosphonic acid		1		0.24 / 0.48	0.12 / 0.24	0.06 / 0.12	0.03 / 0.06
Bromide	**CAL HIGH**	500	MeOH	120 / 240	60 / 120	30 / 60	15 / 30
Chlorate/Perchlorate		1		0.24 / 0.48	0.12 / 0.24	0.06 / 0.12	0.03 / 0.06
Phosphonic acid		10		2.4 / 4.8	1.2 / 2.4	0.6 / 1.2	0.3 / 0.6
Chlorate $^{18}O_3$/ Perchlorate $^{18}O_4$ Phosphonic acid $^{18}O_3$	**IL-IS CAL Mix**	1	MeOH	0.4	0.2	0.1	0.05
				0.4	0.2	0.1	0.05

*These calibration levels correspond to 60 and 120% of the expected level at full recovery.

Obtained by adding 100 µL of "IL-IS for CAL"** to each calibration solution for Carrot, Lemon, Avocado and Rye and 50 µL of IL-IS for CAL to calibration solution for Milk.



E. Sample Preparation

1) WEIGHING OF TEST MATERIAL INTO EXTRACTION VESSELS:

- Lemon, Carrot, Avocado, Whole Fat Milk: 10 g ± 0.1 g,
- Rye Flour: 5 g ± 0.05 g.
- Solvent Blank : 10 mL water

Note: If the study will be performed at 3 spiking levels in quintuplicate it is necessary to prepare 17 extraction vessels for each test material (1 blank for the calibrations, 1 blank with IL-IS and 3x5 spiked).

Close the vessels and put them in the **freezer** (except rye flour). Remove the set of samples to be analyzed early enough from the freezer so that the material is half thawed during spiking.

Note: In practice real samples are processed in frozen condition, but spiking of thawed material allows the residues to better distribute within the sample.

2) SPIKING (5 x per level and commodity):

For each level (LOW, MID, HIGH) 5 portions of blank are spiked with pesticides and IL-IS solutions as shown in Table 8.

3) BLANKS and BLANK CONTROLS

a) "**Matrix-Blank for CAL":** Without any additions to be used for preparing the calibration solutions (CAL) but also for "Intermediate Blank Matrix injections" in LC-MS/MS. For volume adjustment add the same volume of solvent to the blank portion as it was added to the spiked samples (e.g. 300 µL MeOH);

b) "**Matrix-Blank + IL-IS" to quantify analyte levels in blank:** With addition of IL-IS;

c) "**Solvent Blank + IL-IS" (reagent blank):** With addition of IL-IS

Shake the liquid samples gently to distribute the pesticides, solvents (a vortex mixer can be used here). Let the samples stand for 5 min before you continue.

Table 8: Pipetting-Scheme for spiking one Matrix at 3 levels ("[Matrix1]")

Suggested Labelling of Extraction Vessels	Spiking of Matrix Portions with...			Volume of "IL-IS SPIKE" to be added		Volume adjustment MeOH*
	SPIKE LOW	SPIKE MED	SPIKE HIGH	IL-IS SPIKE ChPe	IL-IS SPIKE Ph	
[Matrix 1] LOW (1~5)	100 µL	-	-	100 µL	100 µL	-
[Matrix 1] MED (1~5)	-	100 µL	-	100 µL	100 µL	-
[Matrix 1] HIGH (1~5)	-	-	100 µL	100 µL	100 µL	-
MATRIX BLANK (for CAL)	-	-	-			300 µL
MATRIX BLANK + IL-IS (to quantify levels in matrix)	-	-	-	100 µL	100 µL	100 µL
SOLVENT BLANK + IL-IS (reagent blank)				100 µL	100 µL	100 µL

* to equalize the volume solvent added to the various blank matrix portions

4) ADJUSTMENT OF WATER CONTENT TO 10 mL

Add the following amounts of water to each matrix portion:

- Lemon: add 1.5 mL of water
- Carrot: add 1 mL of water
- Avocado: add 3 mL of water
- Rye Flour: add 10 mL of water
- Whole Fat Milk: add 1.2 mL of water

Note: where IL-ISs are used, volume adjustment is not essential as IL-ISs can correct both volumetric errors and matrix effects. In the case of Bromide, no IL-IS is available. Nevertheless, we additionally intent to evaluate the effectiveness of matrix-matched calibration with-out IL-IS. This is the reason why we ask for volumetric adjustment.

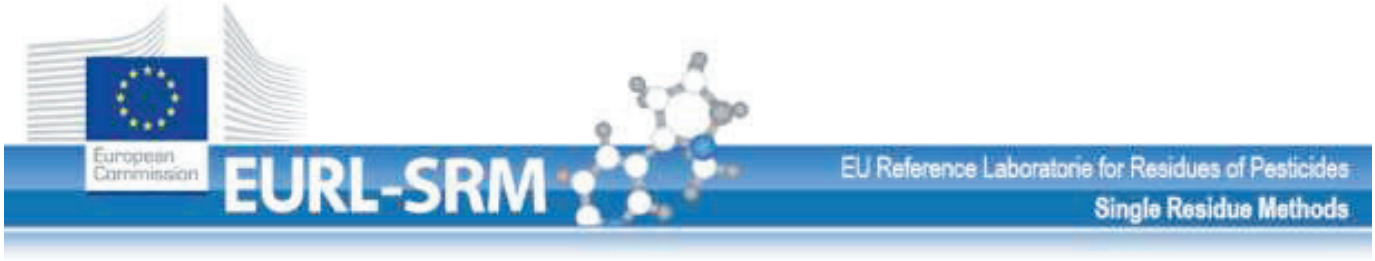
5) ADDITION OF EXTRACTION SOLVENT:

Add **10 mL of acidified methanol (methanol with 1% formic acid v/v)** to all samples (e.g. using a solvent dispenser)

6) EXTRACTION:

Tightly close the extraction vessels and s**hake vigorously for 1 min**. In the case of rye shake mechanically for 15 min.

Note: Fresh samples may also be shaken mechanically for 15 min if this is more convenient. If you do not have a mechanical shaker, shake the rye sample initially by hand for 1 min and repeat this after 15 min of soaking.



7) FREEZING

The extracts of the rye flour may pose difficulties in filtration. To avoid this, place the extraction tubes for at least 3 hours into the freezer and proceed with step 8.

8) CENTRIFUGATION:

Centrifuge **for 5 min.** (at least 4000 rpm).

9) C18-CLEANUP (FOR AVOCADO and MILK):

Transfer a 4 mL aliquot into a 10 mL plastic centrifuge tube, which already contains 200 mg of C18 (ODS) sorbent and additionally 4 mL acetonitrile in case of milk, respectively. Close the tubes and shake 1 min by hand and centrifuge for 5 min (at least 4000 rpm).

10) FILTRATION:

Filter at least 1.5-3 mL of centrifuged extracts into plastic tubes, for example the QuPPe extraction tubes, using cellulose mixed esters with 0.45 µm pore size. Please check the filters for any cross contamination of Chlorate and Perchlorate as especially polyester filters may contain both analytes.

11) PREPARATION OF SOLUTIONS FOR MEASUREMENT:

see pipetting scheme in Table 9.

For the measurement of **Chlorate, Perchlorate and Phosphonic acid a 5-fold dilution of the isolated extract is used**. The dilution can be either made manually by hand (200 µL QuPPe extract + 800 µL MeOH containing 1% Formic acid) or by the instrument (1 µL QuPPe extract + 4 µL MeOH containing 1% Formic acid). Follow the same procedure for the calibration standards, “Solvent blank +IL-IS”, “Matrix Blank +IL-IS” (prepared according to Table 9) and the Blank matrix for “intermediate Blank matrix injection”.
For the measurement of **Bromide a 50-fold dilution of the isolated extract is needed.** We recommend mixing 20 µl of the QuPPe extract with 980 µl MeOH containing 1 % formic acid. Follow the same procedure for the calibration standards (prepared according to Table 9).

Table 9: Pipetting-Scheme for calibration standards for Lemon, Carrot, Avocado, Rye Flour

Solution to be used	Transfer volume to HPLC-Vials	Spiking of Extracts with...				Volume adjustment w. MeOH cont. 1% FA (addition to extracts)	Suggested Labelling of Vials	Final-volume
		CAL LOW	CAL MED	CAL HIGH	„IL-IS CAL-mix“			
Matrix Blank (for CAL)	1 mL	60 µL	-	-	100 µL	60 µL	[Matrix] –LOW CAL- low	1.22 mL
	1 mL	120 µL	-	-	100 µL	-	[Matrix] –LOW CAL- high	1.22 mL
	1 mL	-	60 µL	-	100 µL	60 µL	[Matrix] –MED CAL- low	1.22 mL
	1 mL	-	120 µL	-	100 µL	-	[Matrix] –MED CAL- high	1.22 mL
	1 mL	-	-	60 µL	100 µL	60 µL	[Matrix] –HIGH CAL- low	1.22 mL
	1 mL	-	-	120 µL	100 µL	-	[Matrix] –HIGH CAL- high	1.22 mL
	1 mL	-	-	-	-	-	Interm. Blank Matrix Injection	1.00 mL
Solvent (MeOH with 1% FA)	1 mL	60 µL	-	-	100 µL	60 µL	S- LOW CAL-low	1.22 mL
	1 mL	120 µL	-	-	100 µL	-	S-LOW CAL-high	1.22 mL
	1 mL	-	60 µL	-	100 µL	60 µL	S-MED CAL-low	1.22 mL
	1 mL	-	120 µL	-	100 µL	-	S-MED CAL-high	1.22 mL
	1 mL	-	-	60 µL	100 µL	60 µL	S-HIGH CAL-low	1.22 mL
	1 mL	-	-	120 µL	100 µL	-	S-HIGH CAL-high	1.22 mL
Extracts of spiked samples (LOW, MED and HIGH)	1 mL	-	-	-	-	220 µL	[Matrix]-LOW (1~5) [Matrix]-MED (1~5) [Matrix]-HIGH (1~5)	1.22 mL
Solvent Blank + IL-IS	1 mL	-	-	-	-	220 µL	Solvent Blank + IL-IS	1.22 mL
Matrix Blank + IL-IS	1 mL	-	-	-	-	220 µL	[Matrix] Blank + IL-IS	1.22 mL

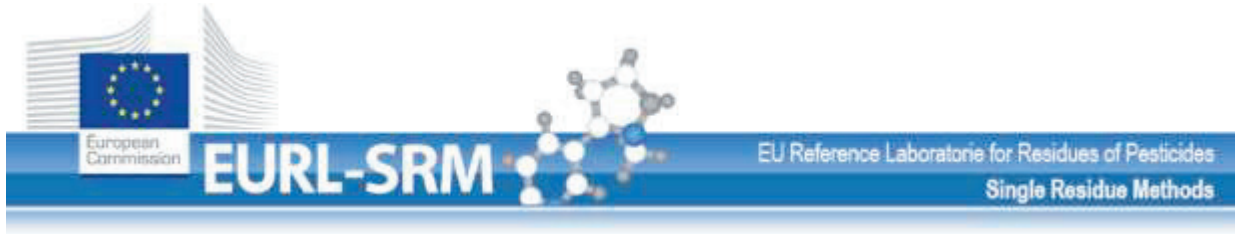


Table 10: Pipetting-Scheme for calibration points for Milk

Solution to be used	Transfer volume to HPLC-Vials	Spiking of Extracts with...				Volume adjustment w. MeOH cont. 1% FA (addition to extracts)	Suggested Labelling of Vials	Final-volume
		CAL LOW	CAL MED	CAL HIGH	„IL-IS for Cal“			
Matrix Blank (for CAL)	1 mL	30 µL	-	-	50 µL	30 µL	Milk-LOW CAL- low	1.11 mL
	1 mL	60 µL	-	-	50 µL	-	Milk-LOW CAL- high	1.11 mL
	1 mL	-	30 µL	-	50 µL	30 µL	Milk-MED CAL- low	1.11 mL
	1 mL	-	60 µL	-	50 µL	-	Milk-MED CAL- high	1.11 mL
	1 mL	-	-	30 µL	50 µL	30 µL	Milk-HIGH CAL- low	1.11 mL
	1 mL	-	-	60 µL	50 µL	-	Milk-HIGH CAL- high	1.11 mL
	1 mL	-	-	-	-	-	Interm. Blank Matrix Injection	1.00 mL
MeOH with 1% FA	1 mL	30 µL	-	-	50 µL	30 µL	S-LOW CAL-low	1.11 mL
	1 mL	60 µL	-	-	50 µL	-	S-LOW CAL-high	1.11 mL
	1 mL	-	30 µL	-	50 µL	30 µL	S-MED CAL-low	1.11 mL
	1 mL	-	60 µL	-	50 µL	-	S-MED CAL-high	1.11 mL
	1 mL	-	-	30 µL	50 µL	30 µL	S-High CAL-low	1.11 mL
	1 mL	-	-	60 µL	50 µL	-	S-HIGH CAL-high	1.11 mL
Extracts of spiked samples (LOW, MED and HIGH)	1 mL	-	-	-	-	110 µL	Milk-LOW (1~5) Milk-MED (1~5) Milk-HIGH (1~5)	1.11 mL
Solvent Blank + IL-IS	1 mL	-	-	-	-	110 µL	Solvent Blank + IL-IS	1.11 mL
Matrix Blank + IL-IS	1 mL	-	-	-	-	110 µL	Milk Blank + IL-IS	1.11 mL

F. Measurement and Data Processing:

Measurement should be conducted by LC-MS/MS following the conditions indicated in Table 11 and

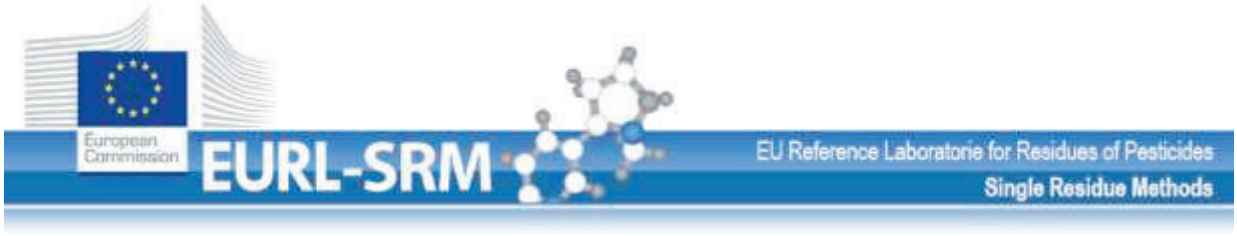


Table 12. We recommend using a well primed Hypercarb column which has been already in routine use.

If you will use a **new column and precolumn priming** by multiple injections of spinach-extracts is necessary. For this please inject 50 times 50 µL QuPPe extract of spinach. If your instrument cannot inject this volume please inject the highest volume possible and increase the number of injections accordingly. If only the pre-column is to be primed 20 injections á 50 µL spinach extract are normally enough. You may use the LC-MS/MS condition shown in Table 13 in Annex 1 for priming. **After the described priming procedure, we recommend to check the condition of the column by injecting a standard mixture before starting the validation. It's important to include additional spinach extract injections during the sequence if the column is freshly primed.**

Further hints for priming and using the Hypercarb column, can be found in Annex 1.

Table 11: LC-MS/MS conditions (Method 1.4 for "PerChloPhos")

Ionisation mode	ESI neg				
Column/ Temperature	**Hypercarb** 2.1 x 100 mm, 5 µm, (P/N 35005-102130); 40°C				
Pre-column	**Hypercarb Guard** 2.1 x 10 mm, 5 µm (P/N 35005-102101)				
Pre-filters	e.g. Supelco column saver 2.0 µm Filter (optional)				
Eluent A	1% Acetic acid in Water + 5% MeOH **Use brown glass bottles to avoid growth of algi!**				
Eluent B	1% Acetic acid in MeOH				
Gradient	**%A**	**Flow [mL/min]**	**Time [min]**		
	100	0.4	0		
	70	0.4	10		
	100	0.4	10.1		
	100	0.4	15		
Injection volume	5 µL				
Dilution	**1:5 dilution MeOH containing 1% Formic acid (1 µL sample extract + 4 µL MeOH containing 1% Formic acid)**				
Calibration standards	See protocol				
Acquired mass transitions	**Compound**	**Mass Transitions (m/z)**			
	Bromide*	81/81	79/79		
	Chlorate	83/67	85/69		
	Chlorate-$^{18}O_3$ (ILIS)	89/71			
	Perchlorate	99/83	101/85		
	Perchlorate-$^{18}O_4$ (ILIS)	107/89			
	Phosphonic acid	81/79	81/63		
	Phosphonic acid $^{18}O_3$ (ILIS)	87/85			

Table 12: Mass Transitions and Instrumental settings for a ABSciex 5500 LC-MS/MS

Substance Name	Parent	Daughter	DP	EP	CE	CXP
Bromid 81/81	80.7	80.7	-35	-10	-60*	-1
Bromid 79/79	78.8	78.8	-35	-10	-70*	-1
Chlorat 83/67	82.8	67.0	-85	-10	-30	-1
Chlorat 85/69	84.8	69.0	-95	-10	-30	-1
Chlorate $^{18}O_3$ IS 89/71	88.9	70.9	-100	-10	-30	-2
Perchlorate 99/83	98.8	82.9	-95	-10	-38	-5
Perchlorate 101/85	100.8	84.9	-95	-10	-36	-13
Perchlorate $^{18}O_4$ IS 107/89	106.8	89.0	-110	-10	-38	-11
Phosphonic acid 81/63	80.8	3.0	-60	-10	-42	-3
Phosphonic acid 81/79	80.8	78.9	-60	-10	-22	-5
Phosphonic acid $^{18}O_3$ IS 87/85	87.0	85.0	-80	-10	-30	-5
Phosphonic acid $^{18}O_3$ IS 87/67	87.0	67.0	-80	-10	-40	-3

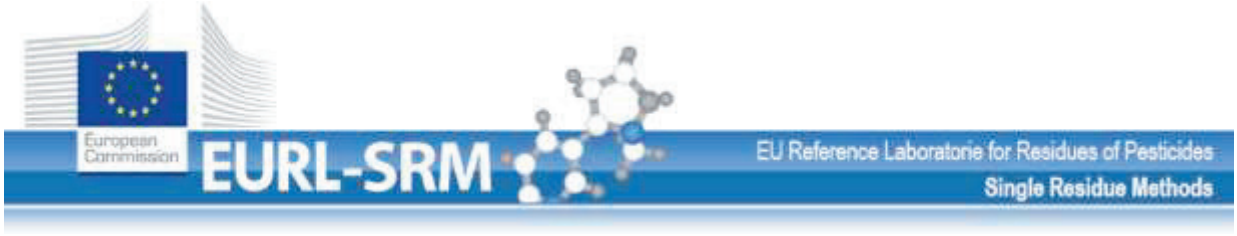
* We recommend not using the collision energy optimized by the instrument software due to strong phosphate interferences, see also Annex 1, chapter 3).

1) INJECTION-SEQUENCES

Note: for simplicity only one exemplary sequence is shown below. Each spiking level requires its own sequence.

a) INJECTIONS FOR CHLORATE, PERCHLORATE AND PHOSPHONIC ACID

1 Solvent-BLANK (check for analytes and interferences and report them)
2 [Matrix]-BLANK + IL-IS (check for analytes and interferences and report them)
3 Solvent-CAL- LOW/MID/HIGH 60%
4 Solvent-CAL-LOW/MID/HIGH 120%
5 Intermediate Blank Matrix injection
6 [Matrix]-CAL-LOW/MID/HIGH 60%
7 [Matrix]-CAL-LOW/MID/HIGH 120%
8 Intermediate Blank Matrix injection
9 [Matrix]-LOW/MID/HIGH1
10 [Matrix]-LOW/MID/HIGH2
11 [Matrix]-LOW/MID/HIGH3
12 [Matrix]-LOW/MID/HIGH4
13 [Matrix]-LOW/MID/HIGH5
14 Intermediate Blank Matrix injection
15 Solvent-CAL-LOW/MID/HIGH 60%
16 Solvent-CAL- LOW/MID/HIGH 120%
17 Intermediate Blank Matrix injection
18 [Matrix]-CAL- LOW/MID/HIGH 60%
19 [Matrix]-CAL- LOW/MID/HIGH 120%
20 MeOH +1% FA (check for carry-over and report if relevant, should be<2%).
21 5 µL Spinach QuPPe Extract



b) INJECTIONS FOR BROMIDE

1	Solvent-BLANK 1:50 (check for analytes and interferences and report them)
2	[Matrix]-BLANK + IL-IS 1:50 (check for analytes and interferences and report them)
3	Solvent-CAL-LOW/MID/HIGH 60% 1:50
4	Solvent-CAL--LOW/MID/HIGH 120% 1:50
5	Intermediate Blank Matrix injection 1:50
6	[Matrix]-CAL-LOW/MID/HIGH 60% 1:50
7	[Matrix Code]-CAL-LOW/MID/HIGH 120% 1:50
8	Intermediate Blank Matrix injection 1:50
9	[Matrix]-LOW/MID/HIGH1 1:50
10	[Matrix]-LOW/MID/HIGH2 1:50
11	[Matrix]-LOW/MID/HIGH3 1:50
12	[Matrix]-LOW/MID/HIGH4 1:50
13	[Matrix]-LOW/MID/HIGH5 1:50
14	Intermediate Blank Matrix injection 1:50
15	Solvent-CAL-LOW/MID/HIGH 60% 1:50
16	Solvent-CAL- LOW/MID/HIGH 120% 1:50
17	Intermediate Blank Matrix injection 1:50
18	[Matrix]-CAL- LOW/MID/HIGH 60% 1:50
19	[Matrix]-CAL- LOW/MID/HIGH 120% 1:50
20	MeOH + 1% FA (check for carry-over and report if relevant, should be <2%).
21	5 µL Spinach QuPPe Extract

2) DATA PROCESSING:

The measured peak areas of the pesticides and IL-ISs should be tracked into an **Excel sheet specially prepared by the Organizer for this study and distributed to the participants**. All recovery calculations are done automatically.

The recovery figures obtained by the participants will be entered in the Method Validation Database within the EURL-Datapool (www.eurl-pesticides-datapool.eu).

ANNEX 1

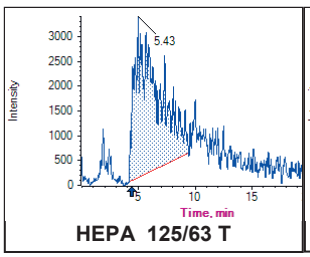
1) Hints for priming and using the Hypercarb column

If you use a new Hypercarb column please prime before start with the measurements of the validation study. The hypercarb column and its pre-column should be thoroughly primed before usage. Priming and reconditioning of the column: before the first use, the Hypercarb columns and pre-columns have to be thoroughly primed to cover certain active sites on the surface. Priming with solutions containing planar molecules such as chlorophyll and anthocyans accelerates the priming period. A recommendable procedure for priming is the injection of QuPPe extracts of spinach (for equilibration of the pre-column inject 10-15 injections spinach extracts, for column and pre-column inject 50 injections spinach extracts, if possible inject 50 µL)

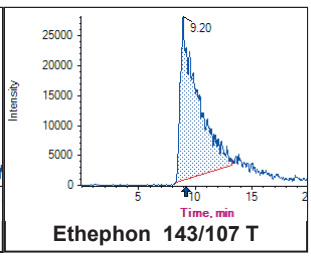
This masking of the active sites is temporary and the activity of the column gradually increases with the injection of solvent or diluted extracts. Following a sequence of injections with low or no matrix load will typically raise the need for intermediate conditioning with extracts to restore the column. The impact of priming on the chromatographic properties of the column is exemplary shown in Figures 10, 11 and 12.:

Table 13: Proposed LC-MS/MS conditions for priming and reconditioning of the Hypercarb column.

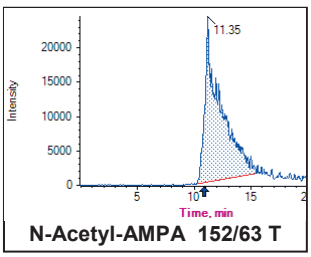
Instrument parameters	**Conditions**		
Ionisation mode	ESI neg		
Column/temperature	**Hypercarb** 2.1 x 100 mm 5 µm (P/N 35005-102130); 40°C		
Pre-column	**Hypercarb Guard** 2.1 x 10 mm 5 µm (P/N 35005-102101)		
Pre-filters	e.g. Supelco column saver 2.0 µm Filter (optional)		
Eluent A	1% Acetic acid in Water + 5% MeOH		
Eluent B	1% Acetic acid in MeOH		
Gradient	**%A**	**Flow [mL/min]**	**Time [min]**
	100	0.3	0
	70	0.3	7
	100	0.3	7.1
	100	0.3	12
Injection volume	50 µL		
MS-System	If possible disconnect the MS-System to prevent contamination of the MS.		



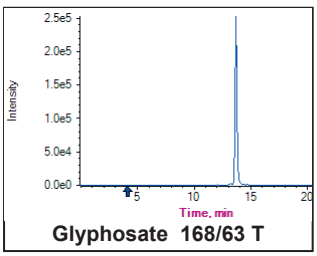
HEPA 125/63 T



Ethephon 143/107 T



N-Acetyl-AMPA 152/63 T



Glyphosate 168/63 T

Figure 1: Chromatograms obtained using a new Hypercarb column, poor chromatographic behavior due to strong interactions of analytes with active sites. Same behavior is observed when the pre-column is new.

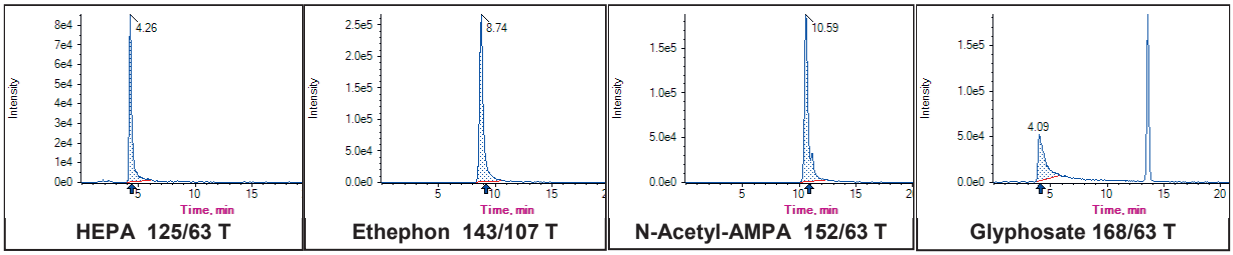


Figure 2: Chromatograms following priming with 25 injections (50 µL) of Spinach QuPPe extracts.

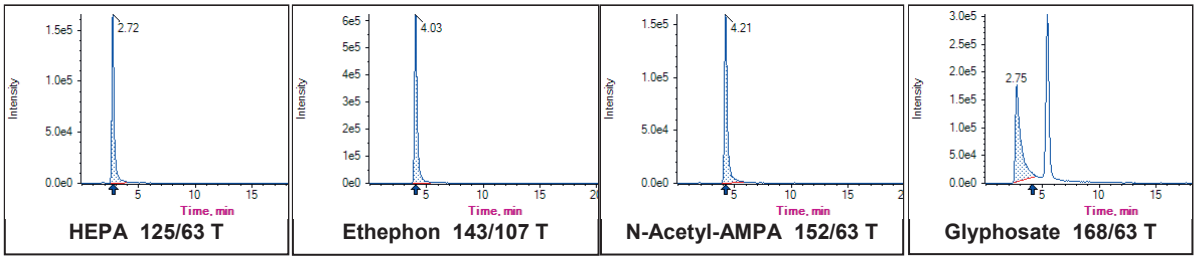


Figure 3: Chromatograms after additional injection of approx 100 QuPPe-extracts of various fruit and vegetables during normal routine use.

Pre-columns (guard columns): The pre-column should be exchanged as soon as a clear deterioration of the separation performance (worsening of peak-shape) is noticed. The pre-column of method 1.3 needs to be clearly less often exchanged compared to the pre-columns of methods 1.1 and 1.2. Any exchange of the pre-column requires priming as described above. For this the pre-column does not have to be attached to the column. Connecting several pre-columns in a row and priming them simultaneously is also an option.

Storage of columns: Following normal operation the column can be stored directly after any normal sequence/run (full gradient). Run system 3-4 times with full gradient to reactivate the column (inject standards in matrix) before starting the sequence. If to be stored for longer periods (e.g. >2 months) it is recommended to recondition the column as described above.

Pre-filters: If pre-filters are used exchange them as soon as backpressure increases significantly. For practical and convenience reasons it is recommended to exchange pre-filters when performing other maintenance operations such as reconditioning or pre-column exchange. If after pre-filter exchange (see above) the pressure does not come back to normal levels, the frit of the pre-column may need to be exchanged. Check the filters for any cross-contamination of Perchlorate and Chlorate.

2) Hints for Phosphonic acid and Phosphoric acid

When extracts containing high levels of Phosphoric acid (which is naturally contained at high concentrations in many samples) are injected, the chromatographic separation of Phosphoric and Phosphonic acid is compromised. This often results in a suppression of the Phosphonic acid signal and in some cases even leads to false negative results. The most important qualifier mass-transition of Phosphonic acid (m/z 81/63) also occurs as a minor transition of the in-source fragment of Phosphoric acid, but as the latter is often present at much higher levels than Phosphonic acid the interference on this mass transition can still be significant, especially if these two elute in close vicinity (exemplarily shown at the chromatograms in **Figure 4**). The chromatographic separation of Phosphoric and Phosphonic acid considerably improves following dilution of the extracts typically allowing proper detection, identification and quantification of Phosphonic acid next to high levels of phosphoric acid. It is thus beneficial to inject smaller volumes of sample extract (e.g. 1-2 µL) or to dilute QuPPe extracts 5-10-fold before injection. Fortunately both, Phosphoric and Phosphonic acid have at least one proper mass-transition (m/z 97/63 and 81/79 respectively, shown in **Figure 4** which in the case of Phosphonic acid can be used for quantitation and to improve identification certainty. The elution time and peak shape of the Phosphonic acid ILIS can also be used to distinguish it from Phosphoric acid and to avoid false positives. Using signals on the m/z 81/63 mass trace it was calculated that approx. 200 mg/kg Phosphoric acid would fake 0.1 mg/kg Phosphonic acid if this mass transition was used for quantification. In an experiment using Differential Mobility Separation (DMS) technique (see Figure 8 and Figure 9) a separation of Phosphoric acid and Phosphonic acid at the mass trace m/z 81/63 was achieved.

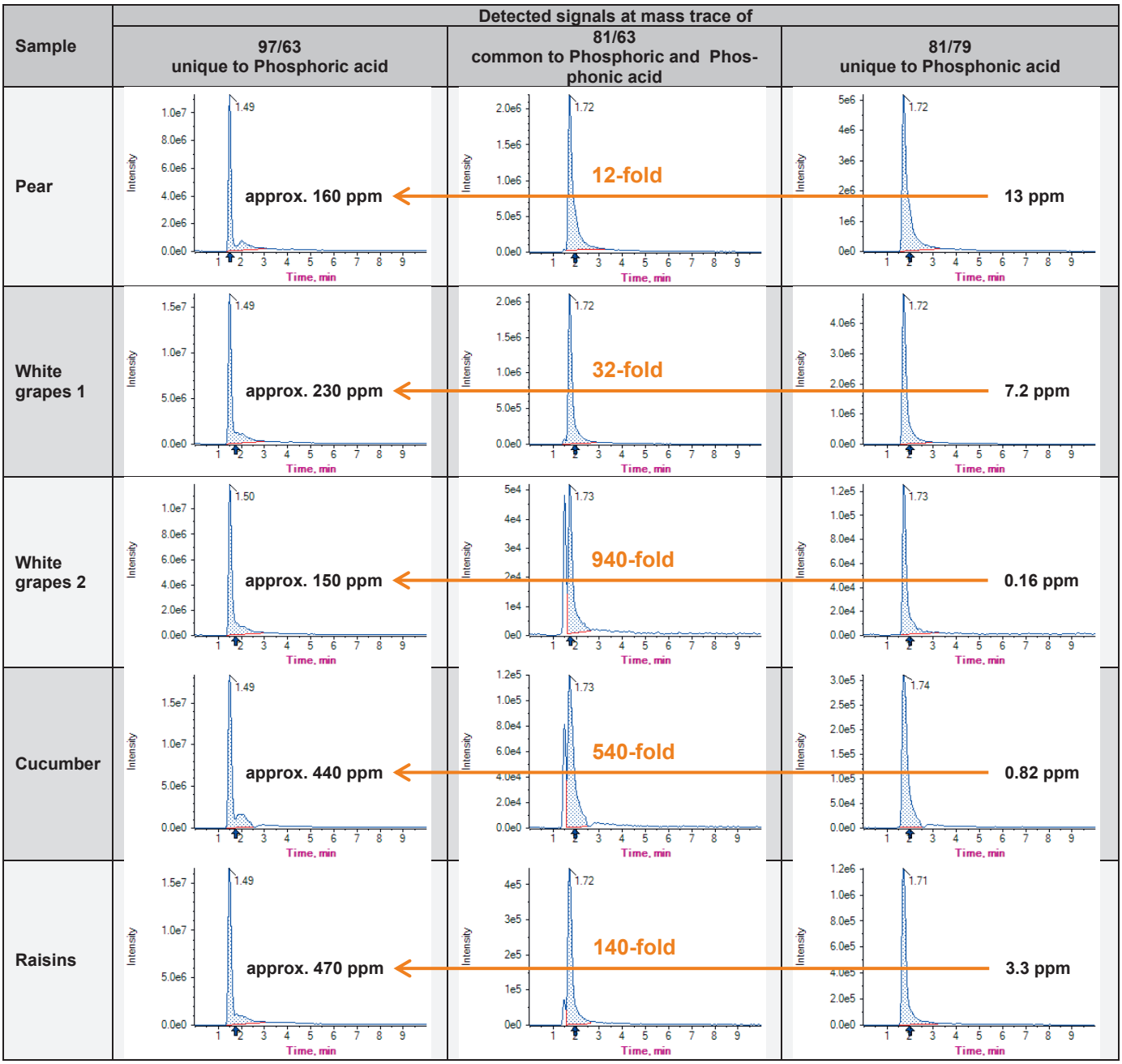


Figure 4: Chromatographic and mass-spectrometric separation of Phosphoric and Phosphonic acid.

3) Hints for the determination of bromide

High levels of Phosphoric acid (which is naturally contained at high concentrations in many samples) or Phosphonic acid (that is used as insecticide) could affect the determination of bromide. Depending on the condition of the column, the separation of these three compounds could be insufficient, resulting in compromised identification and quantification. Bromine is mainly composed of two naturally occurring stable isotopes, that are almost equally frequent (^{79}Br and ^{81}Br). Being an element, no MS/MS fragmentation is possible so that MS/MS analysis has to rely on “parent/parent” analysis. The mass trace m/z 81/81 is recommended for quantifications whereas m/z 79/79 can be used as a qualifier. The mass trace m/z 81/81 is

interfered by Phosphonic acid (m/z of [H2PO3]-=81) whereas m/z 79/79 is highly affected by Phosphoric acid due to in-source fragmentation (Figure 5, the two columns declared as “CE -5 V”), the two left columns). At the mass trace m/z 81/81, 10 ppm Phosphonic acid simulate 7 ppm Bromide. At the mass trace m/z 79/79, 10 ppm Phosphoric acid simulate aprox. 2.5 ppm bromide. In practice the interference by Phosphoric acid is more critical as it is naturally contained at high levels (e.g. 100-2000 mg/kg) in various samples. A 50-fold dilution of QuPPe extracts typically allows better identification and quantification of bromide next to high levels of Phosphoric and Phosphonic acid as this improves chromatographic separation and reduces matrix-effects. To improve selectivity and increase quantification accuracy and identification certainty, the interferences caused by Phosphoric and Phosphonic acid can be further reduced by increasing the Collision Energy (CE) for the m/z 81 and 79 (Figure 5, the two columns declared as “CE -70 V”). While Bromide cannot be fragmented, the interfering quasi-molecular ion of Phosphonic acid (m/z 81) as well as the interfering in-source fragments of Phosphoric and Phosphonic acid (m/z 79) are largely destroyed by increased collision induced dissociation. While losing up to a 100-fold of absolute sensitivity, the interferences were largely decreased resulting in a better signal-to-noise ratio.

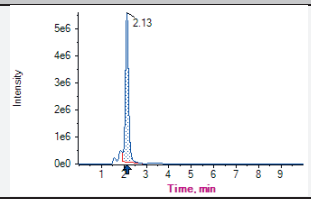
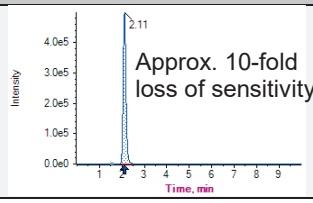
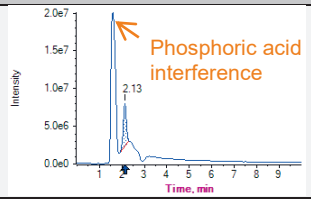
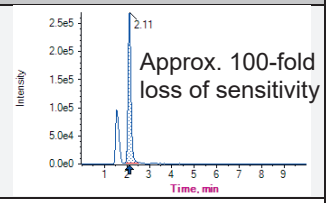
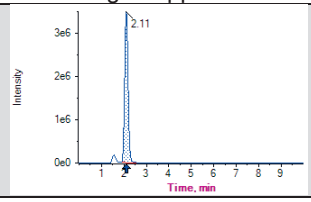
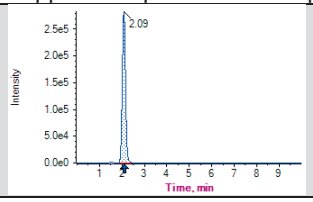
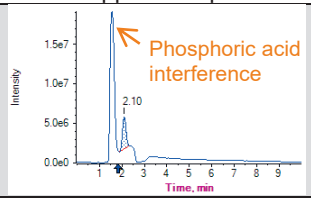
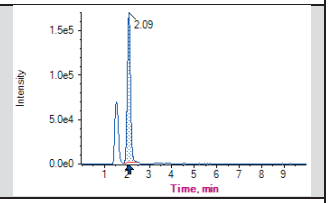
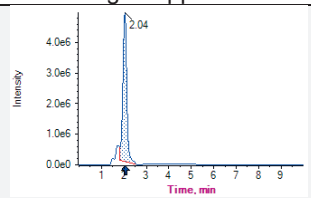
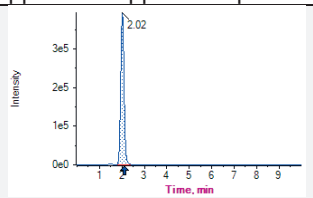
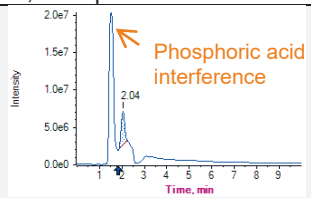
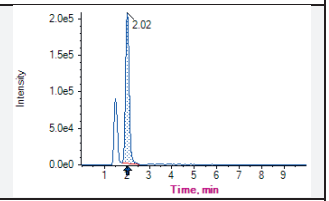
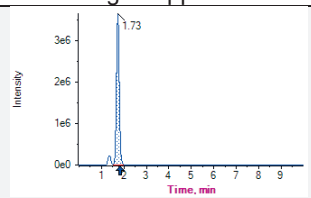
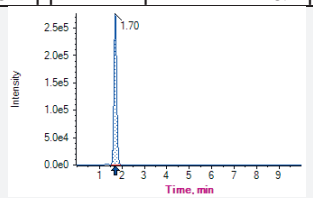
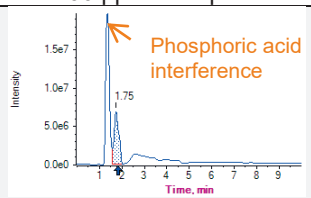
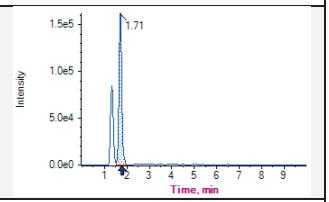
Commodity	m/z 81/81		m/z 79/79	
	CE -5 V	CE -60 V	CE -5 V	CE -70 V
Cucumber				
containing 1.7 ppm Bromide, 2.0 ppm Phosphonic acid & approx. 300 ppm Phosphoric acid				
Mint leaves				
containing 1.1 ppm Bromide, approx. 370 ppm Phosphoric acid; Phosphonic acid n. d.				
Fennel				
containing 2.2 ppm Bromide, 5.4 ppm Phosphonic acid & approx. 400 ppm Phosphoric acid				
Sweet Corn				
Containing 1.1 ppm Bromide, approx. 350 ppm Phosphoric acid; Phosphonic acid n. d.				

Figure 5: Chromatograms of Bromide using non-optimized collision energies (CE -5 V) showing the interference by Phosphoric acid and Phosphonic acid as well as optimized collision energies (CE -60 V and -70 V, the) showing reduced interferences.

4) Hints for Chlorate and Perchlorate measurement

Chlorate can be a minor contaminant of Perchlorate solutions and is also a minor in-source fragment of Perchlorate. In the experiment shown below Perchlorate standard at 0.2 µg/mL was injected resulting in two peaks on the mass traces of Chlorate (see Table 5). One originating from Chlorate contained as impurity in the Perchlorate solution (at approx. 0.35%) and one originating from in-source fragmentation at the retention time of Perchlorate, corresponding to a Chlorate amount of 0.001 µg/mL. This means that calibration solutions containing both chlorate and perchlorate at the same level the chlorate signal will be overestimated by approx. 0.5% which is negligible. Also samples containing perchlorate may fake the presence of chlorate at very low levels normally well below the reporting level of chlorate. When chlorate ILIS is co-injected misidentification is highly unlikely as the two compounds typically separate well chromatographically.

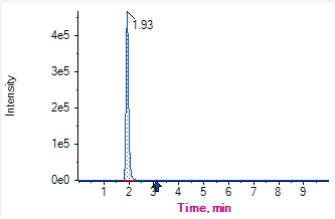
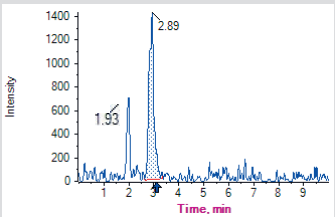
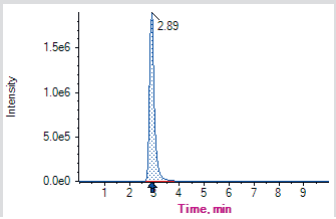
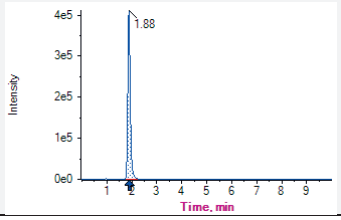
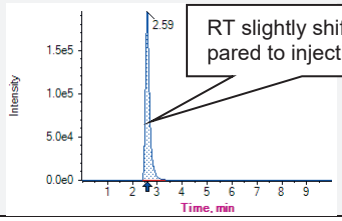
Calibration standard injected	Detected signals at mass trace of …	
Chlorate 0.2 µg/mL	Chlorate 83/67 	
Perchlorate 0.2 µg/mL	Chlorate 83/67 	Perchlorate 99/83 
Chlorate-$^{18}O_3$ (approx. 0.18 µg/mL) + Perchlorate-$^{18}O_4$ (approx. 0.02 µg/mL)	Chlorate-$^{18}O_3$ 89/71 	Perchlorate-$^{18}O_4$ 107/89  RT slightly shifted compared to injection above

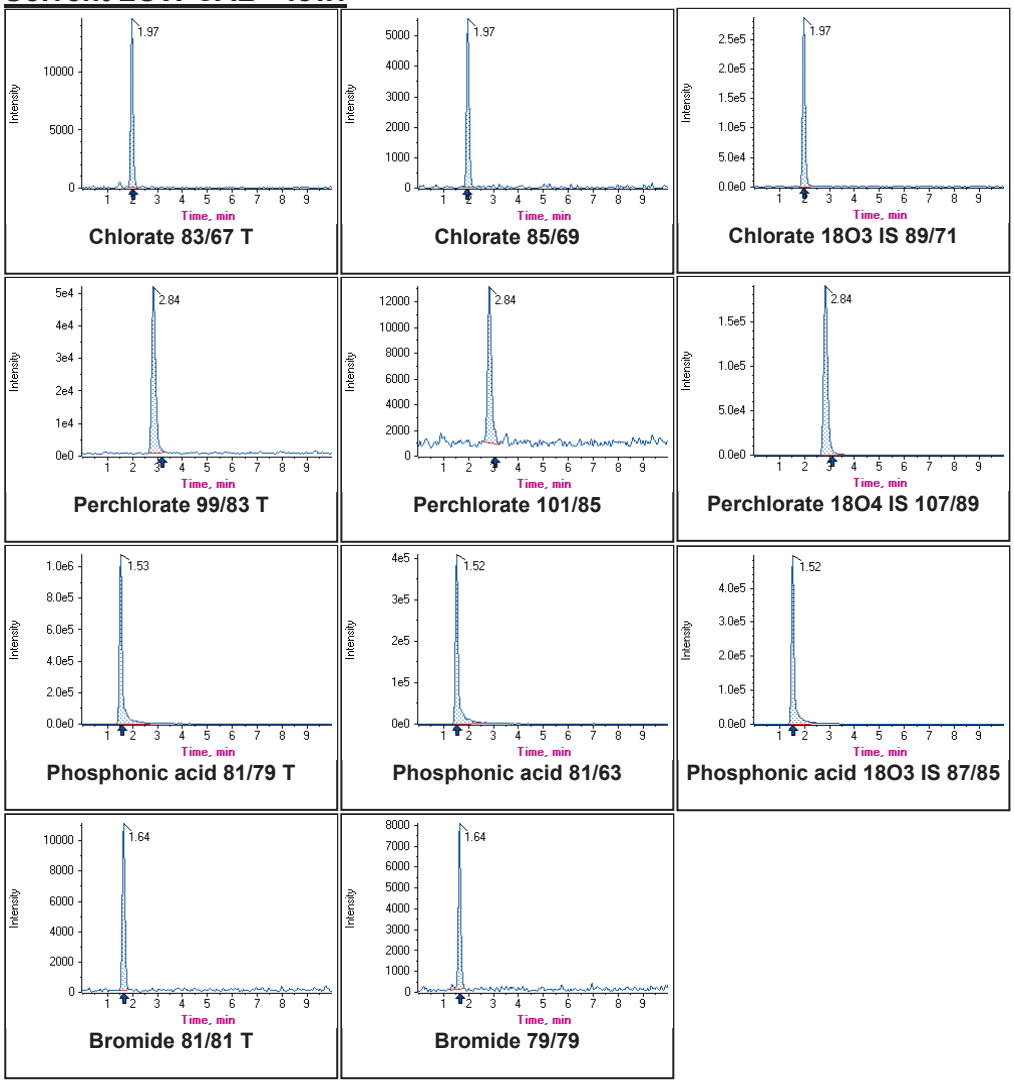
Figure 6: Chromatograms of Chlorate and Perchlorate at 0.2 µg/mL and of a mixture of Chlorate-$^{18}O_3$ and Perchlorate-$^{18}O_4$, containing approx. 0.2 µg/mL Chlorate $^{18}O_3$ and approx. 0.02 µg/mL Perchlorate-$^{18}O_4$.

ANNEX 2

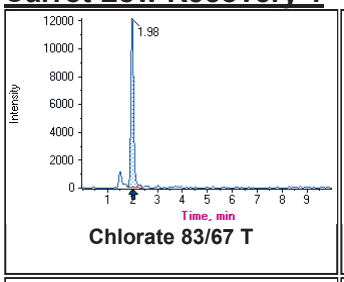
Typical chromatograms of the spiking level 0.01 mg/kg and the corresponding labeled IL-ISs obtained using the condition in Table 11 **and**

Table 12

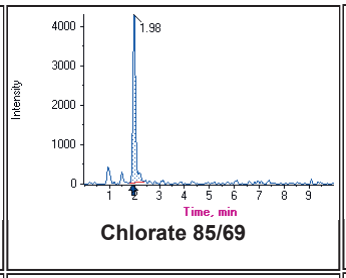
Solvent LOW CAL – low:



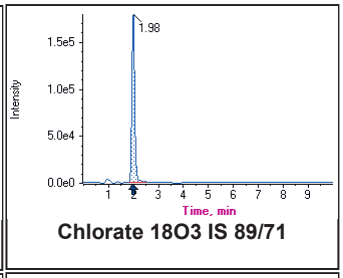
Carrot Low Recovery 1



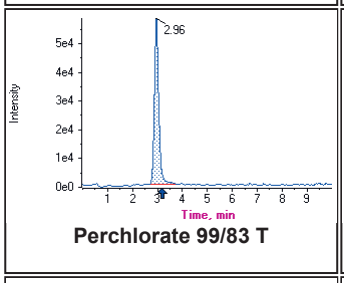
Chlorate 83/67 T



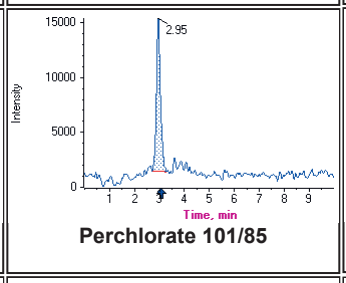
Chlorate 85/69



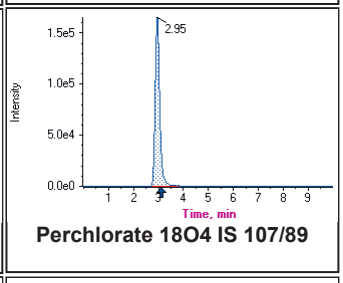
Chlorate 18O3 IS 89/71



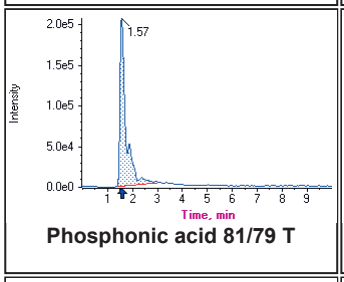
Perchlorate 99/83 T



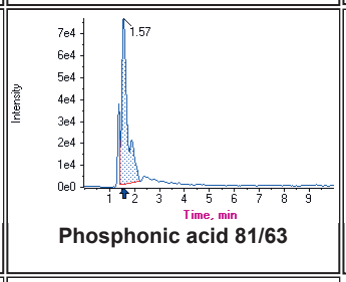
Perchlorate 101/85



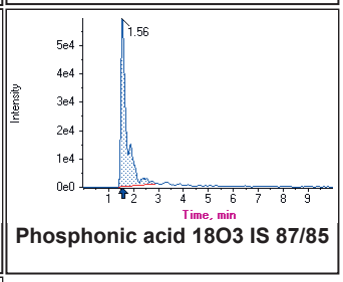
Perchlorate 18O4 IS 107/89



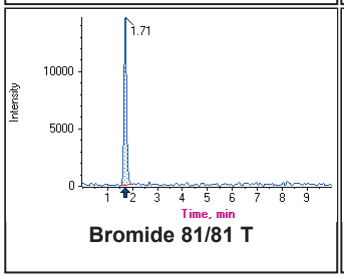
Phosphonic acid 81/79 T



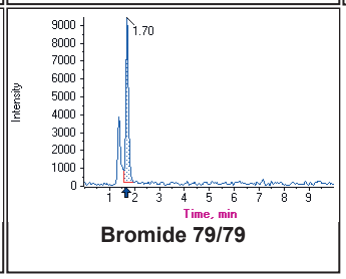
Phosphonic acid 81/63



Phosphonic acid 18O3 IS 87/85

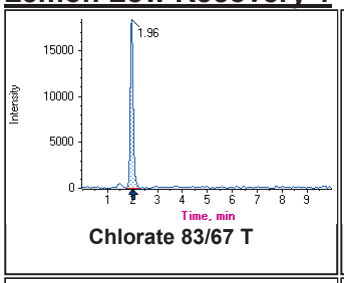


Bromide 81/81 T

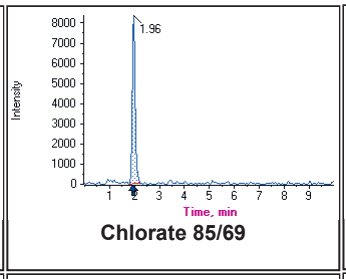


Bromide 79/79

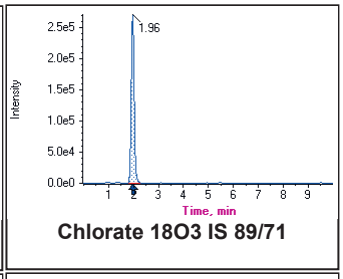
Lemon Low Recovery 1



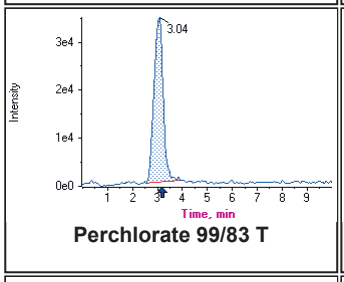
Chlorate 83/67 T



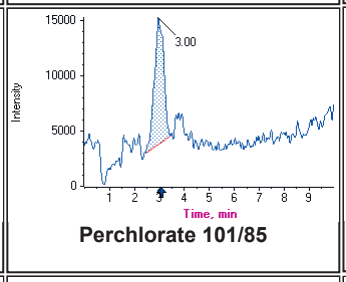
Chlorate 85/69



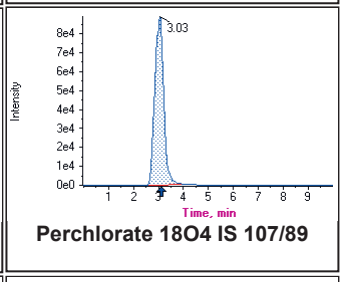
Chlorate 18O3 IS 89/71



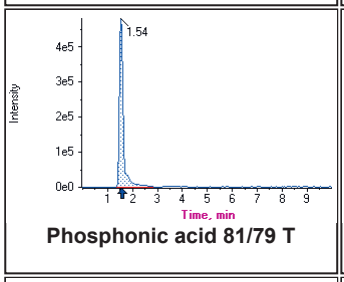
Perchlorate 99/83 T



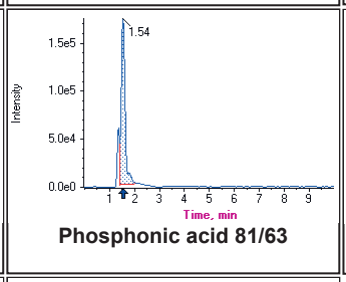
Perchlorate 101/85



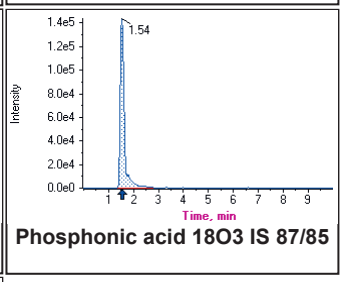
Perchlorate 18O4 IS 107/89



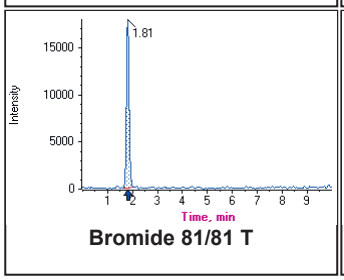
Phosphonic acid 81/79 T



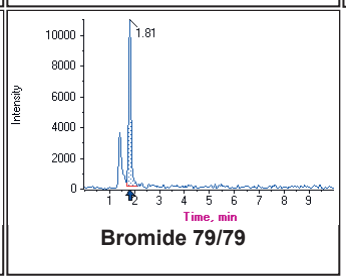
Phosphonic acid 81/63



Phosphonic acid 18O3 IS 87/85

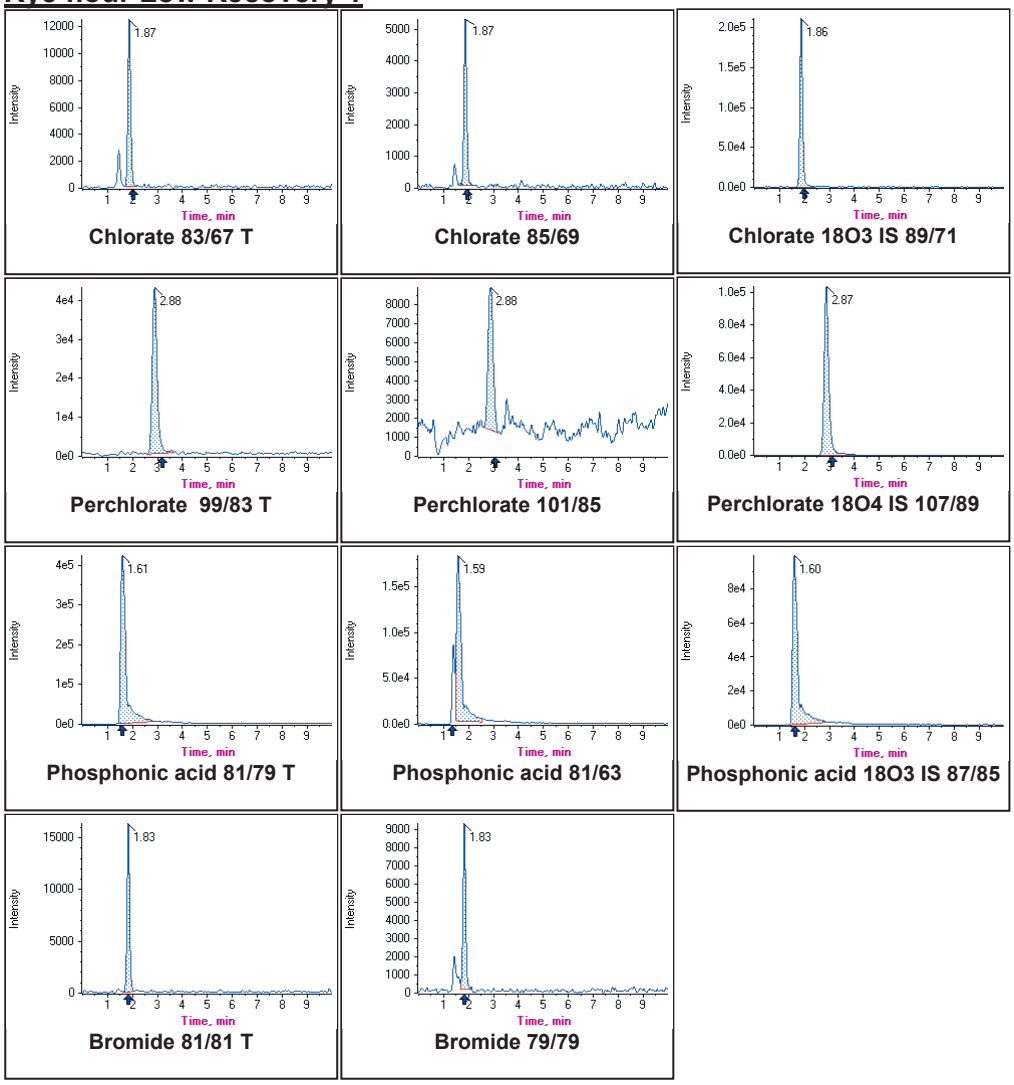


Bromide 81/81 T

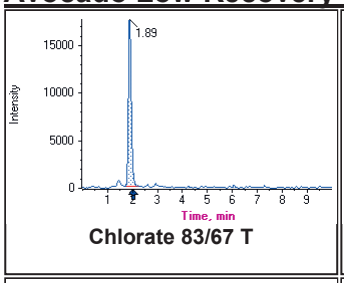


Bromide 79/79

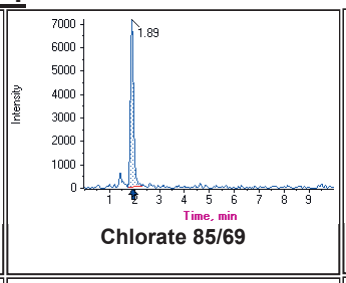
Rye flour Low Recovery 1



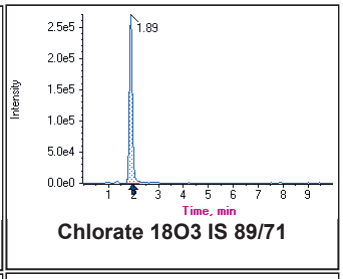
Avocado Low Recovery 1



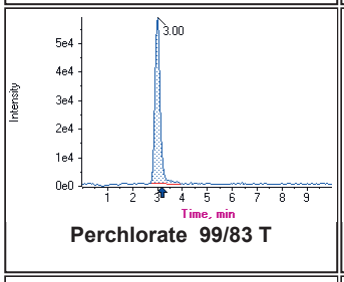
Chlorate 83/67 T



Chlorate 85/69



Chlorate 18O3 IS 89/71



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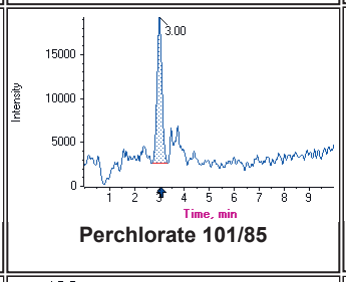
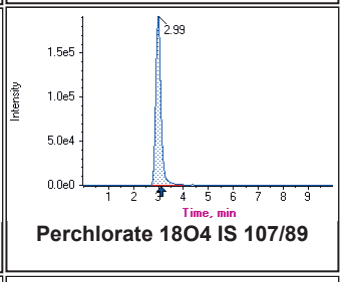
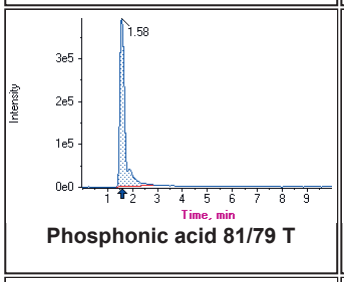


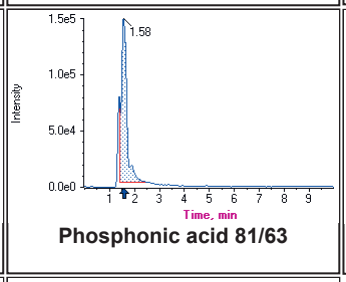
Perchlorate 101/85



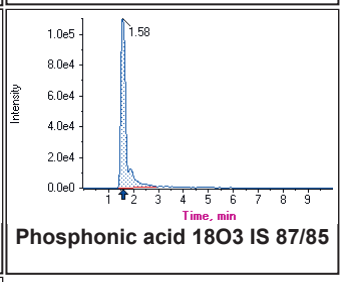
Perchlorate 18O4 IS 107/89



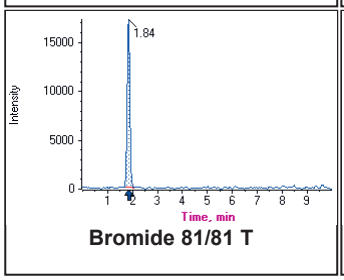
Phosphonic acid 81/79 T



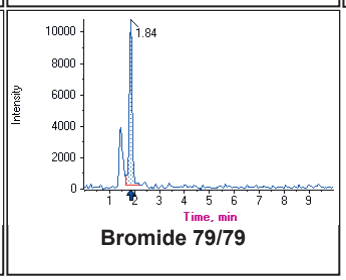
Phosphonic acid 81/63



Phosphonic acid 18O3 IS 87/85

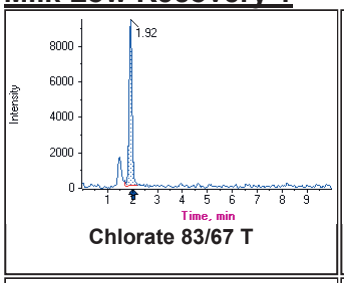


Bromide 81/81 T

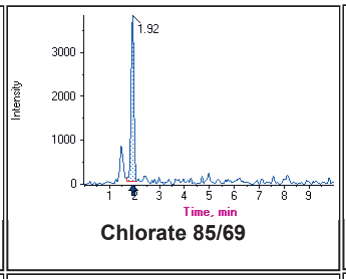


Bromide 79/79

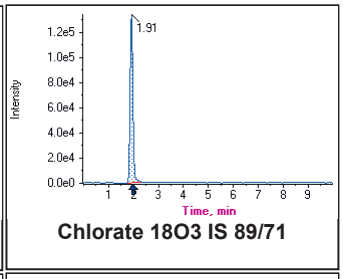
Milk Low Recovery 1



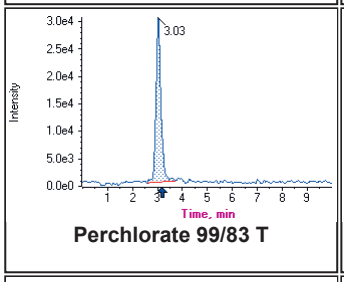
Chlorate 83/67 T



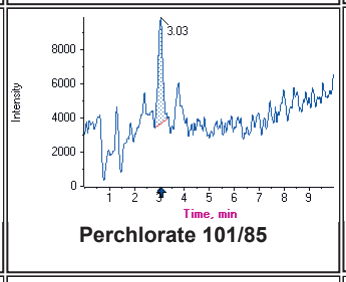
Chlorate 85/69



Chlorate 18O3 IS 89/71



Perchlorate 99/83 T



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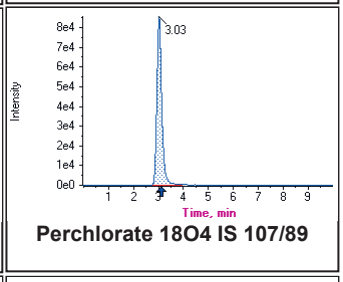
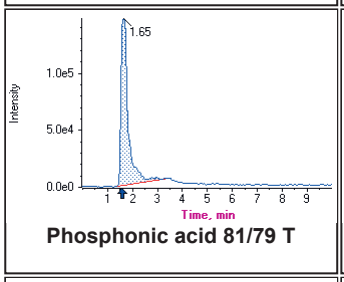
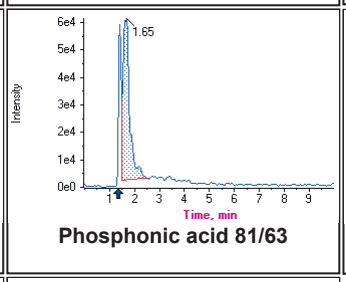


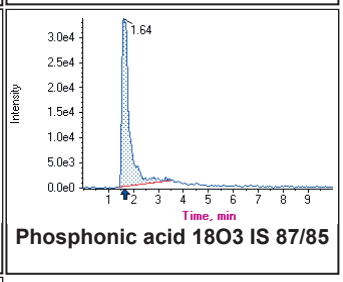
Perchlorate 18O4 IS 107/89



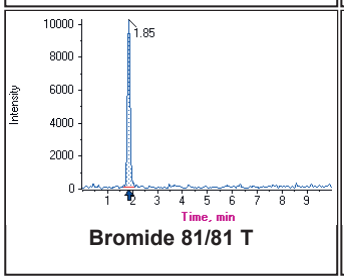
Phosphonic acid 81/79 T



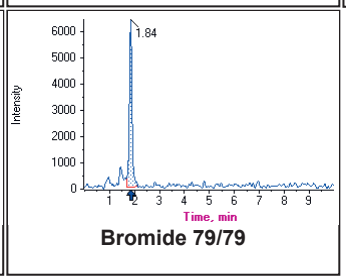
Phosphonic acid 81/63



Phosphonic acid 18O3 IS 87/85



Bromide 81/81 T



Bromide 79/79

5. Protocol Round 3

European Commission
EURL-SRM
EU Reference Laboratories for Residues of Pesticides
Single Residue Methods

Protocol Round 3

for the Interlaboratory Validation of QuPPe "Gly & Co."

(Version 3)

Introduction:

The QuPPe method is widely used by OfLs and commercial laboratories throughout the EU and beyond, various of these labs have expressed their wish to standardize the QuPPe method. A CEN-project has thus been launched aiming to introduce parts of the QuPPe method[1] as a CEN-standard. A central requirement in this process is **to prove that the method is fit for purpose** which can be achieved via **inter-laboratory validation trials**.

Two validation trials, one on QuPPe M4.1 ("Quats&Co.") and one on QuPPe M1.4 ("PerChloPhos"), have been already successfully conducted. The present **3rd validation round**, **focuses on analytes** of the "Gly&Co."-group, which are **analysed by LC-MS/MS in the ESI (neg.) mode**.

The study foresees recovery experiments for:

- **11 analytes** (see Table 1 and Table 2),
- **7 commodities** (Cucumber, Strawberry, Rice, Soybean, Whole fat Milk[2], Liver, Kidney),
- **4 spiking levels** (LOW, MED, HIGH, MAX).

The **number of replicates** per analyte/commodity/spiking level combination **is fixed to n=5**.

THE PARTICIPANTS DO NOT HAVE TO COVER THE FULL RANGE OF EXPERIMENTS, they may choose to process fewer commodities and/or analytes and/or levels.

The main **focus is on the LC-procedures M1.3 and M1.6**, but the use of other HILIC-based LC-methods (e.g. M1.5) is also possible. Nevertheless, **the extraction and clean-up procedures described in this protocol must be followed as closely as possible**. In case of significant deviations, results may have to be excluded from the final joint evaluation. Any relevant deviations from the protocol should be reported!

[1] see EURL-SRM-website[1], http://www.eurl-pesticides.eu/docs/public/tmplt_article.asp?CntID=887&LabID=200&Lang=EN
[2] in the following also referred as "Milk"

ALL REQUIRED SOLUTIONS OF THE ANALYTES AND THEIR CORRESPINDING ILISs WILL BE PROVIDED BY THE ORGANIZER. Due to the different measurement sensitivity exhibited by each analyte (which is also instrument, column and matrix dependent), **the spiking solutions will contain the analytes at different concentrations,** so there will be deviating spiking concentrations for different analytes within the same recovery experiment. Within the Excel data collection template, the calculations are done by setting the spiking concentration to a default value of 1 mg/kg for simplicity.

Table 1 gives an overview of the analytes, the corresponding ILISs and the foreseen spiking levels (LOW, MED, HIGH, MAX). Participating laboratories are free to choose which among these levels they would like to validate. For the commodities where 5 g sample portions are used the spiking concentrations for the same spiking amount are twice as high, and are thus shown in a separate column.

Table 2 gives an overview of the procedures and particularities applying to each matrix-column combination.

Table 1: Overview of analytes, corresponding ILISs and the possible spiking levels of each analyte

Analytes	ILIS	POSSIBLE Spiking Concentrations (mg/kg)	
		In case of 10 g portions (*Cucumber, Strawberry, Milk, Liver, Kidney*)	In case of 5 g portions (*Rice and Soybean*)
Ethephon	D_4	LOW: 0.01* MED: 0.02 HIGH: 0.05 MAX: 0.10	LOW: 0.02* MED: 0.04 HIGH: 0.10 MAX: 0.20
Fosetyl-Al (measured as fosetyl)	D_{15} (Fosetyl-Al) = D_5 (Fosetyl)-		
HEPA	D_4	LOW: 0.02* MED: 0.04 HIGH: 0.10 MAX: 0.20	LOW: 0.04* MED: 0.08 HIGH: 0.20 MAX: 0.40
Maleic hydrazide	D_2		
MPPA	D_3		
N-Acetylglufosinate	D_3		
Glufosinate	D_3	LOW: 0.03* MED: 0.06 HIGH: 0.15 MAX: 0.30	LOW: 0.06* MED: 0.12 HIGH: 0.30 MAX: 0.60
AMPA	$^{13}C,^{15}N$	LOW: 0.05* MED: 0.10 HIGH: 0.25 MAX: 0.50	LOW: 0.10* MED: 0.20 HIGH: 0.50 MAX: 1.00
Cyanuric acid**	$^{13}C_3$		
Glyphosate	$^{13}C_2$, ^{15}N		
N-Acteylglyphosate	$^{13}C_2$, ^{15}N		

*Using the Hypercarb column sensitivity limitations may be noticed for certain analytes at the LOW level

** In the case of cyanuric acid the measurement sensitivity would in principle allow validation at lower levels. Higher spiking concentrations were, however, chosen to account for the typical background levels encountered.

Table 2: Particularities of matrices and columns at a glance

Comments refer to the following matrices...		Cucumber, Strawberry	Rice	Soyabeans	Milk, Liver, Kidney
Procedure is found in ...		**Chapter E**	**Chapter F**		
Particularities as regards **Extraction and Cleanup**		10 g portions	5 g portions	5 g portions	10 g portions
			• Freeze-out • ACN precipitation • Ultrafiltration	• Freeze-out • ACN precipitation and dSPE (C_{18}) • Ultrafiltration	
Particularities as regards **Spiking Levels and Dilutions**	when using **Torus-DEA**	**Spiking Level Options: LOW**, MED, HIGH (same for all commodities)			
	when using **Hypercarb**	**Spiking Level Options: LOW**, MED, HIGH		• **Spiking Levels:** MED, HIGH, **MAX** • Separate ILIS-mix (will be provided) • Dilution indicated (see protocol)	

Pre-tests:

Labs differ in their instrumental setup and the sensitivity they can achieve for different procedure-matrix-analyte combinations. Participating labs are thus **highly advised to CONDUCT PRE-TESTS in order to better choose the most suitable among the spiking levels proposed and to familiarize with the procedure.** If, for example, the "LOW level" is too low for proper validation in the case of AO-commodities or soybean (which is likely if M 1.3 is applied), this level may be skipped and, e.g. the "MAX-level" may be included. A **pre-test** may for example involve spiking of **blank extracts** of commodities to be validated at **60% of lowest intended validation level** (see Table 14, Table 15, and Table 16).

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A. Test Materials (Matrices):

The required **blank commodities are to be acquired by the participating labs**. The test materials employed should be tested to not contain any of the analytes included in this study at levels >20% of the lowest validation level. The use of **organically grown crops is thus recommended** but it should be kept in mind that even organic samples may contain these analytes at certain levels. The commodities can be homogenized the way they are typically homogenized in each lab. The use of dry ice is recommended in all cases but not mandatory. If rice is obtained in form of flour, it can be employed as such. For sample preparation see the latest versions of QuPPe-PO method and QuPPe-AO method published on the EURL-SRM website.

B. Apparatus and Consumables:

See latest versions of QuPPe-PO method and QuPPe-AO method (Link: http://www.quppe.eu).

C. Chemicals for Sample Preparation:

See latest versions of QuPPe-PO method and QuPPe-AO method (Link: http://www.quppe.eu).

D. Standard Solutions:

Standard solutions necessary for this validation study **will be provided by the organizers**. Native analytes and ILISs will be delivered in separate mixtures (see Table 3, Table 6 and Table 8).
Instructions on **how to prepare the analyte working solutions and ILIS working solution** to be used for spiking and for preparing calibration solutions are shown in Table 4, Table 5, Table 7, and Table 9.

The volumes of analyte and ILIS working solutions to be used for the spiking are shown in the pipetting scheme in Table 10. Preparation of calibration solutions are shown in the pipetting schemes in Table 14 for cucumber and strawberry, and in Table 15 and Table 16 for milk, liver, kidney, rice and soybean.

Where to store the solutions?

The provided analyte solutions and ILIS solutions as well as the working solutions prepared thereof are to be stored in the **refrigerator** (ca. 4°C). This also applies to the extracts if measurement is delayed.

Table 3: ANALYTE STOCK MIXTURES (provided by organizer)

Name of solution provided	Analytes contained	Concentration	No. of vials and volume provided
Analytes Stock-Mix I	AMPA Ethephon MPPA N-Acetylglufosinate N-Acetylglyphosate	250 µg/mL 50 µg/mL 100 µg/mL 100 µg/mL 250 µg/mL	1 á 5 mL
Analytes Stock-Mix II	Cyanuric acid Fosetyl Glufosinate Glyphosate HEPA Maleic hydrazide	250 µg/mL 50 µg/mL 150 µg/mL 250 µg/mL 100 µg/mL 100 µg/mL	1 á 5 mL

Table 4: Instructions for preparing ANALYTE WORKING SOLUTIONS FOR SPIKING SAMPLE PORTIONS

Name of solution to be prepared	Dilution factor	How to prepare	End-volume	Volume used for spiking
SPIKE LOW	50	100 µL Analytes Stock-Mix I + 100 µL Analytes Stock-Mix II + 4.8 mL 10 % ACN in H_2O	5 mL **SPIKE LOW**	100 µL
SPIKE MED	25	200 µL Analytes Stock-Mix I + 200 µL Analytes Stock-Mix II + 4.6 mL 10 % ACN in H_2O	5 mL **SPIKE MED**	
SPIKE HIGH	10	500 µL Analytes Stock-Mix I + 500 µL Analytes Stock-Mix II + 4 mL 10 % ACN in H_2O	5 mL **SPIKE HIGH**	
SPIKE MAX	5	1 mL Analytes Stock-Mix I + 1 mL Analytes Stock-Mix II + 3 mL 10 % ACN in H_2O	5 mL **SPIKE MAX**	
Total consumption of SPIKE solutions (LOW/MED/HIGH/MAX): 600 µL per experiment (n=5, 1 matrix, 1 level)				

Table 5: Instructions for preparing ANALYTE WORKING SOLUTIONS FOR CALIBRATION STANDARDS

Name of solution to be prepared*	Dilution factor	How to prepare	End-volume	Volume used for calibration
CAL LOW	20	100 µL **SPIKE LOW** + 1.9 mL 10 % ACN in H_2O	2 mL **CAL LOW**	See **Table 14** (for cucumber and strawberry) See **Table 15** (for liver, kidney, milk, rice and soybean)
CAL MED	20	100 µL **SPIKE MED** + 1.9 mL 10 % ACN in H_2O	2 mL **CAL MED**	
CAL HIGH	20	100 µL **SPIKE HIGH** + 1.9 mL 10% in H_2O	2 mL **CAL HIGH**	
CAL MAX	20	100 µL **SPIKE MAX** + 1.9 mL 10% in H_2O	2 mL **CAL MAX**	
Total consumption of CAL solutions (LOW/MED/HIGH/MAX): ➢ For cucumber, strawberry and rice: 480 µL per experiment (n=5, 1 matrix, 1 level) ➢ For liver, kidney, milk and soybean: 240 µL per experiment (n=5, 1 matrix, 1 level)				
The solutions below are **only needed in case of a 5-fold dilution of the extracts prior to LC-MS/MS measurement (see Table 16)**				
CAL LOW-F5	5	400 µL **CAL LOW** + 1.6 mL 10 % ACN in H_2O	2 mL **CAL LOW-F5**	See **Table 16** (for liver, kidney, milk, rice and soybean in combination with **HYPERCARB column**)
CAL MED-F5	5	400 µL **CAL MED** + 1.6 mL 10 % ACN in H_2O	2 mL **CAL MED-F5**	
CAL HIGH-F5	5	400 µL **CAL HIGH** + 1.6 mL 10 % ACN in H_2O	2 mL **CAL HIGH-F5**	
CAL MAX-F5	5	400 µL **CAL MAX** + 1.6 mL 10 % ACN in H_2O	2 mL **CAL MAX-F5**	
Total consumption : ➢ CAL solutions (LOW/MED/HIGH/MAX): 400 µL per dilution ➢ CAL-F5 (LOW/MED/HIGH/MAX): 240 µL per experiment (n=5, 1 matrix, 1 level).				

* Store all CAL solutions in fridge and use only for 2 weeks.

Table 6: ILIS SPIKE SOLUTION FOR SPIKING SAMPLE PORTIONS
Provided by organizer

Name of ILIS Stock/Spike solution provided	ILISs contained	Concentration	No. of Vials & Volume provided
ILIS SPIKE	AMPA-^{13}C,^{15}N Cyanuric acid $^{13}C_3$ Ethephon-D_4 MPPA-D_3 Glufosinate-D_3 Glyphosate-$^{13}C_2$, ^{15}N Maleic hydrazide-D_2 HEPA-D_4 N-Acetylglufosinate-D_3 N-Acetylglyphosate $^{13}C_2$, ^{15}N Fosetyl-Al-D_{15}	40 µg/mL 20 µg/mL 20 µg/mL 20 µg/mL 20 µg/mL 20 µg/mL 20 µg/mL 20 µg/mL 20 µg/mL 20 µg/mL 20 µg/mL	Depending on scope. 2,5 mL would be provided for 4 recovery experiments (1 Experiment = 1 matrix/1 level; n=5)

The ILIS SPIKE solution has to be **diluted 20-fold** to obtain the ILIS CAL (see Table 7). It is **recommended to freshly prepare** the ILIS CAL solution from the ILIS SPIKE solution on every sample extraction day.

Table 7: Instructions for preparing ILIS CAL WORKING SOLUTIONS FOR CALIBRATION STANDARDS

Name of solution to be prepared	Dilution factor	How to prepare (exemplary)...	End-volume*	Volume used for calibration
ILIS CAL	20	150 µL **ILIS SPIKE*** + 2.85 mL 10 % ACN in H_2O*	3 mL*	See **Table 14** (for cucumber and strawberry) See **Table 15** (for liver, kidney, milk, rice and soybean)
Total consumption of ILIS CAL solution: 500 µL per experiment (n=5, 1 matrix, 1 level)				

* Please adapt the end volume of the **ILIS CAL** to the numbers of experiments conducted on one sample extraction day, please note that for the MATRIX BLANK **additional 100 µL (once per matrix)** are needed.

Additional information about the spiking and calibration solutions as well as the expected concentrations can be found in Table 11.

Table 8: ILIS SPIKE AO HyCarb SOLUTION FOR SPIKING SAMPLE PORTIONS
(to be used for Milk, Liver, Kidney, Rice and Soybeans in combination with M1.3 Hypercarb; see CHAPTER F). Provided by organizer.

Name of ILIS Stock/Spike solution provided	ILISs present	Concentration	No. of Vials & Volume provided
ILIS SPIKE AO HyCarb	AMPA-^{13}C,^{15}N Cyanuric acid $^{13}C_3$ Ethephon-D_4 MPPA-D_3 Glufosinate-D_3 Glyphosate-$^{13}C_2$, ^{15}N Maleic hydrazide-D_2 HEPA-D_4 N-Acetylglufosinate-D_3 N-Acetylglyphosate $^{13}C_2$, ^{15}N Fosetyl-Al-D_{15}	100 µg/mL 20 µg/mL 20 µg/mL 20 µg/mL 60 µg/mL 100 µg/mL 20 µg/mL 40 µg/mL 20 µg/mL 100 µg/mL 20 µg/mL	Depending on scope. 2,5 mL would be provided for 4 recovery experiments (1 Experiment = 1 matrix/1 level; n=5)

The ILIS SPIKE AO HyCarb solution has to be **diluted 20-fold** to obtain the ILIS CAL AO HyCarb (see Table 9). It is **recommended to freshly prepare** the ILIS CAL AO HyCarb solution from the ILIS SPIKE AO HyCarb solution on every sample extraction day.

Table 9: Instructions for preparing ILIS CAL AO HyCarb WORKING SOLUTIONS FOR CALIBRATION STANDARDS (see Chapter F)

Name of solution to be prepared	Dilution factor	How to prepare (exemplary)…	End-volume*	Volume used for calibration
ILIS CAL AO HyCarb	20	150 µL **ILIS SPIKE AO HyCarb*** + 2.85 mL 10 % ACN in H_2O*	3 mL*	See **Table 15** (for liver, kidney, milk, rice and soybean)
The solutions below are only needed **in case of a 5-fold dilution of the extracts before LC-MS/MS (see Table 16)**				
ILIS CAL AO HyCarb-F5	5	400 µL **ILIS CAL AO HyCarb*** + 1.6 mL 10 % ACN in H_2O*	2 mL*	See **Table 16** (for liver, kidney, milk, rice and soybean in combination with **HYPERCARB column**)
Total consumption of ILIS CAL solution: 250 µL per experiment (n=5, 1 matrix, 1 level)				

* Please adapt the end volume of the **ILIS CAL AO HyCarb** and **ILIS CAL AO HyCarb-F5** to the numbers of experiments conducted on one sample extraction day, please note that for the MATRIX BLANK **additional 100 µL (once per matrix)** are needed.

An overview of the analyte spiking concentrations (in matrix and in the final extract) resulting when spiking sample portions with the working solutions (prepation see Table 4), can be found in Table 11.

Table 10: Pipetting scheme for spiking sample portions and suggested labelling (exemplary for "[Matrix1]")

Suggested Labelling of Extraction Vessels	Spiking of Matrix Portions with…				Volume of ILIS solution to be added	Volume of Water to be added (to equalize volumes)
	*SPIKE LOW**	SPIKE MED	SPIKE HIGH	*SPIKE MAX**		
[Matrix 1] LOW (1~5)*	*100 µL*	-	-		100 µL	-
[Matrix 1] MED (1~5)	-	100 µL	-		100 µL	-
[Matrix 1] HIGH (1~5)	-	-	100 µL		100 µL	-
[Matrix 1] MAX (1~5)*				*100 µL*	100 µL	-
[Matrix 1] BLANK (for CAL)	-	-	-			200 µL
[Matrix 1] BLANK + ILIS** (to quantify background levels in matrix)	-	-	-		100 µL	100 µL
Reagent BLANK + ILIS***					100 µL	100 µL

* For **liver, milk, kidney and soybean** in combination with **M1.3** (Hypercarb), it is recommended to validate at the levels MED, HIGH and MAX. For **any matrix** in combination with **M1.6** (Torus DEA), it is recommended to validate at the levels LOW, MED and HIGH.

Prepare **ONE "[Matrix 1] BLANK + ILIS" and use it for **ALL LEVELS OF ONE MATRIX**.

***You may prepare **ONE** "Reagent BLANK + ILIS" and use it for **ALL EXPERIMENTS (all matrices and all levels)**.

Table 11: Analyte concentrations resulting when spiking sample portions with working solutions

Analyte	Solution Name (spiking vol.)	Pesticide conc. [µg/mL]	Corresponding Analyte Spiking Levels [mg/kg]		Expected approx. concentrations of analytes in final extract [µg/mL]	
			Rice Soybean	Cucumber Strawberry Milk Liver Kidney	Cucumber Strawberry	Milk Liver Kidney Soybean Rice
			5 g	10 g		
AMPA	SPIKE LOW (100 µL)	5	0.1	0.05	0.025	0.0125
Cyanuric acid		5	0.1	0.05	0.025	0.0125
Ethephon		1	0.02	0.01	0.005	0.0025
Fosethyl-Al		1	0.02	0.01	0.005	0.0025
Glufosinate		3	0.06	0.03	0.015	0.0075
Glyphosate		5	0.1	0.05	0.025	0.0125
HEPA		2	0.04	0.02	0.01	0.005
Maleic hydrazide		2	0.04	0.02	0.01	0.005
MPPA		2	0.04	0.02	0.01	0.005
N-Acetylglufosinate		2	0.04	0.02	0.01	0.005
N-Acetylglyphosate		5	0.1	0.05	0.025	0.0125
AMPA	SPIKE MED (100 µL)	10	0.2	0.1	0.05	0.025
Cyanuric acid		10	0.2	0.1	0.05	0.025
Ethephon		2	0.04	0.02	0.01	0.005
Fosethyl-Al		2	0.04	0.02	0.01	0.005
Glufosinate		6	0.12	0.06	0.03	0.015
Glyphosate		10	0.2	0.1	0.05	0.025
HEPA		4	0.08	0.04	0.02	0.01
Maleic hydrazide		4	0.08	0.04	0.02	0.01
MPPA		4	0.08	0.04	0.02	0.01
N-Acetylglufosinate		4	008	0.04	0.02	0.01
N-Acetylglyphosate		10	0.2	0.1	0.05	0.025
AMPA	SPIKE HIGH (100 µL)	25	0.5	0.25	0.125	0.0625
Cyanuric acid		25	0.5	0.25	0.125	0.0625
Ethephon		5	0.1	0.05	0.025	0.0125
Fosethyl-Al		5	0.1	0.05	0.025	0.0125
Glufosinate		15	0.3	0.15	0.075	0.0375
Glyphosate		25	0.5	0.25	0.125	0.0625
HEPA		10	0.2	0.1	0.05	0.025
Maleic hydrazide		10	0.2	0.1	0.05	0.025
MPPA		10	0.2	0.1	0.05	0.025
N-Acetylglufosinate		10	0.2	0.1	0.05	0.025
N-Acetylglyphosate		25	0.5	0.25	0.125	0.0625
AMPA	SPIKE MAX (100 µL)	50	1	0.5		0.125
Cyanuric acid		50	1	0.5		0.125
Ethephon		10	0.2	0.1		0.025
Fosethyl-Al		10	0.2	0.1		0.025
Glufosinate		30	0.6	0.3		0.075
Glyphosate		50	1	0.5		0.125
HEPA		20	0.4	0.2		0.05
Maleic hydrazide		20	0.4	0.2		0.05
MPPA		20	0.4	0.2		0.05
N-Acetylglufosinate		20	0.4	0.2		0.05
N-Acetylglyphosate		50	1	0.5		0.125

Table 12: ILIS concentrations resulting when spiking sample portions with ILIS SPIKE

Analyte	Solution Name (spiking vol.)	ILIS conc. [µg/mL]	Corresponding ILIS Spiking Levels [mg/kg]		Expected approx. concentrations of ILISs in final extract [µg/mL]	
			Rice Soybean	Cucumber Strawberry Milk Liver Kidney	Cucumber Strawberry	Milk Liver Kidney Soybean Rice
			5 g	10 g		
AMPA ^{13}C, ^{15}N	ILIS SPIKE (100 µL)	40	0.8	0.4	0.2	0.1
Cyanuric acid $^{13}C_3$		20	0.4	0.2	0.1	0.05
Ethephon-D_4		20	0.4	0.2	0.1	0.05
Fosetyl-Al-D_{15}		20	0.4	0.2	0.1	0.05
Glufosinate-D_3		20	0.4	0.2	0.1	0.05
Glyphosate-$^{13}C_2$, ^{15}N		20	0.4	0.2	0.1	0.05
HEPA-D_4		20	0.4	0.2	0.1	0.05
Maleic hydrazide-D_2		20	0.4	0.2	0.1	0.05
MPPA-D_3		20	0.4	0.2	0.1	0.05
N-Acetylglufosinate-D_3		20	0.4	0.2	0.1	0.05
N-Acetylglyphosate $^{13}C_2$, ^{15}N		20	0.4	0.2	0.1	0.05

Table 13: ILIS concentrations resulting when spiking sample portions with ILIS SPIKE AO HyCarb (see Chapter F)

Analyte	Solution Name (spiking vol.)	ILIS conc. [µg/mL]	Corresponding ILIS Spiking Levels [mg/kg]		Expected approximate concentrations of ILISs in final extract [µg/mL]	
			Soybean Rice	Milk Liver Kidney	Soybean Rice	Milk Liver Kidney
			5 g	10 g		
AMPA ^{13}C, ^{15}N	ILIS SPIKE AO HyCarb (100 µL)	100	2	1	0.25	0.25
Cyanuric acid $^{13}C_3$		20	0.4	0.2	0.05	0.05
Ethephon-D_4		20	0.4	0.2	0.05	0.05
Fosetyl-Al-D_{15}		20	0.4	0.2	0.05	0.05
Glufosinate-D_3		60	1.2	0.6	0.15	0.15
Glyphosate-$^{13}C_2$, ^{15}N		100	2	1	0.25	0.25
HEPA-D_4		40	0.8	0.4	0.1	0.1
Maleic hydrazide-D_2		20	0.4	0.2	0.05	0.05
MPPA-D_3		20	0.4	0.2	0.05	0.05
N-Acetylglufosinate-D_3		20	0.4	0.2	0.05	0.05
N-Acetylglyphosate $^{13}C_2$, ^{15}N		100	2	1	0.25	0.25

E. Sample Prep for Commodities of Plant Origin (except Soybean*)

***Note:** for soybean follow the procedures for commodities of animal origin (see Chapter F).

E.1 WEIGHING OF TEST MATERIAL INTO EXTRACTION VESSELS:

- Cucumber, Strawberry: 10 g ± 0.1 g,
- ~~Rice: 5 g ± 0.05 g.~~ (MOVED TO CHAPTER F)
- Reagent blank: 10 mL water

Note: If the study will be performed at 3 spiking levels (LOW, MED, HIGH) in quintuplicate it is necessary to prepare 17 extraction vessels for each test material (1 blank for the calibrations, 1 blank with ILIS for background control and 3 x 5 spiked samples). In case of **cucumber prepare one additional blank for measurement of matrix effects**.

Close the 17 vessels and put them in the **freezer**. Remove the set of samples to be analysed early enough from the freezer to ensure that the material is half thawed during spiking.

Note: In practice real samples are processed in frozen condition, but spiking of thawed material allows the residues to be better distributed within the sample.

E.2 SPIKING (5 x per level and commodity):

For each level (LOW, MED, HIGH) 5 portions of blank sample are spiked with analytes and ILIS solutions as shown Table 10.

Gently distribute the spiked solutions (e.g. by swinging or shortly vortexing the vial). Let the samples stand for 5 min before you continue.

E.3 BLANKS AND BLANK CONTROLS (see also Table 10):

a) "**Matrix-Blank for CAL":** Extracted as described below but without spiking (**NO** ILIS). To be used for preparing the calibration solutions ("[Matrix] –xxx CAL") but also for "Intermediate Blank Matrix injections" in LC-MS/MS. For volume adjustment during spiking **add 200 µL water**;

b) "**Cucumber-Blank for CAL":** Extracted as described below but without spiking (**NO** ILIS). To be used for preparing the calibration solutions ("C –xxx CAL"). For volume adjustment during spiking **add 200 µL water**;

c) **Spinach-Blank**: Extracted as described below but without spiking (**NO** ILIS). To be used for priming of Hypercarb column. For volume adjustment during spiking **add 200 µL water**;

d) "**Matrix-Blank + ILIS" to quantify analyte levels in blank:** With addition of ILIS. Prepare **ONE** "Matrix-Blank + ILIS" for **ALL LEVELS OF ONE MATRIX**.;

e) "**Reagent Blank + ILIS":** With addition of ILIS: Prepare **ONCE** ONE "Reagent Blank + ILIS" for **ALL EXPERIMENTS**

In case of b) gently distribute the ILIS (e.g. vortex shortly). Let the samples stand for 5 min before you continue.

E.4 ADJUSTMENT OF WATER CONTENT TO 10 mL

Add the following amounts of water to each matrix portion (including portions that will remain unspiked = blanks):

- Cucumber: add 0.5 mL of water
- Strawberry: add 1 mL of water

Note: where ILISs are used, volume adjustment is not essential for cucumber and strawberry as ILISs can correct both volumetric errors and matrix effects, but for dry commodities water addition is essential. The volumetric adjustment for cucumber and strawberry is performed as it is additionally intented to evaluate the suitability of matrix-matched calibration without ILIS.

E.5 ADDITION OF EXTRACTION SOLVENT AND EXTRACTION:

Add **10 mL of acidified methanol (methanol with 1% formic acid v/v)** to all samples (e.g. using a solvent dispenser). Tightly close the extraction vessels and **shake vigorously** either for 1 min by hand or for 2 – 15 min by an automatic shaker.

Note: Fresh samples may also be shaken mechanically for 15 min if this is more convenient.

E.6 FREEZING

Optionally (especially where there is filtration difficulties) place the extraction tubes for at least 90 min in the freezer (-18°C) or for at least 30 min at -80°C, and proceed with step 7.

E.7 CENTRIFUGATION:

Centrifuge **for 5 min.** (at least 4000 rpm).

E.8 FILTRATION:

Filter at least 1.5 - 3 mL of centrifuged extracts into plastic tubes, for example the QuPPe extraction tubes, using H-PTFE filters with 0.2 µm pore size.

E.9 PREPARATION OF SOLUTIONS FOR MEASUREMENT:

For calibration, standard solutions corresponding to 60 and 120 % of the spiked concentration are prepared for each level (MIN/MED/HIGH/MAX). This is done two times:

a) using a blank extract of the corresponding matrix (matrix matched)

b) using blank cucumber extract (generic matrix-based)

For calculation of matrix effects a solvent-based standard at 120 % of the spiked concentration is prepared for each level. For detailed descriptions see pipetting schemes in Table 14.

E.10 PROCEDURE AT A GLANCE FOR CUCUMBER AND STRAWBERRY

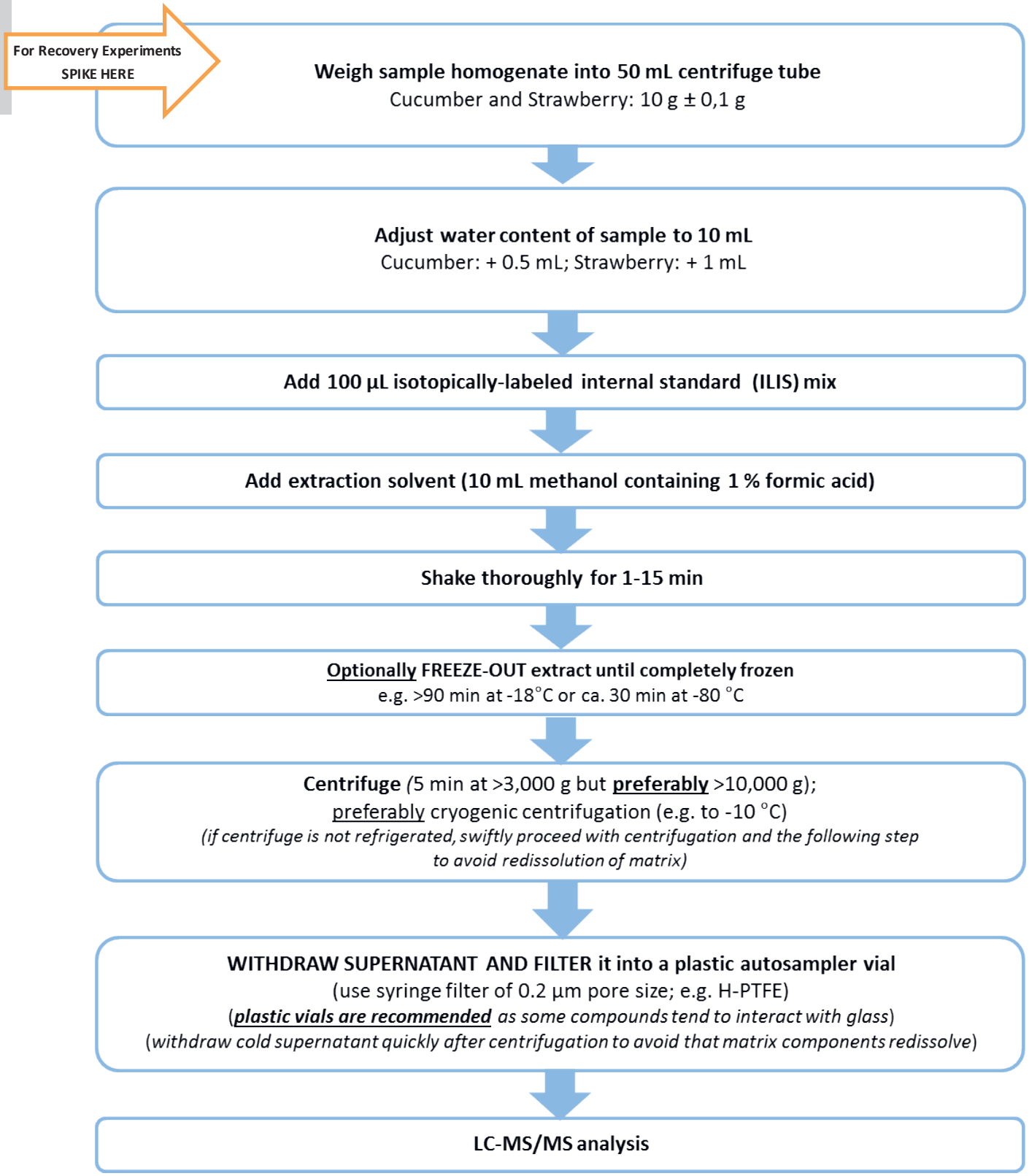

Figure 1: Method at a glance for cucumber and strawbeery

Table 14: Pipetting Scheme for preparing Calibration Standards for Cucumber and Strawberry

Extract/Solution to be used	Transfer volume to HPLC-Vials	Add the following solutions or solvents to the Extracts/Solution...					Suggested Vial Labelling	Final-volume
		CAL LOW	CAL MED	CAL HIGH	„ILIS CAL-mix“	Water (volume adjustm.)		
Matrix Blank (for CAL)	1 mL	60 µL	-	-	100 µL	60 µL	[Matrix] –LOW CAL- 60%	1.22 mL
	1 mL	120 µL	-	-	100 µL	-	[Matrix] –LOW CAL- 120%	1.22 mL
	1 mL	-	60 µL	-	100 µL	60 µL	[Matrix] –MED CAL- 60%	1.22 mL
	1 mL	-	120 µL	-	100 µL	-	[Matrix] –MED CAL- 120%	1.22 mL
	1 mL	-	-	60 µL	100 µL	60 µL	[Matrix] –HIGH CAL- 60%	1.22 mL
	1 mL	-	-	120 µL	100 µL	-	[Matrix] –HIGH CAL- 120%	1.22 mL
	1 mL	-	-	-	-	-	[Matrix] –BLANK	1 mL
Cucumber Extract	1 mL	60 µL	-	-	100 µL	60 µL	C- LOW CAL-60%	1.22 mL
	1 mL	120 µL	-	-	100 µL	-	C-LOW CAL-120%	1.22 mL
	1 mL	-	60 µL	-	100 µL	60 µL	C-MED CAL-60%	1.22 mL
	1 mL	-	120 µL	-	100 µL	-	C-MED CAL-120%	1.22 mL
	1 mL	-	-	60 µL	100 µL	60 µL	C-HIGH CAL-60%	1.22 mL
	1 mL	-	-	120 µL	100 µL	-	C-HIGH CAL-120%	1.22 mL
Solvent (1:1 H_2O:MeOH cont. 1% FA*)	1 mL	120 µL	-	-	100 µL	-	S-LOW CAL-120%	1.22 mL
	1 mL	-	120 µL	-	100 µL	-	S-MED CAL-120%	1.22 mL
	1 mL	-	-	120 µL	100 µL	-	S-HIGH CAL-120%	1.22 mL
	1 mL	-	-	-	-	-	S-BLANK	1 mL
Extracts of spiked samples (LOW, MED and HIGH)	1 mL	-	-	-	-	220 µL	[Matrix]-LOW (1~5) [Matrix]-MED (1~5) [Matrix]-HIGH (1~5)	1.22 mL
Reagent Blank + ILIS	1 mL	-	-	-	-	220 µL	Reagent Blank + ILIS	1.22 mL
Matrix Blank + ILIS	1 mL	-	-	-	-	220 µL	[Matrix] Blank + ILIS	1.22 mL

* Corresponds to the solvent-composistion of the final extract

F. Sample Preparation for Commodities of Animal Origin, Rice and Soybean

F.1 WEIGHING OF TEST MATERIAL INTO EXTRACTION VESSELS:

- Milk, liver, kidney: 10 g ± 0.1 g,
- Soybean and Rice: 5 g ± 0.05 g

Note: in case only M1.3 (using a hypercarb column) is applied, it is recommended to skip the LOW levels and add the MAX levels (validation of MED, HIGH and MAX). In case M1.6 (using a torus column) is applied the MAX levels may be skipped instead of the LOW levels (validation of LOW, MED and HIGH).
If the study will be performed at 3 spiking levels in quintuplicate it is necessary to prepare 17 extraction vessels for each test material (1 blank for the calibrations, 1 blank with ILIS and 3 x 5 spiked), in case the study will be performed at 4 spiking levels in quintuplicate it is necessary to prepare 22 extraction vessels for each test material.

Close the 17 vessels and put them in the **freezer** (soybean and rice may be kept at RT). Remove the set of samples to be analysed early enough from the freezer to ensure that the material is half thawed during spiking.

Note: In practice real samples are processed in frozen condition, but spiking of thawed material allows the residues to be better distributed within the sample.

F.2 SPIKING (5 x per level and commodity):

For each level (LOW, MED, HIGH applying method 1.6; MID, HIGH, MAX applying method 1.3) 5 portions of blank are spiked with analytes and ILIS working solutions as shown in Table 10.
Gently distribute the spiked solutions (e.g. by swinging or shortly vortexing the vial). Let the samples stand for 5 min before you continue.

F.3 BLANKS AND BLANK CONTROLS (see also Table 10):

a) "**Matrix-Blank for CAL":** Extracted as described below but without spiking (**NO** ILIS). To be used for preparing the calibration solutions ("[Matrix] –xxx CAL") but also for "Intermediate Blank Matrix injections" in LC-MS/MS. For volume adjustment during spiking **add 200 µL water**;

b) "**Cucumber-Blank for CAL":** Extracted as described above (see chapter E) but without spiking (**NO** ILIS). To be used for preparing the calibration solutions ("C –xxx CAL"). For volume adjustment during spiking **add 200 µL water**;

c) **Spinach-Blank**: Extracted as described above (see chapter E) but without spiking (**NO** ILIS). To be used for priming of Hypercarb column. For volume adjustment during spiking **add 200 µL water**;

d) "**Matrix-Blank + ILIS" to quantify analyte levels in blank:** With addition of ILIS. Prepare **ONE** "Matrix-Blank + ILIS" for **ALL LEVELS OF ONE MATRIX**.;

e) "**Reagent Blank + ILIS":** With addition of ILIS: Prepare **ONCE ONE** "Reagent Blank + ILIS" for **ALL EXPERIMENTS**

In case of b) gently distribute the ILIS (e.g. vortex shortly). Let the samples stand for 5 min before you continue.

F.4 ADJUSTMENT OF WATER CONTENT

Add the following amounts of water to each matrix portion (including portions that will remain un-spiked = blanks):

- Whole Fat Milk: add 0.5 mL of water
- Liver: add 2 mL of water
- Kidney: add 1 mL of water
- Soybean and rice: add 9 mL of water

Note: where ILISs are used, volume adjustment is not essential for cow's milk as ILISs can correct both volumetric errors and matrix effects, but for liver, kidney, rice and soybean water addition is essential. The volumetric adjustment for milk is performed as it is additionally intended to evaluate the suitability of matrix-matched calibration without ILIS.

F.5 ADDITION OF EXTRACTION SOLVENT AND EXTRACTION:

Add **10 mL of acidified methanol (methanol with 1% formic acid v/v)** and an extra amount of **100 µL formic acid** to all samples (e.g. using a solvent dispenser). Tightly close the extraction vessels and shake for a few seconds to distribute the acid and allow proteins to coagulate. Add **1 mL 10% aqueous EDTA solution** and shake vigorously either for 1 min by hand or for 2 – 15 min by an automatic shaker. In case of soybean and rice shake mechanically for 15 min.

Note: If you do not have a mechanical shaker, shake soybean and rice sample initially by hand for 1 min and repeat this after 15 min of soaking.

F.6 FREEZE-OUT AND CENTRIFUGATION

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

(1) Centrifugation following freeze-out: place the tubes containing the extract and sample material 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 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.

Note: Low temperatures reduce the solubility of matrix compounds resulting in increased precipitation. It is recommended to proceed immediately with the next steps to avoid redissolving of matrix compounds.

F.7 REMOVAL OF LIPIDS AND PRECIPITATION OF PROTEINS:

Transfer a **2 mL aliquot** of the supernatant into a 10 mL plastic centrifuge tube, which already contains **2 mL of acetonitrile and 100 mg of C_{18}** (ODS) sorbent. Close the tubes, shake 1 min by hand and centrifuge for 5 min at > 3,000 g).

Alternatively, mix equal volumes of the supernantant and acetonitrile, pass 4 mL slowly through a C_{18} SPE cartridge (e.g. Agilent Bond Elut C18, 200 mg, Order No. 12113024 or Macherey-Nagel, SPE-Cartridge, CHROMAFIX C18 ec (S) 270 mg; Product-Nr.: MN731804) and collect the cleaned-up extract in a vial or in the reservoir of the ultrafiltration unit.

REMARK: The treatment with C_{18} may be skipped for rice (but the dilution with acetonitrile is to be done).

F.8 FILTRATION:

Transfer a 3 mL aliquot of the supernatant from F.7 into the ultrafiltration unit[3] and centrifuge at 3,000 g until enough filtrate is accumulated in the reservoir (5 min are typically enough when using the Vivaspin filters and 10-15 min when using the Amicon filters).

Transfer an aliquot of the filtrate into an autosampler vial but be aware that a dilution of the extract in the vial may be needed (see below).

F.9 PREPARATION OF SOLUTIONS FOR MEASUREMENT:

See pipetting schemes in Table 15.

Different columns and instrument configurations will have different optimal combinations of dilution factor and injection volume. The settings below proved suitable for the instrument configuration employed at the EURL-SRM (see QuPPe PO document) and are thus recommended. Adjustments to fit other instrumental configurations are possible but **the details of the procedure actually used need to be reported in the excel sheet**.

Recommended procedures for the extracts of milk, kidney, liver and soja:

Using **M1.6** (Torus): No further preparation of the extracts is typically needed. The extracts are **injected directly** into the LC-MS/MS system.

[3] e.g. Vivaspin® 6 mL 5 kDa entailing Polyethersulfone membranes or alternatively Amicon® Ultra-15 10K entailing Ultracel® low binding regenerated cellulose

Using **M1.3** (Hypercarb): **Dilute the extracts 5-fold.** This applies to all spiking levels and to all calibration solutions and the "Matrix Blank +ILIS". Dilution may be skipped for the "Reagent Blank+ILIS"). **Alternatively inject 1 µL of undiluted extract**.

The 5-fold dilution can either be conducted …

a) in the LC-vial (200 µL QuPPe extract + 800 µL H_2O) dilution procedure according to Table 16 OR
b) in a second LC-vial (200 µL undiluted QuPPe solution according to Table 15 + 800 µL H_2O) OR
c) in the LC-injector (1 µL QuPPe extract + 4 µL MeOH containing 1% Formic acid)

A **pipetting scheme in case of no dilution** is given in Table 15.

A **pipetting scheme in case of 5-fold dilution** is given in Table 16.

For calculation, calibration solutions corresponding to 60 and 120 % of the spiked concentration are prepared for each level (MIN/MED/HIGH/MAX). Two different sets of calibration solutions are prepared:

a) using a blank extract of the corresponding matrix (matrix matched)
b) using blank cucumber extract (generic matrix-based)

For calculation of matrix effects a solvent-based standard at 120 % of the spiked concentration is prepared for each level. For detailed descriptions see pipetting schemes in Table 15 and Table 16.

F.10 PROCEDURE AT A GLANCE FOR SOYBEAN, RICE, LIVER, KIDNEY AND MILK

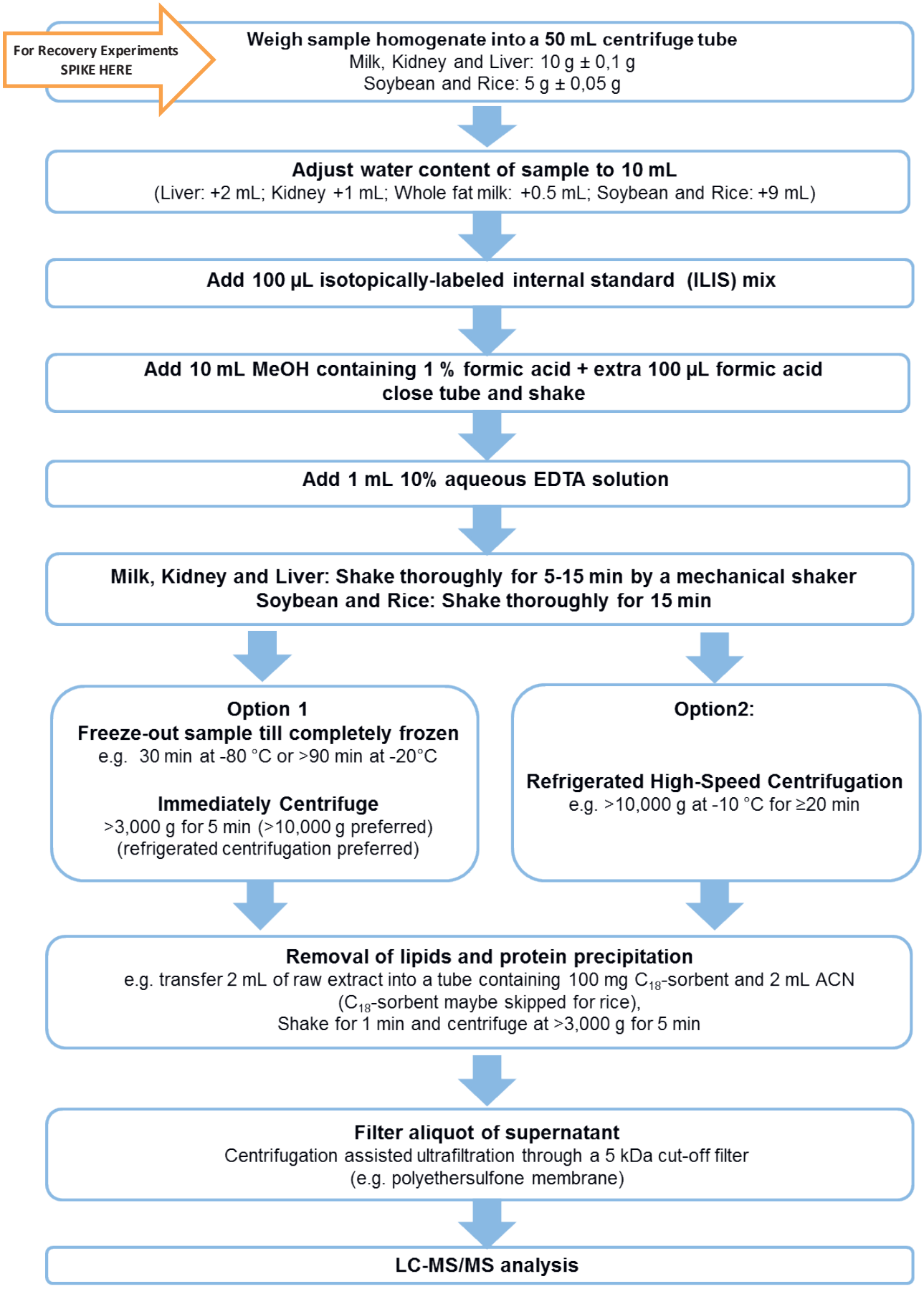

Figure 1: Method at a glance for liver, kidney, milk, rice and soybeans

Table 15: Pipetting Scheme for preparing Calibration Standards for Milk, Liver, Kidney, Rice and Soybean -

IN CASE OF NO DILUTION (RECOMMENDED FOR M1.6)

Extract/Solution to be used	Transfer volume to HPLC-Vials	Add the following solutions or solvents to the Extracts/Solution...*						Suggested Vial Labelling	Final-volume
		CAL LOW	CAL MED	CAL HIGH	*CAL MAX**	„ILIS CAL-mix“	Water (volume adjustm.)		
Matrix Blank (for CAL)	1 mL	30 µL	-	-		50 µL	30 µL	[Matrix] –LOW CAL- 60%	1.11 mL
	1 mL	60 µL	-	-		50 µL	-	[Matrix] –LOW CAL- 120%	1.11 mL
	1 mL	-	30 µL	-		50 µL	30 µL	[Matrix] –MED CAL- 60%	1.11 mL
	1 mL	-	60 µL	-		50 µL	-	[Matrix] –MED CAL- 120%	1.11 mL
	1 mL	-	-	30 µL		50 µL	30 µL	[Matrix] –HIGH CAL- 60%	1.11 mL
	1 mL	-	-	60 µL		50 µL	-	[Matrix] –HIGH CAL- 120%	1.11 mL
	1 mL				*30 µL*	50 µL	30 µL	[Matrix] –MAX CAL- 60%	1.11 mL
	1 mL				*60 µL*	50 µL	-	[Matrix] –MAX CAL- 120%	1.11 mL
	1 mL	-	-	-		-	-	[Matrix] –BLANK	1 mL
Cucumber Extract	1 mL	30 µL	-	-		50 µL	30 µL	C-LOW CAL-60%	1.11 mL
	1 mL	60 µL	-	-		50 µL	-	C-LOW CAL-120%	1.11 mL
	1 mL	-	30 µL	-		50 µL	30 µL	C-MED CAL-60%	1.11 mL
	1 mL	-	60 µL	-		50 µL	-	C-MED CAL-120%	1.11 mL
	1 mL	-	-	30 µL		50 µL	30 µL	C-HIGH CAL-60%	1.11 mL
	1 mL	-	-	60 µL		50 µL	-	C-HIGH CAL-120%	1.11 mL
	1 mL				*30 µL*	50 µL	30 µL	C–MAX CAL-60%	1.11 mL
	1 mL				*60 µL*	50 µL	-	C–MAX CAL-120%	1.11 mL
Solvent (2:1:1 ACN:H_2O:MeOH cont. 1% FA)**	1 mL	60 µL	-	-		50 µL	-	S-LOW CAL-120%	1.11 mL
	1 mL	-	60 µL	-		50 µL	-	S-MED CAL-120%	1.11 mL
	1 mL	-	-	60 µL		50 µL	-	S-HIGH CAL-120%	1.11 mL
	1 mL				*60 µL*	50 µL	-	S–MAX CAL-120%	1.11 mL
	1 mL							S-BLANK	1 mL
Extracts of spiked samples (LOW, MED, HIGH and MAX)	1 mL	-	-	-		-	110 µL	[Matrix]-LOW (1~5) [Matrix]-MED (1~5) [Matrix]-HIGH (1~5) [Matrix]-MAX (1~5)	1.11 mL
Reagent Blank + ILIS	1 mL	-	-	-		-	110 µL	Reagent Blank + ILIS	1.11 mL
Matrix Blank + ILIS	1 mL	-	-	-		-	110 µL	[Matrix] Blank + ILIS	1.11 mL

* In combination with **M1.6** (Torus DEA)), it is recommended to validate at the levels LOW, MED and HIGH (i.e. skip MAX), but this will also depend on instrument sensitivity

** Corresponds to the solvent-composition of the final extract

Table 16: Pipetting Scheme for preparing Calibration Standards for Milk, Liver, Kidney, Rice and Soybean -

IN CASE OF 5-FOLD DILUTION (RECOMMENDED FOR M1.3)

Extract/Solution to be used	Transfer volume to HPLC-Vials	Add the following solutions or solvents to the Extracts/Solution...*						Suggested Vial Labelling	Final-volume
		*CAL LOW - F5**	CAL MED - F5	CAL HIGH-F5	CAL MAX - F5*	„ILIS CAL AO HyCarb -F5“	Water (volume adjustm.)		
Matrix Blank (for CAL)	200 µL	*30 µL*	-	-		50 µL	830 µL	[Matrix] –LOW CAL- 60%-F5	1.11 mL
	200 µL	*60 µL*	-	-		50 µL	800 µL	[Matrix] –LOW CAL- 120%-F5	1.11 mL
	200 µL	-	30 µL	-		50 µL	830 µL	[Matrix] –MED CAL- 60%-F5	1.11 mL
	200 µL	-	60 µL	-		50 µL	800 µL	[Matrix] –MED CAL- 120%-F5	1.11 mL
	200 µL	-	-	30 µL		50 µL	830 µL	[Matrix] –HIGH CAL- 60%-F5	1.11 mL
	200 µL	-	-	60 µL		50 µL	800 µL	[Matrix] –HIGH CAL- 120%-F5	1.11 mL
	200 µL				30 µL	50 µL	830 µL	[Matrix] –MAX CAL- 60%-F5	1.11 mL
	200 µL				60 µL	50 µL	800 µL	[Matrix] –MAX CAL- 120%-F5	1.11 mL
	200 µL	-	-	-		-	800 µL	[Matrix] –BLANK	1 mL
Cucumber Extract	1 mL	*30 µL*	-	-		50 µL	30 µL	C-LOW CAL-60%-F5	1.11 mL
	1 mL	*60 µL*	-	-		50 µL	-	C-LOW CAL-120%-F5	1.11 mL
	1 mL	-	30 µL	-		50 µL	30 µL	C-MED CAL-60%-F5	1.11 mL
	1 mL	-	60 µL	-		50 µL	-	C-MED CAL-120%-F5	1.11 mL
	1 mL	-	-	30 µL		50 µL	30 µL	C-HIGH CAL-60%-F5	1.11 mL
	1 mL	-	-	60 µL		50 µL	-	C-HIGH CAL-120%-F5	1.11 mL
	1 mL				30 µL	50 µL	30 µL	C–MAX CAL-60%-F5	1.11 mL
	1 mL				60 µL	50 µL	-	C-MAX CAL-120%-F5	1.11 mL
Solvent (2:1:1 ACN:H_2O:MeOH cont. 1% FA)**	1 mL	*60 µL*	-	-		50 µL	-	S-LOW CAL-120%-F5	1.11 mL
	1 mL	-	60 µL	-		50 µL	-	S-MED CAL-120%-F5	1.11 mL
	1 mL	-	-	60 µL		50 µL	-	S-HIGH CAL-120%-F5	1.11 mL
	1 mL				60 µL	50 µL	-	S-MAX CAL-120%-F5	1.11 mL
	200 µL	-	-	-		-	800 µL	S-BLANK	1 mL
Extracts of spiked samples (LOW, MED, HIGH and MAX)	200 µL	-	-	-		-	910 µL	[Matrix]-LOW (1~5) -F5 [Matrix]-MED (1~5) -F5 [Matrix]-HIGH (1~5) -F5 [Matrix]-MAX (1~5) -F5	1.11 mL
Reagent Blank + ILIS	200 µL	-	-	-		-	910 µL	Reagent Blank + ILIS	1.11 mL
Matrix Blank + ILIS	200 µL	-	-	-		-	910 µL	[Matrix] Blank + ILIS	1.11 mL

* For liver, milk, kidney, rice and soybean in combination with **M1.3** (Hypercarb), it is recommended to validate at the levels MED, HIGH and MAX (i.e. skip LOW), but this will also depend on instrument sensitivity.

** Corresponds to the solvent-composition of the final extract.

G. Measurement and Data Processing:

Measurement should be conducted by LC-MS/MS. **Exemplary Chromatograms and peaks shapes** of all analytes in several matrixes can be found in the latest versions of QuPPe-PO method and QuPPe-AO method (Link: http://www.quppe.eu).

HEPA is heavily interfered in liver (especially bovine liver), thus validation data of HEPA in liver will be excluded from the evaluation.

G.1 MEASUREMENTS BY M1.3:

It is highly recommended using a well primed Hypercarb column, which has been already in routine use.

Exemplary measurement settings are shown in **Table 17, Table 20 and Table 21.**

If you intent to use a **new column and pre-column, priming** by multiple injections of spinach-extracts is necessary. For this please inject 50 times 50 µL QuPPe extract of spinach (see also Table 18). If your instrument cannot inject this volume please inject the highest volume possible and increase the number of injections accordingly. If only the pre-column is to be primed 20 injections á 50 µL spinach extract are usually enough. **After the priming procedure, we recommend to check the condition of the column by injecting a standard mixture before starting the validation. If the column is freshly primed, it's important to include additional spinach extract injections during a long sequence and inbetween sequences.**

Further hints for priming and using the Hypercarb column can be found in our official QuPPe PO[4] document (see also ANNEX 1).

[4] http://www.quppe.eu

Table 17: LC-MS/MS Conditions (Method 1.3 for "Glyphosate & Co. Hypercarb")

Ionisation mode	ESI neg		
Column	**Hypercarb** 2.1 x 100 mm, 5 µm, (P/N 35005-102130)		
Temperature	40°C		
Pre-column	**Hypercarb Guard** 2.1 x 10 mm, 5 µm (P/N 35005-102101)		
Pre-filters	e.g. Supelco column saver 2.0 µm Filter (optional)		
Eluent A	1% Acetic acid in Water + 5% Methanol		
Eluent B	1% Acetic acid in Methanol		
Gradient	**%A**	**Flow [mL/min]**	**Time [min]**
	100	0.2	0
	70	0.2	10
	70	0.4	11
	70	0.4	18
	10	0.4	19
	10	0.4	22
	100	0.2	22.1
	100	0.2	30
Injection volume / Dilution within Injector	**Cucumber and Strawberry**: 5 µL (without additional dilution in the injector) **Liver, Kidney, Milk, Rice and Soya (previously DILUTED in vial)** 5 µL (without additional dilution in the injector) **Liver, Kidney, Milk, Rice and Soya (NOT diluted in vial)** **1 µL+4 µL** MeOH containing 1% Formic acid (5-fold dilution in injector)		
Acquired mass transitions	**Analyte**	**Mass Transitions (m/z)**	
	Glyphosate : Glyphosate-$^{13}C_2$, ^{15}N (ILIS) :	168/63, 168/124, 168/150, 168/81 171/63, (alternative 171/126)	
	AMPA: AMPA-^{13}C, ^{15}N (ILIS):	110/63, 110/79, 110/81 112/63, (alternative 112/81)	
	N-Acetyl-Glyphosate: N-Acetyl-Glyphosate-$^{13}C_2$, ^{15}N (ILIS):	210/63, 210/150, 210/79, 210/148 213/63, (alternative 213/153)	
	Ethephon: Ethephon-D_4 (ILIS):	143/107, 143/79, 145/107 147/111, (alternative 147/79)	
	HEPA: HEPA-D_4 (ILIS)	125/79, 125/95, 125/63 129/79, (alternative 129/97)	
	Glufosinate: Glufosinate-D_3 (ILIS):	180/63, 180/136, 180/85, 180/95 183/63, (alternative 183/98)	
	N-Acetyl-Glufosinate: N-Acetyl-Glufosinate-D_3 (ILIS):	222/63, 222/59, 222/136 225/63, (alternative 225/137)	
	MPPA: MPPA-D_3 (ILIS):	151/63, 151/107, 151/133 154/63, (alternative 154/136)	
	Fosetyl-Al: (detected as Fosetyl) Fosetyl-Al-D_{15} (ILIS):	109/81, 109/63 114/82, (alternative 114/63)	
	Maleic Hydrazide: Maleic Hydrazide-D_2 (ILIS):	111/82, 111/42, 111/55, 111/83 113/42, (alternative 113/85)	
	Cyanuric acid: Cyanuric acid $^{13}C_3$ (ILIS):	128/42, 128/85 131/43	

Table 18: Proposed LC-MS/MS Conditions for Priming and Reconditioning of the Hypercarb Column

Instrument parameters	Conditions		
Ionisation mode	ESI neg		
Column/temperature	**Hypercarb** 2.1 x 100 mm 5 µm (P/N 35005-102130); 40°C		
Pre-column	**Hypercarb Guard** 2.1 x 10 mm 5 µm (P/N 35005-102101)		
Pre-filters	e.g. Supelco column saver 2.0 µm Filter (optional)		
Eluent A	1% Acetic acid in Water + 5% MeOH		
Eluent B	1% Acetic acid in MeOH		
Gradient	**%A**	**Flow [mL/min]**	**Time [min]**
	100	0.3	0
	70	0.3	7
	100	0.3	7.1
	100	0.3	12
Injection volume	50 µL		
MS-System	If possible disconnect the MS-System to prevent contamination of the MS.		

G.2 MEASUREMENTS BY M1.6:

It is recommended conditioning the Torus column according to the Start-up Guide[5].

Exemplary measurement settings are shown in **Table 19, Table 20 and Table 21.**

Maleic hydrazide and Cyanuric acid show a very poor retention behaviour on this column, with retention times close to the dead-time, and are therefore heavyly interference by matrix components causing poor detection signals, peak shapes and peak intensities (signal suppression). Proper evaluation of the peaks is often not possible.

Table 19: LC-MS/MS Conditions (Method 1.6 for "Glyphosate & Co. on Torus DEA")

Ionisation mode	ESI neg		
Column	**Waters Torus™DEA** 2.1 mm x 100 mm; 1.7 µm		
Temperature	50°C		
Pre-column	**Waters Torus™DEA VanGuard™** 2.1 mm x 5 mm; 1.7 µm		
Pre-filters	Waters ACQUITY UPLC Column In-Line Filter Kit [205000343]		
Eluent A	1.2 % formic acid in water		
Eluent B	0.5 % formic acid in acetonitrile		
Gradient	**%A**	**Flow [mL/min]**	**Time [min]**
	10	0.5	0
	10	0.5	0.5
	80	0.5	1.5
	90	0.5	4.5
	90	0.5	17.5
	10	0.5	17.6
	10	0.5	23
Injection volume	10 µL		
Acquired mass transitions	**Analyte**	**Mass Transitions (m/z)**	
	Glyphosate :	168/63, 168/124, 168/150, 168/81	
	Glyphosate-$^{13}C_2$, ^{15}N (ILIS) :	171/63, (alternative 171/126)	
	AMPA:	110/63, 110/79, 110/81	
	AMPA-^{13}C, ^{15}N (ILIS):	112/63, (alternative 112/81)	
	N-Acetyl-Glyphosate:	210/63, 210/150, 210/79, 210/148	
	N-Acetyl-Glyphosate-$^{13}C_2$,^{15}N (ILIS):	213/63, (alternative 213/153)	
	Ethephon:	143/107, 143/79, 145/107	
	Ethephon-D_4 (ILIS):	147/111, (alternative 147/79)	
	HEPA:	125/79, 125/95, 125/63	
	HEPA-D_4 (ILIS)	129/79, (alternative 129/97)	
	Glufosinate:	180/63, 180/136, 180/85, 180/95	
	Glufosinate-D_3 (ILIS):	183/63, (alternative 183/98)	
	N-Acetyl-Glufosinate:	222/63, 222/59, 222/136	
	N-Acetyl-Glufosinate-D_3 (ILIS):	225/63, (alternative 225/137)	
	MPPA:	151/63, 151/107, 151/133	
	MPPA-D_3 (ILIS):	154/63, (alternative 154/136)	
	Fosetyl-Al:	109/81, 109/63 (detected as Fosetyl)	
	Fosetyl-Al-D_{15} (ILIS):	114/82, (alternative 114/63)	

[5] https://www.waters.com/webassets/cms/support/docs/720006156en.pdf

G.3 EXEMPLARY MS/MS TRANSITIONS AND POTENTIALS

Here is a compilation of MS/MS settings for two models of MS/MS instruments by AB Sciex.

Table 20: Exemplary MS/MS Transitions and Potentials* for AB Sciex QTRAP® 5500

Name	Q1 (Da)	Q2 (Da)	Dwell time (msec)	DP (V)	EP (V)	CE (V)	CXP (V)
AMPA 110/63	110	63	20	-70	-10	-26	-5
AMPA 110/79	110	79	20	-70	-10	-36	-7
AMPA 110/81	110	81	20	-70	-10	-18	-7
AMPA-^{13}C, ^{15}N IS 112/63	112	63	20	-75	-10	-24	-5
AMPA-^{13}C, ^{15}N IS 112/81	112	81	20	-75	-10	-36	-9
Cyanuric acid 128/42	128	42	20	-35	-10	-24	-13
Cyanuric acid 128/85	128	85	20	-35	-10	-12	-7
Cyanuric acid $^{13}C_3$ 131/43	131	42	20	-60	-10	-24	-7
Ethephon 143/107	143	107	20	-60	-10	-12	-5
Ethephon 143/79	143	79	20	-60	-10	-24	-1
Ethephon 145/107	145	107	20	-60	-10	-12	-5
Ethephon-$D_4$147/111	147	111	20	-60	-10	-12	-7
Ethephon-$D_4$147/79	147	79	20	-60	-10	-26	-7
Fosetyl 109/63	109	63	20	-60	-10	-16	-7
Fosetyl 109/81	109	81	20	-60	-10	-38	-5
Fosetyl-Al-D_{15} 114/82	114	82	20	-65	-10	-18	-7
Fosetyl-Al-D_{15} 114/63	114	63	20	-65	-10	-38	-7
Glufosinate 180/136	180	136	20	-65	-10	-24	-5
Glufosinate 180/63	180	63	20	-65	-10	-50	-1
Glufosinate 180/85	180	85	20	-65	-10	-26	-5
Glufosinate 180/95	180	95	20	-65	-10	-24	-5
Glufosinate-D_3 183/63	183	63	20	-70	-10	-62	-9
Glufosinate-D_3 183/98	183	98	20	-70	-10	-24	-7
Glyphosate 168/124	168	124	20	-60	-10	-16	-7
Glyphosate 168/150	168	150	20	-60	-10	-14	-9
Glyphosate 168/63	168	63	20	-60	-10	-32	-3
Glyphosate 168/81	168	81	20	-60	-10	-22	-1
Glyphosate-$^{13}C_2$, ^{15}N 171/63	171	63	20	-60	-10	-32	-7
Glyphosate-$^{13}C_2$, ^{15}N 171/126	171	126	20	-60	-10	-14	-9
HEPA 125/63	125	63	20	-70	-10	-72	-1
HEPA 125/79	125	79	20	-70	-10	-28	-3
HEPA 125/95	125	95	20	-70	-10	-20	-5
HEPA-D_4 129/79	129	79	20	-80	-10	-30	-3
HEPA-$D_4$129/97	129	97	20	-8	-10	-20	-5
Maleic hydrazid 111/42	111	42	20	-70	-10	-40	-5
Maleic hydrazid 111/55	111	55	20	-70	-10	-22	-7
Maleic hydrazid 111/82	111	82	20	-70	-10	-26	-3
Maleic hydrazid 111/83	111	83	20	-70	-10	-20	-5
Maleic Hydrazide-D_2 113/42	113	42	20	-75	-10	-50	-5
Maleic Hydrazide-D_2 113/85	113	85	20	-35	-10	-18	-5
MPPA 151/107	151	107	20	-75	-10	-22	-5
MPPA 151/133	151	133	20	-75	-10	-18	-7
MPPA 151/63	151	63	20	-75	-10	-48	-9
MPPA-D_3 154/63	154	63	20	-40	-10	-44	-9
MPPA-D_3 154/136	154	136	20	-40	-10	-18	-7
N-acetylglufosinate 222/136	222	136	20	-70	-10	-30	-9
N-acetylglufosinate 222/59	222	59	20	-70	-10	-20	-9
N-acetylglufosinate 222/63	222	63	20	-70	-10	-82	-9
N-Acetyl-Glufosinate-D_3 225/63	225	63	20	-80	-10	-72	-29
N-Acetyl-Glufosinate-D_3 225/137	225	137	20	-80	-10	-30	-7
N-Acetylglyphosate 210/150	210	150	20	-50	-10	-20	-7
N-Acetylglyphosate 210/63	210	63	20	-50	-10	-42	-1
N-Acetylglyphosate 210/79	210	79	20	-50	-10	-58	-1
N-Acetylglyphosate 210/148	210	148	20	-50	-10	-22	-5
N-Acetyl-Glyphosate-$^{13}C_2$,^{15}N 213/153	213	153	20	-60	-10	-20	-7
N-Acetyl-Glyphosate-$^{13}C_2$,^{15}N 213/63	213	63	20	-60	-10	-46	-1

Table 21: Exemplary MS/MS Transitions and Potentials* for AB Sciex QTRAP® 6500+

Name	Q1 (Da)	Q2 (Da)	Dwell time (msec)	DP (V)	EP (V)	CE (V)	CXP (V)
AMPA 110/63	110	63	20	-40	-10	-24	-9
AMPA 110/81	110	81	20	-40	-10	-18	-11
AMPA 110/79	110	79	20	-40	-10	-32	-9
AMPA-^{13}C, ^{15}N 112/63	112	63	20	-50	-10	-24	-9
AMPA-^{13}C, ^{15}N 112/81	112	81	20	-50	-10	-18	-9
Cyanuric acid 128/42	128	42	20	-55	-10	-24	-13
Cyanuric acid 128/85	128	85	20	-55	-10	-12	-7
Cyanuric acid $^{13}C_3$ 131/43	131	42	20	-60	-10	-24	-7
Ethephon 143/107	143	107	20	-10	-10	-10	-15
Ethephon 143/79	143	79	20	-10	-10	-22	-9
Ethephon 145/107	145	107	20	-5	-10	-10	-13
Ethephon-D_4 147/111	147	111	20	-15	-10	-10	-13
Ethephon-$D_4$147/79	147	79	20	-15	-10	-24	-9
Fosetyl 109/81	109	81	20	-30	-10	-16	-9
Fosetyl 109/63	109	63	20	-30	-10	-34	-9
Fosetyl-Al-D_{15} 114/82	114	82	20	-35	-10	-18	-9
Fosetyl-Al-D_{15} 114/63	114	63	20	-35	-10	-32	-9
Glufosinate 180/95	180	95	20	-30	-10	-22	-13
Glufosinate 180/136	180	136	20	-30	-10	-22	-15
Glufosinate 180/85	180	85	20	-30	-10	-24	-11
Glufosinate 180/63	180	63	20	-30	-10	-52	-9
Glufosinate-D_3 183/98	183	98	20	-45	-10	-22	-11
Glufosinate-D_3 183/63	183	63	20	-45	-10	-60	-9
Glyphosate 168/150	168	150	20	-30	-10	-14	-7
Glyphosate 168/63	168	63	20	-30	-10	-24	-7
Glyphosate 168/81	168	81	20	-30	-10	-20	-9
Glyphosate 168/124	168	124	20	-30	-10	-16	-13
Glyphosate-$^{13}C_2$, ^{15}N 171/63	171	63	20	-25	-10	-28	-7
Glyphosate-$^{13}C_2$, ^{15}N 171/126	171	126	20	-25	-10	-16	-13
HEPA 125/79	125	79	20	-35	-10	-26	-9
HEPA 125/95	125	95	20	-35	-10	-18	-13
HEPA 125/63	125	63	20	-35	-10	-70	-15
HEPA-D_4 129/79	129	79	20	-30	-10	-26	-9
HEPA-D_4 129/97	129	97	20	-30	-10	-18	-13
Maleic hydrazid 111/42	111	42	20	-70	-10	-40	-5
Maleic hydrazid 111/55	111	55	20	-70	-10	-22	-7
Maleic hydrazid 111/82	111	82	20	-70	-10	-26	-3
Maleic hydrazid 111/83	111	83	20	-70	-10	-20	-5
Maleic Hydrazide-D_2 113/42	113	42	20	-75	-10	-50	-5
Maleic Hydrazide-D_2 113/85	113	85	20	-35	-10	-18	-5
MPPA 151/133	151	133	20	-35	-10	-18	-9
MPPA 151/107	151	107	20	-35	-10	-22	-5
MPPA 151/63	151	63	20	-35	-10	-44	-7
MPPA-D_3 154/136	154	136	20	-25	-10	-18	-17
MPPA-D_3 154/63	154	63	20	-25	-10	-46	-15
N-Acetylglufosinate 222/136	222	136	20	-40	-10	-28	-15
N-Acetylglufosinate 222/59	222	59	20	-40	-10	-18	-9
N-Acetylglufosinate 222/63	222	63	20	-40	-10	-70	-15
N-Acetyl-Glufosinate-D_3 225/137	225	137	20	-15	-10	-30	-9
N-Acetyl-Glufosinate-D_3 225/63	225	63	20	-15	-10	-74	-27
N-Acetylglyphosate 210/150	210	150	20	-20	-10	-18	-9
N-Acetylglyphosate 210/148	210	148	20	-20	-10	-22	-9
N-Acetylglyphosate 210/63	210	63	20	-20	-10	-44	-9
N-Acetylglyphosate 210/79	210	79	20	-20	-10	-64	-9
N-Acetyl-Glyphosate-$^{13}C_2$,^{15}N 213/153	213	153	20	-20	-10	-18	-9
N-Acetyl-Glyphosate-$^{13}C_2$,^{15}N 213/63	213	63	20	-20	-10	-46	-9

*The settings will differ with other models of the same manufacturer and instruments by other manufacturers.

G.4 INJECTION-SEQUENCES

Note: for simplicity only one exemplary sequences are shown below. Each matrix/spiking level combination requires its own sequence.

a) Exemplary Measurement Sequence for M1.3 (Hypercarb column)

1 5 µL Spinach Blank
2 5 µL Spinach Blank
3 5 µL Spinach Blank
4 Reagent-BLANK + ILIS (check for analytes / interferences and report under "Any further remarks" in "Enter General Info here!" within the Excel template)*
5 [Matrix]-BLANK + ILIS (check for analytes / interferences, see Excel template "Enter Peak Areas here!" "BLANK")**
6 ***Solvent-CAL-LOW/MID/HIGH/MAX 120%***
7 ***Cucumber-CAL- LOW/MID/HIGH/MAX 60%***
8 ***Cucumber -CAL-LOW/MID/HIGH/MAX 120%***
9 [Matrix]-BLANK
10 ***[Matrix]-CAL-LOW/MID/HIGH/MAX 60%***
11 ***[Matrix]-CAL-LOW/MID/HIGH/MAX 120%***
12 [Matrix]-BLANK (check for carry-over and report under "Any further remarks" in "Enter General Info here!" within the Excel template if relevant. Carry over should be <2%).
13 **[Matrix]-LOW/MID/HIGH/MAX1**
14 **[Matrix]-LOW/MID/HIGH/MAX2**
15 **[Matrix]-LOW/MID/HIGH/MAX3**
16 **[Matrix]-LOW/MID/HIGH/MAX4**
17 **[Matrix]-LOW/MID/HIGH/MAX5**
18 [Matrix]-BLANK
19 ***Cucumber -CAL-LOW/MID/HIGH/MAX 60%***
20 ***Cucumber -CAL- LOW/MID/HIGH/MAX 120%***
21 [Matrix]-BLANK
22 ***[Matrix]-CAL- LOW/MID/HIGH/MAX 60%***
23 ***[Matrix]-CAL- LOW/MID/HIGH/MAX 120%***
24 S-BLANK
25 5 µL Spinach Blank

* if reagents remain the same, **Reagent-BLANK + ILIS**: may be measured **once for all experiments** (for all matrices and all levels)

****[Matrix]-BLANK + ILIS**: only to be measured **once for each matrix** (for all levels)

b) Exemplary Measurement Sequence for <u>M1.6 or other</u>

1 S-BLANK
2 Reagent-BLANK + ILIS (check for analytes / interferences and report under "Any further remarks" in "Enter General Info here!" within the Excel template)*
3 [Matrix]-BLANK + ILIS (check for analytes / interferences, see Excel template "Enter Peak Areas here!" "BLANK")**
4 ***Solvent-CAL-LOW/MID/HIGH/MAX 120%***
5 ***Cucumber-CAL- LOW/MID/HIGH/MAX 60%***
6 ***Cucumber -CAL-LOW/MID/HIGH/MAX 120%***
7 S-BLANK
8 ***[Matrix]-CAL-LOW/MID/HIGH/MAX 60%***
9 ***[Matrix]-CAL-LOW/MID/HIGH/MAX 120%***
10 Matrix]-BLANK (check for carry-over and report under "Any further remarks" in "Enter General Info here!" within the Excel template if relevant. Carry over should be <2%).
11 **[Matrix]-LOW/MID/HIGH/MAX1**
12 **[Matrix]-LOW/MID/HIGH/MAX2**
13 **[Matrix]-LOW/MID/HIGH/MAX3**
14 **[Matrix]-LOW/MID/HIGH/MAX4**
15 **[Matrix]-LOW/MID/HIGH/MAX5**
16 S-BLANK
17 ***Cucumber -CAL-LOW/MID/HIGH/MAX 60%***
18 ***Cucumber -CAL- LOW/MID/HIGH/MAX 120%***
19 S-BLANK
20 ***[Matrix]-CAL- LOW/MID/HIGH/MAX 60%***
21 ***[Matrix]-CAL- LOW/MID/HIGH/MAX 120%***
22 S-BLANK

* if reagents remain the same, **Reagent-BLANK + ILIS**: may be measured **once for all experiments** (for all matrices and all levels)

****[Matrix]-BLANK + ILIS**: only to be measured **once for each matrix** (for all levels)

G.5 DATA PROCESSING:

The measured peak areas of the analytes and ILISs should be tracked into an **Excel template specially prepared by the organizer for this study and distributed to the participants**. All recovery calculations are done automatically.

Instructions on how to use the provided Excel template are given on the first sheet of the template called "READ this!". Please follow the instructions thoroughly. Hints on how to prepare your validation data before entering them into the sheet "Enter Peak areas" and how to set up the analyte list within the sheet "Enter Analyte Info" can be found within the sheets *'DEMO "Enter Analyte Info"'* and "*DEMO 'Enter Peak Areas!'*". Please describe the procedure applied in the validation experiment within the sheet *'Enter General Info!'* using the drop down menus. Please note that the fields highlighted in yellow are mandatory.

Please save the file indicating Lab acronym, method, spiking level and matrix e.g.:
CVUAS_QUPPE_TORUS_LOW_LIVER.xlsx

For submitting the completed Excel templates use the **online upload tool** (https://wetransfer.com/).
To avoid sending many E-mails with large attachments.

The recovery figures obtained by the participants will be entered in the Method Validation Database within the EURL-Datapool (www.eurl-pesticides-datapool.eu).