EURL-SRM - Analytical Observations Report

concerning the following…

- **Compound(s)**: Dimethoate, Omethoate and their metabolites
- **Commodities**: Fruit and vegetables, cereals
- **Extraction Method(s)**: Citrate QuEChERS, QuPPe
- **Instrumental analysis**: LC-MS/MS

Analysis of Dimethoate and Omethoate Metabolites - Method Development and Pilot Monitoring

Version 1 (last update: 28.04.2019)

Background information:

Dimethoate and omethoate are systemically acting organophosphate pesticides used to control various types of insects and mites. Omethoate, the oxygen analogon of dimethoate, is also one of the main metabolites of dimethoate. It is a more potent AChE inhibitor than dimethoate and presents a much higher acute toxicity towards both insects and humans. Omethoate is considered toxicologically critical not only due to its acute toxicity but also because of concerns regarding its mutagenicity potential. Omethoate is thus not registered for use within the EU.

In 2016 France decided to take emergency measures suspending at a national level the import and marketing of cherries from countries in which the use of dimethoate is allowed. Thereafter, EFSA was requested by the Commission to urgently evaluate the risks arising from the use of dimethoate in agriculture, considering its metabolites. In its "Prioritized review of the existing maximum residue levels for dimethoate and omethoate according to Article 43 of Regulation (EC) No 396/2005" [4], EFSA highlighted the following 6 metabolites of dimethoate (in addition to omethoate) that were found at significant levels in metabolism studies or residue trials:

- Metabolite III: dimethoate carboxylic acid
- Metabolite X: O-desmethyl-dimethoate
- Metabolite XI: O-desmethyl-omethoate
- Metabolite XII: O-desmethyl isodimethoate
- Metabolite XX: O-desmethyl omethoate carboxylic acid
- Metabolite XXIII: O-desmethyl-N-desmethyl omethoate

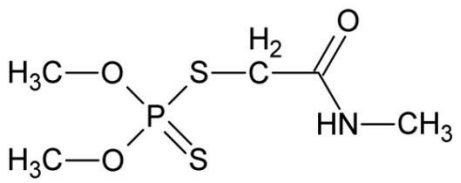
Some of these metabolites formed a major part of the residue and in many cases they seemed to be better markers than dimethoate or omethoate. In its report EFSA identified data gaps regarding the toxicological properties of the 6 metabolites but also as regards their occurrence in primary crops. Thus only tentative MRLs could be proposed at this stage.

Considering the large toxicological difference between dimethoate and omethoate, EFSA also proposed to change the residue definition from 'sum of dimethoate and omethoate expressed as dimethoate' into two separate residue definitions 'dimethoate' and 'omethoate'. This proposal was legally adopted by Regulation 2017/1135/EU.

Following the publication of the EFSA report, the EURL-SRM deemed it as very useful to start collecting residue data from market samples. This would ultimately help in drawing a more complete picture about the residue situation in the market, and in identifying whether any of the 6 metabolites is suited as a residue marker for controlling compliance with GAP rules for dimethoate.

Compound details:

Table 1: General information on dimethoate and omethoate

Name: Dimethoate (CAS: 60-51-5) **IUPAC: O,O-dimethyl S-methylcarbamoylmethyl phosphorodithioate or 2-dimethoxyphosphinothioylthio-N-methylacetamide**		
Parameter	**Value**	
Molecular Mass	229.2 g/mol	
Formula	$C_5H_{12}NO_3PS_2$	
Exact mass	228.99962 Da	
Pka	not ionized	
LogD	0.34 (calculated by chemicalize.org)	
Residue definition EU	Dimethoate	
Dimethoate is approved in…	AT, BE, BG, CY, CZ, DE, EE, EL, ES, FI, HR, HU, IE, IT, LU, MT, NL, PL, PT, RO, SI, SK, UK	
ADI / ARfD	0.001 mg/kg bw per day / 0.010 mg/kg bw (EFSA 2013)	

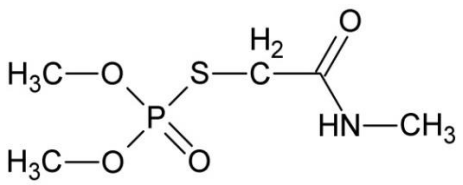
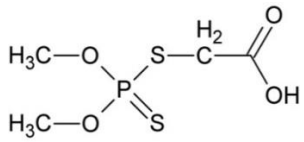
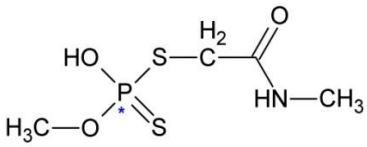
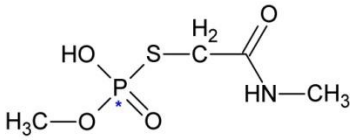
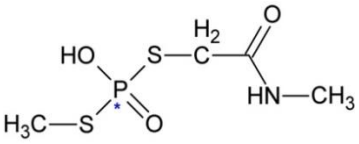
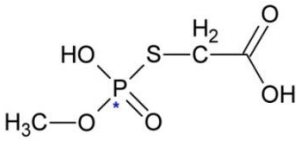
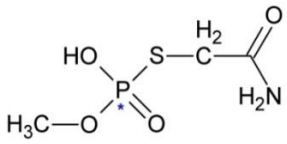
Name: Omethoate (CAS) **IUPAC: 2-dimethoxyphosphinoylthio-N-methylacetamide or O,O-dimethyl S-methylcarbamoylmethyl phosphorothioate**		
Parameter	**Value**	
Molecular Mass	213.2 g/mol	
Formula	$C_5H_{12}NO_4PS$	
Exact mass	213.02246 Da	
Pka	not ionized	
LogD	-0.55 (calculated by chemicalize.org)	
Residue definition EU	Omethoate	
Omethoate is approved in…	No authorization in place within the EU	
ADI / ARfD	0.0003 mg/kg bw per day / 0.002 mg/kg bw (EFSA 2013)	

Table 2: General information on the metabolites

Metabolites of Dimethoate and Omethoate			
Metabolites	**Formula/Molecular mass**	**Computed properties** (calculated by Chemicalize.org)	
		pKa	**LogP**
Dimethoate carboxylic acid **(Metabolite III)** $C_4H_9O_4PS_2$ 216.2 g/mol		pKa1: 4.41 at carboxy group	**pH3: 0,91** **pH4: 0,78** **pH5: 0,24** **pH6: -0,67** pH7: -1,62
O-desmethyl dimethoate **(Metabolite X)** $C_4H_{10}NO_3PS_2$ 215.2 g/mol		pKa1: 2.80 at phosphorus-bound OH group	**pH2: -0.1** **pH2.5: -0.21** **pH3: -0.45** pH4: -1.24 pH5: -2,0 pH6: -2.35 pH>7: -2.41
O-desmethyl omethoate **(Metabolite XI)** $C_4H_{10}NO_4PS$ 199.1 g/mol		pKa1: 2.23 at phosphorus-bound OH group	pH3: -1,75 pH4: -2,61 pH5: -3,16 pH6: -3,29 pH>7: -3,30
O-desmethyl isodimethoate **(Metabolite XII)** $C_4H_{10}NO_3PS_2$ 215.2 g/mol		pKa1: 2.57 at phosphorus-bound OH group	**pH2: -0.51** **pH2.5: -0.67** pH3: -0.97 pH4: -1.81 pH5: -2,51 pH6: -2.75 pH>7: -2.78
O-desmethyl-omethoate carboxylic acid **(Metabolite XX)** $C_3H_7O_5PS$ 186.1 g/mol		pKa1: 2.21 at phosphorus-bound OH group pKa2: 4.08 at carboxy group	pH3: -1.22 pH4: -2.30 pH5: -3,55 pH6: -4,63 pH>9: -6,24
O-desmethyl-N-desmethyl omethoate **(Metabolite XXIII)** $C_3H_8NO_4PS$ 185.1 g/mol		pKa1: 2.23 at phosphorus-bound OH group	pH3: -1,98 pH4: -2,83 pH5: -3,38 pH6: -3,51 pH>7: -3,53

Materials

Table 3: Sources of analytical standards

Substance	Purity	CAS	Source
Dimethoate	98.5%	60-51-5	LGC
Omethoate	98%	1113-02-6	
Dimethoate carboxylic acid (Metabolite III)	92.5%	1113-01-5	Cheminova-FMC (friendly donation)
O-desmethyl dimethoate (Metabolite X)	51.5% (free acid)	20253-71-8 (free acid)	
O-desmethyl omethoate (Metabolite XI)	81.1% (free acid)	86263-80-1	
O-desmethyl isodimethoate (Metabolite XII)	53.9% (free acid)	44988-12-7 (anion)	
O-desmethyl-omethoate carboxylic acid (Metabolite XX)	98.3%	159776-76-8 (free acid)	
O-desmethyl-N-desmethyl omethoate (Metabolite XXIII)	93.9%	159776-77-9 (free acid)	
Cyanuric acid $^{13}C_3$	95% (isotopic purity 89%)	201996-37-4	TRC

Disclaimer: Names of companies are given for the convenience of the reader and do not indicate any preference by the EURL-SRM towards these companies and their products

All other materials and chemicals used as listed in EN 15662 or the QuPPe-PO[1] method

EXPERIMENTS AND OBSERVATIONS

a) Establishment of measurement conditions

Looking at the molecular formulas of the 6 metabolites it was obvious that measurements should be in all cases conducted by LC-MS/MS in the ESI-negative mode. First experiments focused on establishing the MS/MS settings to ensure a sensitive analysis. The selected mass-transitions and elaborated MS/MS settings are given in Table 4.

The next step was the establishment of an LC-separation method taking into consideration that analysis would most probably involve extraction by the QuPPe method (see discussion below) and that the method developed should be incorporated in routine analysis. The efforts thus mainly focused on QuPPe-M1.3 method, which uses a carbon-based LC-column (Hypercarb by Thermo Scientific). In CVUA Stuttgart QuPPe-M1.3 is routinely used for the analysis of many QuPPe-amenable compounds which are measured in the ESI-negative mode.

[1] http://www.eurl-pesticides.eu/docs/public/tmplt_article.asp?CntID=887&LabID=200&Lang=EN

Table 4: MRM details for Dimethoate/Omethoate metabolites (ESI-neg. mode using Sciex 4000QTrap)

Compound	Intensity ranking	Q 1	Q 3	DP**	CE	CXP
Dimethoate carboxylic acid (Metabolite III)	1	140.9*	125.9	-60	-22	-5
	2	140.9*	95.8	-60	-30	-3
	3 (target)	214.9	140.8	-30	-10	-1
	4	214.9	125.8	-30	-34	-5
O-desmethyl dimethoate (Metabolite X)	1 (target 1)	213.9	104	-50	-24	-5
	2	213.9	94.9	-50	-22	-5
	3 (target 2)****	213.9	135.9	-50	-20	-5
O-desmethyl omethoate (Metabolite XI)	1	197.9	103.9	-55	-24	-5
	2	197.9	166.9	-55	-18	-7
	3	197.9	72.9	-55	-40	-1
O-desmethyl isodimethoate (Metabolite XII)	1	213.9	103.8	-50	-26	-5
	2 (target)	213.9	165.9	-50	-18	-7
	3	213.9	72.9	-50	-42	-1
O-desmethyl omethoate carboxylic acid (Metabolite XX)	1	184.9	110.9	-40	-18	-5
	2	184.9	90.9	-40	-24	-3
	3	184.9	78.9	-40	-32	-1
O-desmethyl N-desmethyl omethoate (Metabolite XXIII)	1	183.9	89.9	-50	-26	-3
	2	183.9	166.8	-50	-18	-7
	3	183.9	151.8	-50	-18	-7
Cyanuric acid $^{13}C_3$ *****	-	131.0	43.0	-60***	-24	-7

* In-source fragment
** DP is typically increased by 20 for measurements by Sciex 5500 QTrap
*** DP was optimized on a Sciex 5500QTrap
**** Although less intensive, this transition is more specific to metabolite X and allows to distinguish metabolite X from XII
***** Cyanuric acid $^{13}C_3$ was not used in routine analyses. It was only used for determinations based on standard additions approach or in validation experiments with matrix-matched calibration,

Two additional HILIC-based QuPPe methods were also briefly tested at a later stage and were shown to be also suitable for the analysis of these compounds. These were QuPPe-M1.5 (employing a Trinity Q1 column by Thermo Fisher Scientific) and the QuPPe-M1.6 (employing a Torus DEA column by Waters). For details on the measurement conditions please refer to the latest version of the QuPPe method[2]. Analysis by a reversed-phase column (BEH-C_{18} by Waters) was also briefly tested but retention and separation of critical compounds was poor (see also Figure 4).

Table 5 shows details of QuPPe-M1.3. For the measurement of the 6 metabolites the Hypercarb column has to be well conditioned (see QuPPe protocol). Otherwise the later eluting compounds, the important metabolite X (desmethyl dimethoate) and the metabolite III (dimethoate carboxylic acid) may not elute within the given method run time and no data will be acquired. Periodic conditioning of the Hybercarb column with matrix extracts (e.g. routine samples) and, if needed, with blank spinach extracts, will typically help to avoid this situation.

[2] http://www.eurl-pesticides.eu/docs/public/tmplt_article.asp?CntID=887&LabID=200&Lang=EN

Table 5: Instrumentation details (same as QuPPe M1.3 'Glyphosate & Co.')

LC	Agilent 1200 Series		
MS/MS	Sciex 5500QTrap, run in ESI negative mode		
Column	Thermo Hypercarb 100x2.1 mm 5um (P/N 35005-102130)		
Pre-column	Thermo Universal Uniguard Holder for 2.1/3.0mm ID (P/N 852-00) Thermo Hypercarb 5um 10x2.1mm Drop-In Guards (P/N 35005-012101)		
Mobile Phase	A: 10 mL acetic acid (1%) plus 50 mL methanol plus 940 mL water B: 10 mL acetic acid (1%) plus 990 mL methanol		
Gradient	**Time** (min)	**Mobile Phase A** (%)	**Flow (mL/min)**
	0	100	0.2
	10	70	0.2
	11	70	0.4
	18	70	0.4
	19	10	0.4
	22	10	0.4
	22.1	100	0.2
	30	100	0.2
Injection volume	5 µL		
Column temperature	40°C		

Figure 1 shows some exemplary chromatograms of the 6 metabolites at 0.001; 0.005 and 0.01 µg/mL, corresponding to 0.002; 0.01 and 0.02 mg/kg when 10 g analytical portions are used in the QuPPe method. Calibration curves in the range between 0.001 µg/mL and 1 µg/mL are shown in Figure 2a and between 0.001 µg/mL and 0.2 µg/mL in Figure 2b. For all 6 metabolites calibration curves showed good linearity up to a concentration of 0.2 µg/mL, but saturation effects were observed at higher levels for the three metabolites (X, XI and XII) that showed the highest detection response.

Figure 1: *Chromatograms obtained from injections of differently labelled standards in pure solvent (measured by Sciex 5500QTrap)*

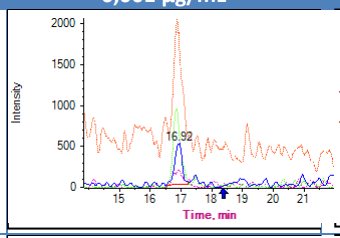
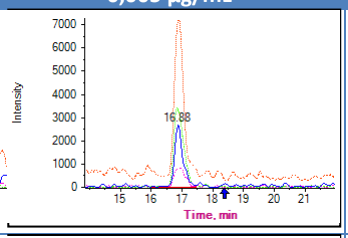
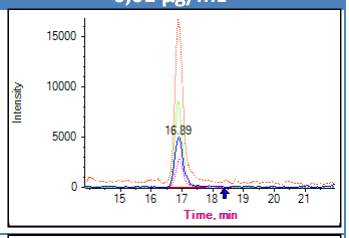
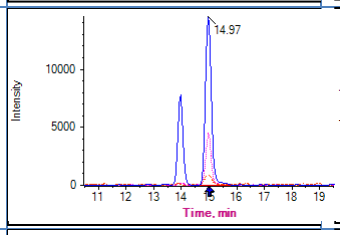
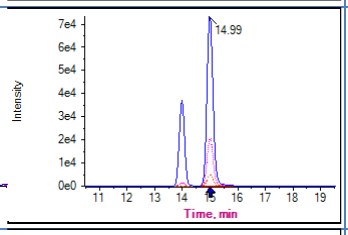
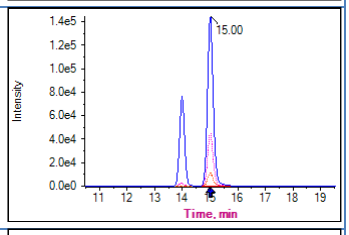
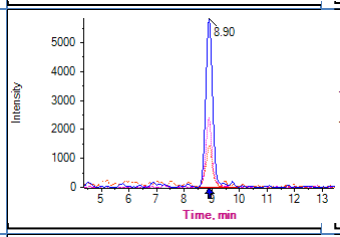
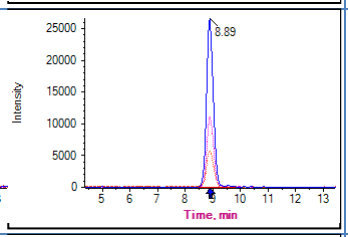
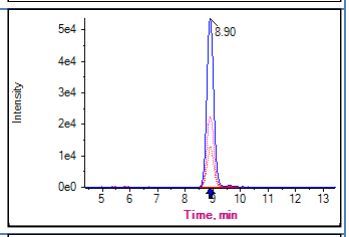
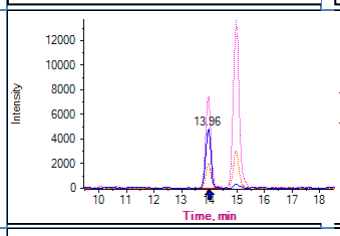
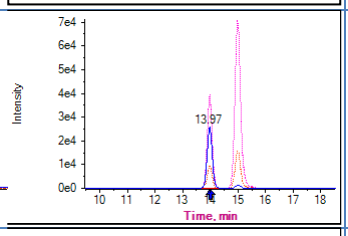
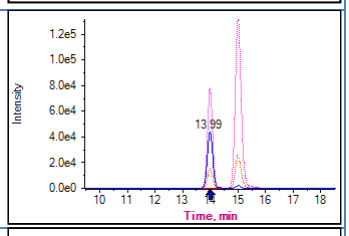
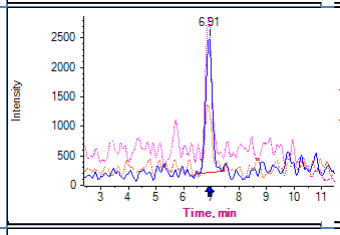
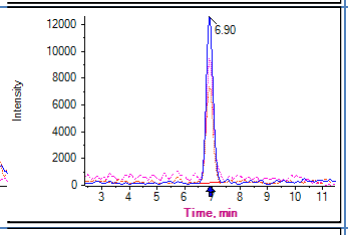
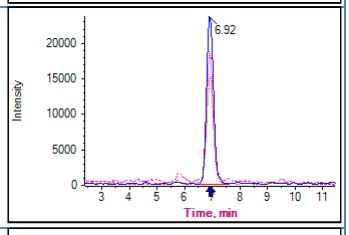
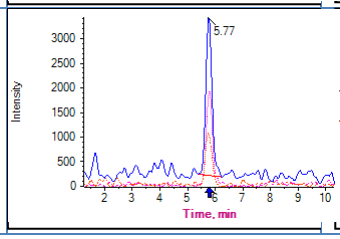
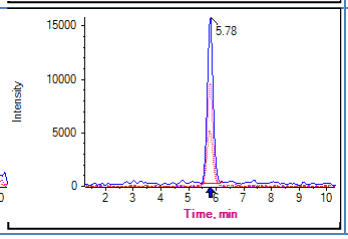
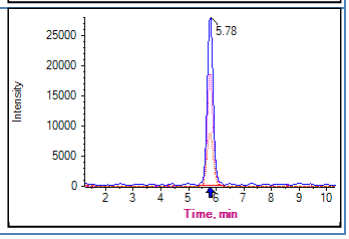
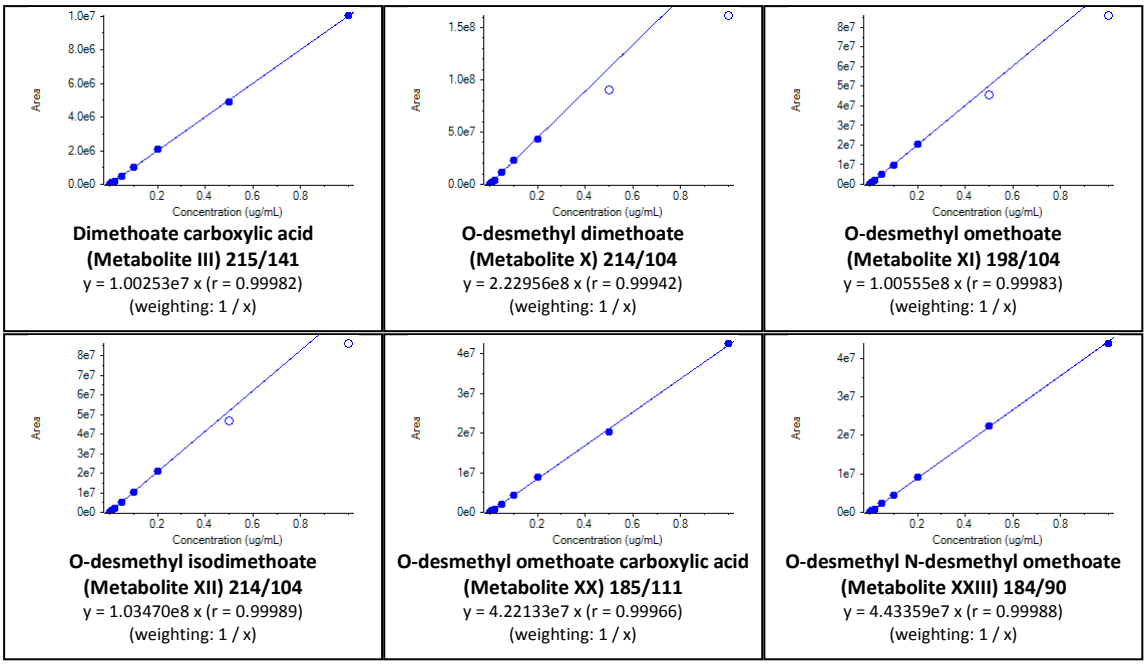
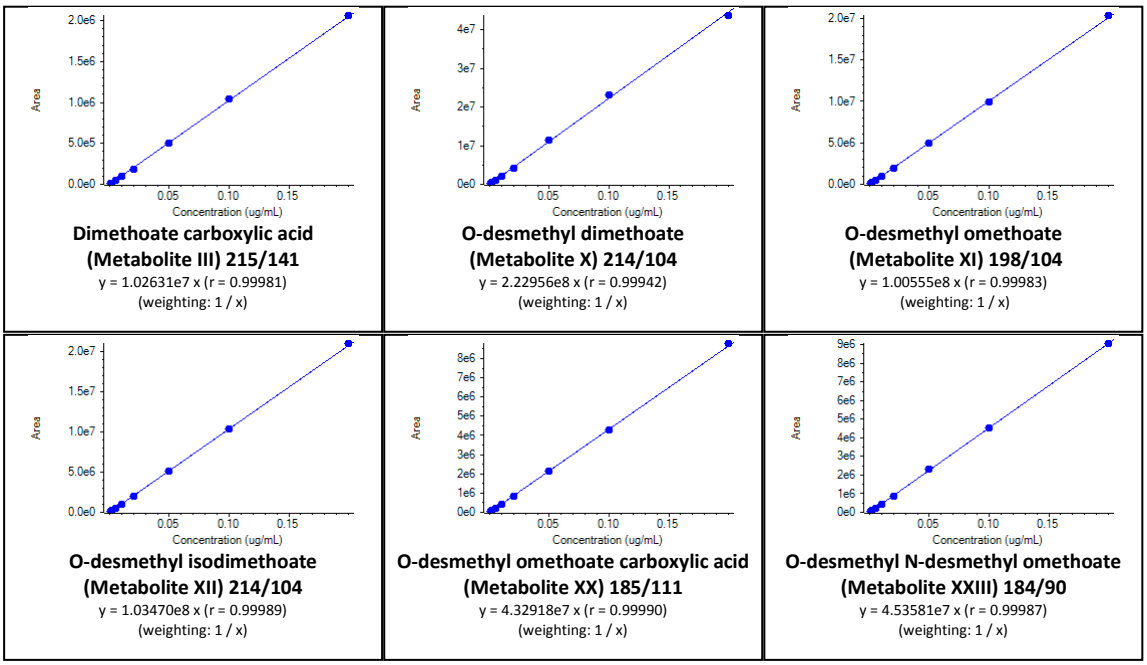
	Solvent Standard 0,001 µg/mL	Solvent Standard 0,005 µg/mL	Solvent Standard 0,01 µg/mL
Dimethoate carboxylic acid (Metabolite III) 215/141			
O-desmethyl dimethoate (Metabolite X) 214/104			
O-desmethyl omethoate (Metabolite XI) 198/104			
O-desmethyl isodimethoate (Metabolit XII) 214/166			
O-desmethyl omethoate carboxylic acid (Metabolite XX) 185/111			
O-desmethyl N-desmethyl omethoate (Metabolite XXIII) 184/90			

Figure 2: *Calibration curves based on standards in pure solvent (measured by Sciex 5500QTrap)*

a) In the range between 0.001 µg/mL and 1 µg/mL



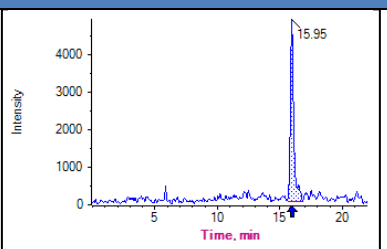
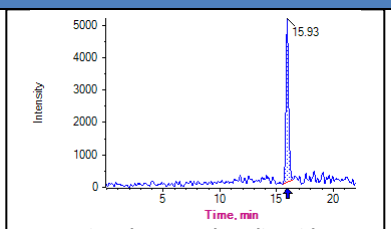
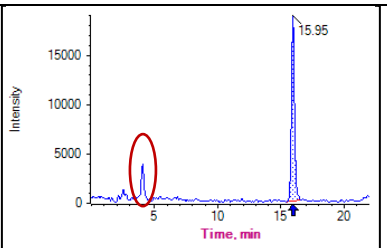
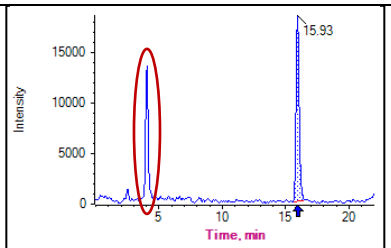
b) In the range between 0.001 µg/mL and 0.2 µg/mL



b) In-source fragmentation

While establishing the ESI-(neg.)-MS/MS settings for the measurement of metabolite III (dimethoate carboxylic acid), two intensive mass-transitions (141/126 and 141/96) were observed and included in the acquisition method. Following LC-separation (Hypercarb column), it became clear that these two mass transitions were partly due to in-source fragmentation of metabolite III and partly due to an additional early eluting compound within the metabolite III solution (see Table 3). In further experiments (see also next chapter), it could be shown that the concentration of this additional compound increases during storage of diluted working standard mixtures. Interestingly, the concentration of metabolite III did not markedly decline in these mixtures, so it was assumed that one of the other metabolites or the impurities contained in the standards used to prepare the mixture may be degrading to this additional compound. Further tests with individual working solutions are required to elucidate this aspect. Furthermore, it remained unclear whether this additional compound, formed during storage, is identical to the in-source fragment of metabolite III, or whether it is the in-source fragment of another compound present in the working-mixture.

Figure 3: *In-source fragmentation of dimethoate carboxylic acid in a standard mixture containing 0.01 µg/mL (prepared in methanol, finally diluted in acidified methanol/H_2O 50:50), (Sciex 5500QTrap)*

Calib Mixture "new"	Calibration Mixture "old"
 **Dimethoate carboxylic acid (Metabolite III) 215/141**	 **Dimethoate carboxylic acid (Metabolite III) 215/141**
 **Dimethoate carboxylic acid (Metabolite III) insource fragment 141/126**	 **Dimethoate carboxylic acid (Metabolite III) insource fragment 141/126**

c) Stability of stock solutions and impurities

The stability of the metabolites during the storage of stock solutions was tested by comparing stored stock solutions against freshly prepared ones. Table 6 gives an overview of these experiments. Overall, the compounds showed a good stability over a period of more than 20 months in individual stock solutions.

Table 6: Stability of stock solutions *of the six metabolites at 1 mg/mL each. The stock solutions were separately freshly diluted just before measurement. The deviation was measured against equivalent standards that were freshly prepared (n=3 each)*

Compound	Solvent of stock solutions (old and new)	Storage time in fridge	Deviation of conc.
Dimethoate carboxylic acid (III)	**ACN**	21 months	- 2.7 %
O-desmethyl dimethoate (X)	**ACN** (but new solution in MeOH)	25 months	9.7 %
O-desmethyl omethoate (XI)	**MeOH**	25 months	0.6 %
O-desmethyl isodimethoate (XII)	**MeOH**	25 months	1.6 %
O-desmethyl omethoate carboxylic acid (XX)	**ACN** (w.~ 10% H_2O)	21 months	3.9 %
O-desmethyl N-desmethyl omethoate (XXIII)	**ACN** (w.~ 10% H_2O)	25 months	7.2 %

Some of the standards provided by the applicant were assigned with a low purity, e.g. metabolite X 51.5%; metabolite XI 81.1% and metabolite XII 53.9%. It was thus interesting to check whether any of the standards contains any significant amounts of the other target metabolites. Only two of the standards were found to contain levels of the other metabolites:

- The provided standard of **metabolite X** (desmethyl-dimethoate) was found to contain traces (< 1 %) of metabolite XI (desmethyl-omethoate) and around 3% of metabolite XII (O-desmethyl-isodimethoate).
- The provided standard of **metabolite XII** (O-desmethyl-isodimethoate) was found to contain traces (< 1 %) of metabolite XI (desmethyl-omethoate).

Furthermore the stability of the compounds in a diluted working mixture was briefly tested. Initially the formation of an additional peak was noticed (see previous chapter). In this case the mixture was prepared from the stock solution in methanol and was stored in the refrigerator. Prior to injection the mixture was further diluted with "QuPPe-solvent" (1:1 mixture of H_2O and methanol with 1% FA) and stored over 1 week at room temperature before injection. The comparison was made against a freshly prepared mixture.

In a follow-up experiment mixtures of the 6 metabolites (at 0.05 µg/mL) in different solvents were studied: a) methanol, b) methanol with 1% formic acid and c) the "QuPPe solvent". The following observations were made:

a) **Methanol:** No significant degradations were noticed
b) **Methanol containing 1% formic acid**: Metabolite X disappeared completely after just 2 days. Metabolite XX showed a strong decline. Metabolites XII and XXIII showed a minor decrease. The additional unknown compound giving an early eluting peak in the mass trace of the in-source fragment of metabolite III (see previous chapter) also increased. Metabolite III did not show any significant decrease and was thus considered as not being the direct source of this additional product.
c) **QuPPe-solvent:** No significant degradations were noticed

More experiments will be necessary to study the stability of the compounds in individual and mixed working standards diluted in different solvents. In our experiments dilutions in methanol or methanol with 1% formic acid / water (50/50) were mainly used.

d) Initial recovery tests

As shown in Table 2, all 6 metabolites entail acidic groups (-P-OH or –COOH) and are characterized by a high polarity, which increases with increasing pH as the share of the deprotonated ionic forms increases. The phosphorous-bound hydroxy group is strongly acidic (pKa ~2.2-2,5); and predominantly deprotonated (anionic) at pH>2.5. The carboxy-group exhibits a weaker acidity (pKa 4-4.5) and is predominantly deprotonized at pH >4.5. Metabolite III is the only of the 6 metabolites that does not entail a free phosphoric acid group. At pH between 4 and 5, the typical range of citrate buffered QuEChERS, only metabolite III (dimethoate carboxylic acid) exhibits logP values (0,78 at pH4 and 0,24 at pH5), that would result in an extensive partitioning into the organic phase and satisfactory recoveries. All other metabolites are by far more polar and thus not expected to show satisfactory recoveries by the citrate buffered QuEChERS. This approach requires compounds to show logP values > -0.5 for having satisfactory recovery rates. Only the logP curve of Metabolites X and XII suggest promising recoveries under acidic conditions. Metabolite X shows calculated logP values of -0.1 at pH2; -0.45 at pH3 and -1.24 at pH4. Metabolite XII shows calculated logP values of -0.51 at pH2; -0.67 at pH 2.5; and -0.97 at pH3. Satisfactory recovery rates for these two compounds would theoretically only be achievable using strongly acidified variants of QuEChERS, such FA-QuEChERS, involving addition of formic acid or SA-QuEChERS involving addition of sulfuric acid (both without addition of buffering salts).

To check the behavior of the 6 compounds during sample preparation, preliminary recovery experiments were conducted for each of them separately using blank leek homogenate for spiking. Extractions (n=1 each, 12 in total) were performed using both methods QuEChERS and QuPPe. The spiking level was chosen at 0.5 mg/kg for the two less sensitive carboxylic acid metabolites and at 0.1 mg/kg for the remaining four metabolites. Matrix effects were equalized using matrix-matched calibration in each case. As no isotopically labeled standards were available matrix-matched calibration was used.

Table 7 shows the recovery data obtained for each metabolite using the QuEChERS and QuPPe method. All extracts were run on the Hypercarb column as well as on a reversed phase column (BEH C18 by Waters). Using the QuPPe method, which does not involve any liquid-liquid-partitioning step, acceptable recoveries were achieved for all 6 metabolites. Using QuEChERS, however, acceptable recoveries were, as expected, only achieved for Metabolite III. Metabolite X and XII were recovered at only ca. 25% and ca. 5% respectively, whereas the remaining 3 extremely polar metabolites were not recovered at any measurable amounts.

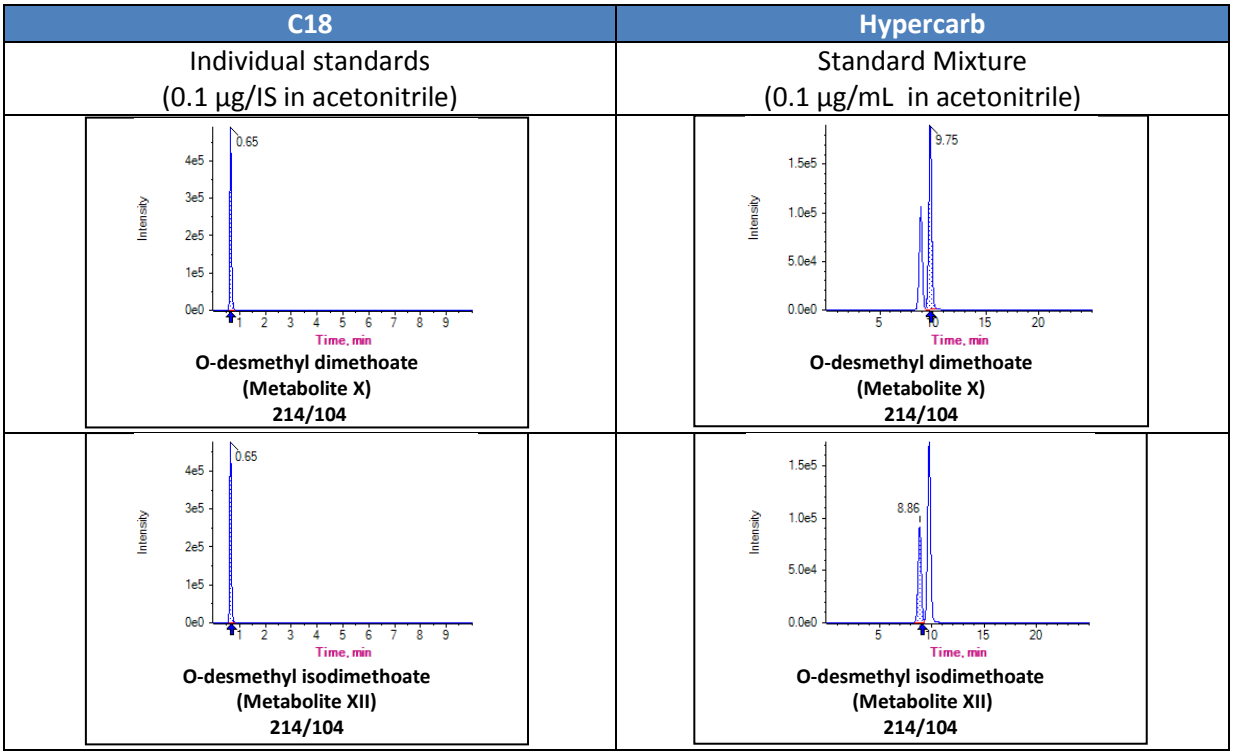
Table 7: Overview on recovery data on leek using QuPPe and QuEChERS sample preparation and different measurement methods (matrix-matched calibration was used in each case)

Compound	Spiking level (mg/kg)	Extraction method			
		QuEChERS*		QuPPe*	
		Column used			
		C18	Hypercarb	C18	Hypercarb
		Recovery rates in %			
Metabolite III	0.5	**88.6**	**108.3**	**93.2**	**100.0**
Metabolite X	0.1	24.6	25.1	**103.7**	**91.2**
Metabolite XI	0.1	-	-	**103.7**	**98.9**
Metabolite XII	0.1	5.1	5.8	**105.7**	**90.4**
Metabolite XX	0.5	-	-	**89.6**	**96.4**
Metabolite XXIII	0.1	-	-	**99.3**	**95.7**

* Average recovery of several MRM transitions and matrix-matched calibration

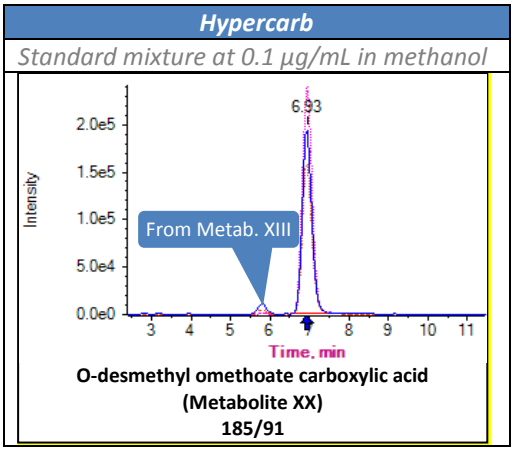
Figure 4 shows the poor retention and separation of the substances on the C_{18} column. For example O-desmethyl-dimethoate (X) and O-desmethyl isodimethoate (XII), which share the important mass-transition 214/104, showed the same retention time and very similar peak forms on this column. On the Hypercarb column these two metabolites were chromatographically sufficiently separated.

Figure 4: Chromatographic behavior of O-desmethyl-dimethoate and O-desmethyl isodimethoate (which share the mass transition 214/104) on a C_{18} and Hypercarb column (measured on a Sciex 4000QTrap)



Another pair of metabolites sharing the same mass transition were XIII (O-desmethyl N-desmethyl omethoate) and XX (O-desmethyl omethoate carboxylic acid). These metabolites were not separated by the C_{18} column. Figure 5 shows the interference of XII in the mass trace typically used for XX when employing the Hypercarb column. Despite the full chromatographic separation, care should still be taken by checking signal ratios of different mass transitions, to avoid peak misassignments and false positive results.

Figure 5: Chromatographic behavior of O-desmethyl-omethoate carboxylic acid and O-desmethyl N-desmethyl omethoate (which share the mass transition 185/91) on a Hypercarb column (measured by Sciex 5500 QTrap)



e) Main validation experiments

Validation experiments were conducted using the QuPPe procedure as described in the QuPPe protocol. Validation studies were performed on cherries and onions, each at 0.005 mg/kg and 0.05 mg/kg. Matrix-matched calibration solutions were prepared using blank extract of these commodities, at the 60% and 120% level of the spiked concentration. The results were evaluated using cyanuric acid $^{13}C_3$ as internal standard, which served to correct for volume deviations; matrix effects on the target analytes were addressed by the use of matrix-matched calibrations. A summary of all validation experiments conducted on both matrices is given in Table 8. Table 9 shows more detailed validation data on onion with each mass transition being evaluated separately. Some exemplary chromatograms for onion are shown in Figure 6.

Table 8: Recovery data of the 6 metabolites on cherry and onion evaluated with matrix-matched calibration (ESI-neg. mode using Sciex 5500QTrap). Only data of the target transition is shown.

Matrix Type	Matrix	Sample Weight	Spiking Level (mg/kg)	Metabolite	QuPPe		
					N	Mean Rec. (%)	RSD (%)
High water	**Onion**	10 g	0.005	**Metabolite III**	5	107	18.4*
				Metabolite X	5	120	5.2
				Metabolite XI	5	102	6.8
				Metabolite XII	5	104	6.1
				Metabolite XX	5	94	3.7
				Metabolite XXIII	5	106	6.3
		10 g	0.05**	**Metabolite III**	5	104	1.8
				Metabolite X	5	111	5.2
				Metabolite XI	5	109	3.2
				Metabolite XII	5	116	5.5
				Metabolite XX	5	106	3.9
				Metabolite XXIII	5	106	2.7
High water + low pH	**Cherry**	10 g	0.005	**Metabolite III**	5	119	17.4*
				Metabolite X	5	101	2.1
				Metabolite XI	5	96	4.5
				Metabolite XII	5	101	8.8
				Metabolite XX	5	94	12.8
				Metabolite XXIII	5	105	5.9
		10 g	0.05	**Metabolite III**	5	95	9.5
				Metabolite X	5	94	12.4
				Metabolite XI	5	98	8.9
				Metabolite XII	5	102	8.6
				Metabolite XX	5	99	10.4
				Metabolite XXIII	5	101	7.9

* High RSD attributed to insufficient sensitivity

** Recovery calculated without internal standard (only via area)

Table 9: Validation data for each mass-transition for the 6 metabolites spiked on onion at 0.005 mg/kg and 0.05 mg/kg; evaluated using matrix-matched calibration (ESI-neg. mode using Sciex 5500QTrap)

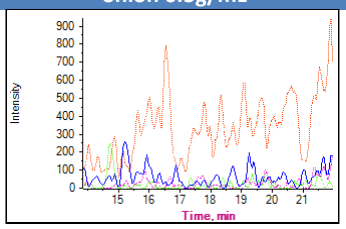
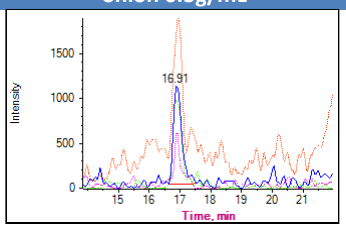
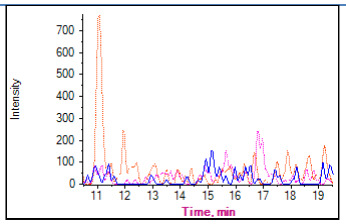
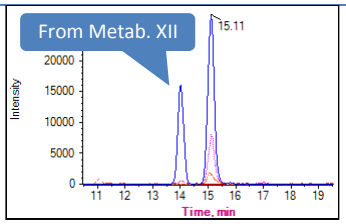
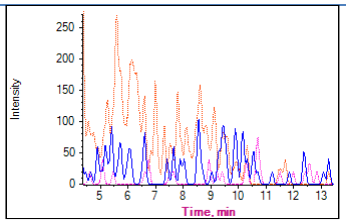
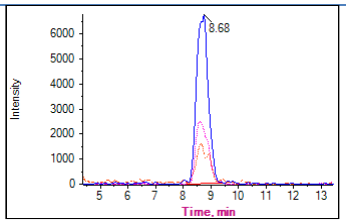
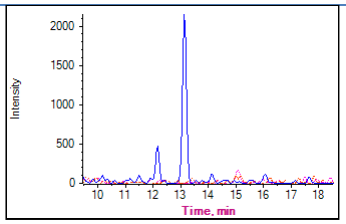
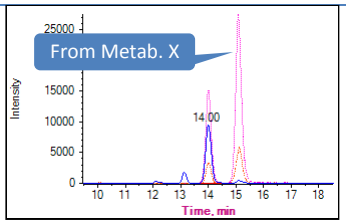
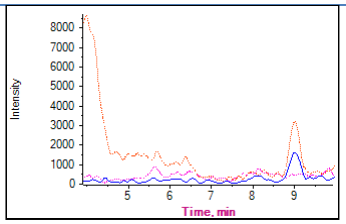
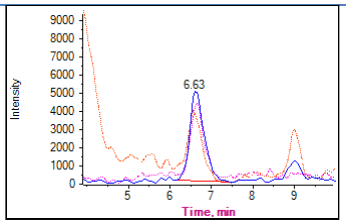
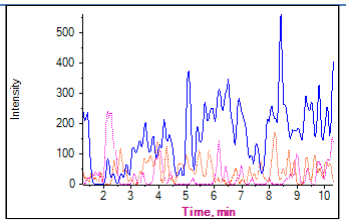
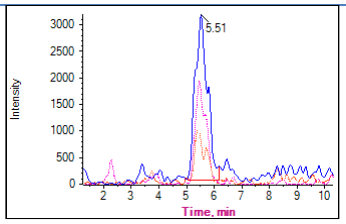
Compound/Transition	MRM	No. of repetitions	Spiking Level (mg/kg)	Mean Recov. (%)	RSD (%)
Validation at 0.005 mg/kg					
Dimethoate carboxylic acid (Metabolite III)	215/141	5	0.005	107	18.4**
	141/126*	5	0.005	103	12.9**
	141/96*	5	0.005	105	12.0**
O-desmethyl dimethoate (Metabolite X)	214/104	5	0.005	120	5.2
	214/95	5	0.005	117	9.4
	214/136	5	0.005	110	3.6
O-desmethyl omethoate (Metabolite XI)	198/104	5	0.005	102	6.8
	198/167	5	0.005	105	7.2
	198/73	5	0.005	108	3.5
O-desmethyl isodimethoate (Metabolit XII)	214/166	5	0.005	104	6.1
	214/104	5	0.005	109	8.6
	214/73	5	0.005	107	11.0
O-desmethyl omethoate carboxylic acid (Metabolite XX)	185/111	5	0.005	94	3.7
	185/91	5	0.005	95	7.2
	185/79	5	0.005	100	12.4***
O-desmethyl N-desmethyl omethoate (Metabolite XXIII)	184/90	5	0.005	106	6.3
	184/167	5	0.005	114	6.1
	184/152	5	0.005	101	13.2**
Validation at 0.05 mg/kg (Recoveries calculated without internal standard; only via peak areas)					
Dimethoate carboxylic acid (Metabolite III)	215/141	5	0.05	104	1.8
	141/126*	5	0.05	104	3.1
	141/96*	5	0.05	102	3.3
O-desmethyl dimethoate (Metabolite X)	214/104	5	0.05	111	5.2
	214/95	5	0.05	106	5.1
	214/136	5	0.05	111	6.6
O-desmethyl omethoate (Metabolite XI)	198/104	5	0.05	109	3.2
	198/167	5	0.05	107	2.7
	198/73	5	0.05	110	2.5
O-desmethyl isodimethoate (Metabolit XII)	214/166	5	0.05	116	5.5
	214/104	5	0.05	108	7.3
	214/73	5	0.05	109	6.5
O-desmethyl omethoate carboxylic acid (Metabolite XX)	185/111	5	0.05	106	3.9
	185/91	5	0.05	105	1.7
	185/79	5	0.05	105	6.5
O-desmethyl N-desmethyl omethoate (Metabolite XXIII)	184/90	5	0.05	106	2.7
	184/167	5	0.05	106	3.5
	184/152	5	0.05	101	4.1

* Mass-transition belongs to in-source fragment of metabolite III

** High RSD attributed to insufficient sensitivity

*** High RSD attributed to high baseline at this mass transition (fragment is common for phosphates and phosphonates)

Figure 6: *Chromatograms of onion (measured by Sciex 5500QTrap)*

	Blank onion 0.5g/mL	Recovery 0.005 mg/kg onion 0.5g/mL
Dimethoate carboxylic acid (Metabolite III) 215/141		
O-desmethyl dimethoate (Metabolite X) 214/104		
O-desmethyl omethoate (Metabolite XI) 198/104		
O-desmethyl isodimethoate (Metabolite XII) 214/166		
O-desmethyl omethoate carboxylic acid (Metabolite XX) 185/111		
O-desmethyl N-desmethyl omethoate (Metabolite XXIII) 184/90		

f) Pilot monitoring

In order to collect information about the residue situation of the metabolites in samples from the market, a total of 1778 samples were analyzed for dimethoate and omethoate using QuEChERS and for the six metabolites using QuPPe. The number of findings exceeding 1 µg/kg and 10µg/kg are shown in Table 10.

Table 10: Findings of dimethoate and omethoate and their metabolites in samples from the German market

Commodity Group	Samples analyzed	Dimethoate	Omethoate	Metabolite Code					
				III	X	XI	XII	XX	XXIII
		Number of findings Detected / ≥RL / ≥0.01 mg/kg							
Baby and infant foods	7	0	0	0	0	0	0	0	0
Beer and ingredients	3	0	0	0	0	0	0	0	0
Beverages, non-alcoholic	19	**1/1/0**	0	0	**4/3/0**	**4/2/0**	0	0	0
Cereals (incl. processed c.)	61	0	0	0	0	0	0	0	0
Coffee	3	0	0	0	0	0	0	0	0
Dry fruits and seeds	90	0	0	0	**3/3/1**	**2/2/1**	0	0	0
Food supplements	2	0	0	0	0	0	0	0	0
Fruit	572	**2/2/1**	**3/3/2**	0	**16/16/5**	**11/5/0**	0	**2/2/0**	0
Fruit, processed	39	0	**1/1/0**	0	**4/4/0**	**1/0/0**	0	0	0
Mushrooms	34	0	0	0	0	0	0	0	0
Mushrooms, processed	5	0	0	0	0	0	0	0	0
Potatoes & starchy veg.	23	0	0	0	0	0	0	0	0
Spices, seasonings	18	0	0	0	**1/1/0**	0	0	0	0
Tea and herbal infusions	9	0	0	0	0	0	0	0	0
Vegetables	775	**5/5/2**	**9/9/2**	0	**68/67/33**	**42/27/3**	0	**10/9/3**	0
Vegetables, processed	56	0	0	0	**2/2/0**	**1/1/0**	0	0	0
Wine and wine products	62	0	0	0	0	0	0	0	0
Total	**1778**	**8/8/3**	**13/13/4**	0	**98/96/39**	**61/37/4**	0	**12/11/3**	0

Among a total of 1778 market samples analysed, 98 (5.5%) were found to contain at least one of the 8 dimethoate-related compounds with 96 of them containing at least one of these compounds above the quantitative reporting limits (RLs), which were as follows for the main groups of raw commodities:

- Omethoate and metabolites X, XI, XII, XXIII: 0.001 mg/kg
- Dimethoate, and metabolite XX: 0.002 mg/kg;
- Metabolite III: 0.005 mg/kg

Dimethoate was quantified at levels ≥RL in only 8 samples (0.45 %), at levels >0.005 mg/kg in 4 samples (0.23 %), and at levels ≥0.01 mg/kg in 3 samples (0.17 %). The highest residue levels were found in kohlrabi (0.17 mg/kg), cherries (0.063 mg/kg) and radish (0.049 mg/kg).

Omethoate was quantified at levels ≥RL in 13 samples (0.73 %), at levels >0.005 mg/kg in 5 samples (0.28 %), and at levels ≥0.01 mg/kg in 4 samples (0.23 %). The highest residue levels were found in cherries (0.085 mg/kg), kohlrabi (0.021 mg/kg), cherries (0.020 mg/kg), and radish (0.015 mg/kg).

Metabolite X was the **most frequently found** among the 8 dimethoate-relevant compounds and was detected in 98 samples (5.5%). It was quantified at levels ≥RL in 95 samples (5.3 %), at levels ≥0.005 mg/kg in 51 samples (2,9 %) and at levels ≥0.01 mg/kg in 39 samples (2.2 %). The highest residue levels were found in kohlrabi (0.54 mg/kg), garlic (0.35 mg/kg), Brussel's sprouts (0.34 mg/kg), dried beans (0.26 mg/kg) and onions (0.23 mg/kg).

Metabolite XI was the **second most frequently found** among the dimethoate-relevant compounds but mostly present at low levels. It was detected in 61 samples (3.5 %). It was quantified at levels ≥RL in 37 samples (2.0 %), at levels ≥0.005 mg/kg in 7 samples (0.39 %) and at levels ≥0.01 mg/kg in only 4 samples (0.23 %).The highest residue levels were found in kohlrabi (0.035 mg/kg), broccoli (0.029 mg/kg), Brussel's sprouts (0.022 mg/kg) and dried beans (0.013 mg/kg).

Metabolite XX was detected in 12 samples (0.68 %). It was quantified at levels ≥RL in 11 samples (0.62 %), at levels ≥0.005 mg/kg in 6 samples (0.34 %) and at levels ≥0.01 mg/kg in 3 samples (0.17 %). The highest residue levels were found in radish (0.28 mg/kg), broccoli (0.011 mg/kg) and kohlrabi (0.011 mg/kg).

Metabolites III, XII and XXIII were not encountered in any of the samples at levels ≥RL.

There were 3 cases where dimethoate but no omethoate was encountered and 8 cases where omethoate was encountered but no dimethoate. Nevertheless, in all 13 cases where omethoate was encountered, either dimethoate or dimethoate-characteristic metabolites (X, XX) were encountered, which **strongly indicates that omethoate was not sprayed in the production of these samples**.

Out of the 98 samples containing dimethoate-related residues only 16 samples (16 %) were found to contain quantifiable residues of the current markers dimethoate or omethoate. Residues of dimethoate or omethoate at levels ≥0.005 mg/kg were only encountered in 6 of those 98 samples (6 %) and residues ≥0.01 mg/kg in only 4 samples (4.1 %). In 82 of these samples (84 %) no residues of dimethoate or omethoate were found. This raises the question whether any of the other metabolites would be a suitable marker for residue controls.

Taking the 98 samples containing dimethoate-relevant residues as a basis for calculations, and assuming a routine LOQ of 0.005 mg/kg; dimethoate and omethoate combined would be suitable markers for only 6 (6 %) of those samples. In contrast, metabolite X would be a suitable marker for 51 samples (52 %) at an LOQ of 0.005 mg/kg. **Metabolite X was actually the only of the tested compounds that was encountered in all samples where any of the other dimethoate-relevant compounds was detected**.

Among the 92 samples not containing any omethoate or dimethoate residues at levels ≥0.005 mg/kg there were 45 cases (46 % of 98) where metabolite X was detected at levels ≥0.005 mg/kg and 34 cases (35 % of 98) where it was detected at levels ≥0.01 mg/kg. Based on these figures **it makes sense to consider the possibility of introducing metabolite X (O-desmethyl dimethoate) as an additional marker for monitoring the proper use of dimethoate in agriculture**.

Metabolite X gives a very strong indication for the use of dimethoate but, looking at the residue profile of the analyzed samples, its absence might also provide some useful information. Providing a sensitive analysis (e.g. LOQ at 0.001 mg/kg), **the non-detection of metabolite X in a sample in combination with the presence of omethoate and/or O-desmethyl omethoate (XI) seems to point towards the use of omethoate**, which is not approved within the EU. But this assumption will need to be experimentally verified.

Metabolites III, XII and XXIII seem to be of no use as residue markers as they were not found at quantifiable levels in any of the samples tested.

Metabolites XI and XX seem to be of limited use as residue markers. All 7 samples containing residues of **metabolite XI** at levels ≥0.005 mg/kg also contained metabolite X at levels >0.01 mg/kg. Dimethoate and omethoate were present at levels ≥0.005 mg/kg in 2 of those 7 samples. Similarly, all 6 samples containing residues of **metabolite XX** at levels ≥0.005 mg/kg also contained metabolite X at levels ≥0.01 mg/kg. Dimethoate and omethoate were both present at levels ≥0.01 mg/kg in 2 out of those 6 samples.

Table 11 gives an overview of the findings of dimethoate, omethoate and the metabolites X, XI and XX in fruits and vegetables.

Table 11: Findings of dimethoate, omethoate and their metabolites in fruits and vegetables from the market

Commodity group	No. Samples	Dimethoate	Omethoate	Metabolite X	Metabolite XI	Metabolite XX
		Number of findings (Detected / ≥RL / ≥0.01 mg/kg)				
FRUITS						
Berries	118	0	0	1/1/**1** **(grapes)**	1/1/0	0
Citrus fruits	88	0	0	6/6/0	5/0/0	0
Exotic fruits	114	0	0	3/3/**1** **(figs)**	0	0
Pome fruits	131	0	0	0	0	0
Stone fruits	121	2/2/**1** **(cherries)**	**3/3/2** **(2x cherries)**	6/6/**4** **(3x cherries, 1x plums)**	5/4/0	2/2/0
VEGETABLES						
Fruiting vegetables	320	0	0	4/3/0	0	0
Leafy vegetables	300	2/2/0	3/3/0	39/24/**19** **(11x brassica, 8x allium)**	23/15/**1** **(Brussel's spr.)**	4/4/0
Root vegetables	79	2/2/**1** **(radishes)**	5/4/**1** **(radishes)**	13/13/**5** **(2x beet, 2x brassica, celeriac)**	8/4/0	**2/2/1** **(radish)**
Sprout vegetables	75	1/1/**1** **(kohlrabi)**	1/1/**1** **(kohlrabi)**	12/12/**9** **(6x allium, 3x brassica)**	12/9/**2** **(2x brassica)**	**4/4/2** **(2x brassica)**
Vegetable mixtures	1	0	0	0	0	0

Table 12 gives an overview of the residue levels of metabolite X (O-desmethyl dimethoate), which was encountered in all 98 samples in which dimethoate-relevant compounds were detected.

Table 12: Findings of O-desmethyl dimethoate (metabolite X)

Group	Commodity	Analyzed	Positive	Mean of positives (mg/kg)	Median of positives (mg/kg)	Residue levels encountered (mg/kg)
Processed food	Bean	42	1			**0.262**
	Bell pepper, frozen	2	1			0.006
	Blackberry, frozen	8	1			0.001
	Juices	31	4	0.001	<0.001	<0.001; <0.001; 0.001; 0.001
	Lentil	28	1			0.004
	Pinto bean	5	1			0.004
	Raspberry, frozen	29	3	0.001	0.001	0.001; 0.001; 0.002
	Olives preserved		1			0.006
	Spices	62	1			0.005
Berries	Grapes	1	1			0.01
Citrus fruits	Clementine	50	4	0.003	0.003	0.002; 0.002; 0.004; 0.006
	Grapefruit	39	1			0.002
	Satsuma	7	1			0.002
Exotic fruits	Figs	16	2	0.006		0.002; 0.01
	Mango	64	1			0.002
Fruiting vegetables	Bell peppers	205	3	0.002	0.002	<0.001; 0.002; 0.003
	Green beans	103	1			0.005
Leafy vegetables	Borecole	28	1			**0.021**
	Brussels sprout	45	5	0.11	0.03	0.001; 0.006; **0.030; 0.17; 0.34**
	Chinese cabbage	15	1			0.004
	Chives	17	1			0.007
	Leek	29	10	0.027	0.016	0.001; 0.001; 0.003; 0.004; 0.007; **0.025; 0.030; 0.049; 0.070; 0.077**
	Parsley	70	1			0.002
	Red cabbage	16	5	0.038	0.017	0.006; 0.007; **0.017; 0.028; 0.13**
	Savoy cabbage	7	1			**0.023**
	Spring onion	27	6	0.046	0.012	0.001; 0.001; 0.003; **0.021; 0.067; 0.183**
	White cabbage	43	8	0.008	0.004	0.001; 0.001; 0.002; 0.003; 0.004; **0.015; 0.017; 0.023**
Root vegetables	Beetroots	22	2	0.017		**0.017; 0.017**
	Carrots	56	4	0.004	0.005	0.001; 0.005; 0.005; 0.005
	Celeriac	23	3	0.017	0.003	0.002; 0.003; **0.047**
	Radish	19	2	0.017		0.002; **0.032**
	Radish, small ~	60	2	0.034		0.006; **0.062**
Sprout vegetables	Broccoli	42	1			0.014
	Cauliflower	21	2	0.01		0.001; **0.019**
	Garlic	2	1			**0.349**
	Kohlrabi	34	2	0.274		0.006; 0541
	Onion	39	6	0.089	0.080	0.003; 0.042; 0.073; 0.086; **0.010; 0.226**
Stone fruits	Avocado	34	1			0.003
	Cherry	58	4	0.033	0.033	0.002; **0.020; 0.042; 0.066**
	Plum	115	1			**0.042**
Total of positiv samples			**98**	**0.037**		

g) Matrix effects and re-analysis of selected samples via standard additions approach

As QuPPe extracts are used to determine the 6 metabolites, matrix effects were to be expected. Matrix effects in LC-MS/MS strongly depend on sample type and the chromatographic separation of analytes and matrix components. In the case of the Hypercarb column the condition of the column was also a factor to be considered as it influences the separation performance. Unfortunately, matrix effects could not be equalized though isotope labelled internal standard of each of the 6 metabolites as none of these were available. In such cases matrix-matched calibration or calibration based on the standard additions approach can be employed.

14 of the routinely analyzed samples, that were found to contain residues of dimethoate-relevant metabolites, were selected for re-analysis via the standard addition approach. In parallel, the residue levels were also determined via solvent-based calibration in order to get an impression of the errors that would be associated with this more simple and efficient.

The standard addition experiments conducted concerned the metabolites X (O-desmethyl dimethoate), XI (O-desmethyl omethoate and XX (O-desmethyl omethoate carboxylic acid). For each approach 10 g of the respective homogenized sample materials were used. Considering the results of the routine analysis approach standard additions were selected to be at levels 2- and 5-fold the expected analyte amount in the sample, and were done onto analytical portions prior to analysis. Furthermore, a non-spiked sample portion was also extracted and was used both for the standard additions calculations as well as for quantification via external solvent-based calibration to obtain an estimate of the matrix effects and also to estimate errors of the first routine analysis. Cyanuric acid $^{13}C_3$ was used as isotopically labeled internal standard for volumetric corrections within the standard additions approach only. Table 13 shows a) the results derived based on a solvent-based calibration curve; and b) the results obtained via standard addition.

None of the metabolites not found in the first analysis were found in the second extraction, but for many samples a decrease of desmethyl-dimethoate (X) along with an increase of desmethyl-omethoate (XI) was observed, which indicates an oxidative transformation. It is suspected that this was a result of the long storage period of the homogenates in the freezer. These effects as well as possible transformations during extract storage should be considered in future analyses.

Fortunately, the differences in the concentrations between the standard additions approach and solvent-based calibration were in almost all cases minor for the relevant compounds. Only in case of Brussel's sprouts a significant deviation was observed.

Table 13: Results of the standard addition approach compared to the result obtained from a solvent calibration

Year	Commodity	Parent conc. (from initial analysis) (mg/kg)	Metabolite code	Result via solvent-based external calibration*	Result via standard addition approach
2017	**Broccoli**	Omethoate: n.d. Dimethoate: n.d.	**X**	**0.003**	**0.004**
			XI	**0.016**	**0.015**
			XX	**0.003**	**0.003**
2017	**Sweet Cherry**	**Omethoate: 0.085** Dimethoate: n.d.	**X**	**0.022**	**0.019**
			XI	**0.019**	**0.019**
			XX	**0.004**	**0.004**
2017	**Red Cabbage**	Omethoate: n.d. Dimethoate: n.d.	**X**	**0.018**	**0.019**
			XI	**0.038**	**0.024**
			XX	**n.n.**	**n/a**
2017	**Spring Onion**	Omethoate: n.d. Dimethoate: n.d.	**X**	**0.090**	**0.087**
			XI	**0.001**	**0.001**
			XX	**n.n.**	**n/a**
2017	**Beetroot**	**Omethoate: 0.001** Dimethoate: n.d.	**X**	**0.020**	**0.018**
			XI	**n.b. < 0,001**	**n.b. < 0,001**
			XX	**n.n.**	**n/a**
2018	**Garlic**	Omethoate: n.d. Dimethoate: n.d.	**X**	**0.57** (diluted 10x)	**0.59** (diluted 10x)
			XI	**0.003**	**0.002**
			XX	**0.008**	**0.006**
2018	**Radish**	Omethoate: n.d. Dimethoate: n.d.	**X**	**0.028**	**0.027**
			XI	**0.002**	**0.001**
			XX	**0.011**	**0.009**
2018	**Sweet Cherry**	**Omethoate: 0.020** **Dimethoate: 0.063**	**X**	**0.012**	**0.012**
			XI	**0.006**	**0.006**
			XX	**n.n.**	**n/a**
2018	**Cauliflower**	Omethoate: n.d. Dimethoate: n.d.	**X**	**0.002**	**0.002**
			XI	**0.006**	**n/a**
			XX	**n.n.**	**n/a**
2018	**Leek**	Omethoate: n.d. Dimethoate: n.d.	**X**	**0.077**	**0.076**
			XI	**n.b. < 0,001**	**n/a**
			XX	**n.n.**	**n/a**
2018	**White Cabbage**	Omethoate: n.d. Dimethoate: n.d.	**X**	**0.002**	**0.002**
			XI	**0.012**	**0.009**
			XX	**n.n.**	**n/a**
2018	**Brussel's sprouts**	**Omethoate: 0.002** Dimethoate: n.d.	**X**	**0.086** (diluted 10x)	**0.075** (diluted 10x)
			XI	**0.078**	**0.10**
			XX	**n.n.**	**n/a**
2018	**Kale**	**Omethoate: 0.008** **Dimethoate: 0.002**	**X**	**0.0050**	**0.0050**
			XI	**0.003**	**0.002**
			XX	**n.n.**	**n/a**
2018	**Onion**	Omethoate: n.d. Dimethoate: n.d.	**X**	**0.051**	**0.058**
			XI	**0.001**	**n/a**
			XX	**n.n.**	**n/a**

* calculation without internal standard, calibration range 0.001-0.2 µg/mL

Conclusions

An analytical method for 6 dimethoate metabolites (III, X, XI, XII, XX and XXIII) was developed and the residue situation was studied based on the analysis of 1778 market samples. Only one of those 6 very polar metabolites included in the study showed good recoveries by the citrate-buffered QuEChERS method, thus the QuPPe method was used. Due to the lack of suitable isotope labelled internal standards, validation was based on matrix-matched calibration. Fortunately matrix effects were typically small. The method was used for the analysis of market samples to assess the residue situation of each metabolite in various crops. 98 out of 1778 samples (5.5% overall) were found to contain dimethoate-related residues. Their residue profiles suggested that in all cases dimethoate was the active substance employed in the field. Metabolite X was encountered in all these 98 samples, in 39 cases thereof at levels ≥ 0.01 mg/kg. In contrast, dimethoate and omethoate were encountered at levels ≥ 0.01 mg/kg in only 3 and 4 samples respectively. Based on these results **the inclusion of Metabolite X (O-desmethyl dimethoate) as an additional marker for controlling proper use of dimethoate should be considered**. Metabolites XI was also very often encountered (61 % of samples) but typically at very low levels and only 4 samples were found to contain it at levels ≥ 0.01 mg/kg. Metabolite XX was found in 12 % of the samples with 3 of them containing it at levels ≥ 0.01 mg/kg. The other three metabolites (III, XII, XIII), were not encountered in any sample. This information should hopefully be of use in future risk assessment and risk management actions on dimethoate.

Literature

[1] Chemicalize.org
[2] The pesticide manual, 15th edition, BCPC 2009
[3] CEN method EN 15662 (citrate buffered), see also brief description under www.quechers.de
[4] EFSA 2016, Reasoned opinion on the prioritized review of the existing maximum residue levels for dimethoate and omethoate according to Art. 43 of Reg. (EC) No 396/2005. EFSA Journal 2016;14(11):4647)
[5] EFSA 2016, Assessment of the human health through the pesticide active substance for dimethoate and its metabolites in food. EFSA Journal 2016;14(4):4461
[6] EFSA 2018, Peer review of the pesticide risk assessment of the active substance dimethoate, EFSA Journal 2018;16(10):5454

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History

Action	When	Document Version
Initial Experiments	July 2017 and January-June 2018	
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