EURL-SRM - Analytical Observations Report

concerning the following…

- **Compound(s)**: Captan, Folpet, Phthalimide, Tetrahydrophthalimide
- **Commodities**: Fruit and vegetables, cereals
- **Extraction Method(s)**: Citrate buffered QuEChERS,
- **Instrumental analysis**: LC-MS/MS

Analysis of Captan, Folpet and their respective metabolites Phthalimide and Tetrahydrophthalimide via LC-MS/MS either directly or following hydrolysis

Version 1 (last update: 28.06.2019)

Background information and previous work:

Analyzing captan and folpet as such is quite challenging, as these compounds tend to degrade during sample comminution, extraction, cleanup as well as during the storage of homogenates, sample extracts and standard solutions. In all cases keeping pH and temperatures low reduces degradation. dSPE cleanup with PSA as sorbent is particularly critical, as the contact of QuEChERS raw extracts with PSA causes the pH of the extracts to raise from ~4-4.5 to ~7-9, which results in losses of alkali-labile compounds. This is why QuEChERS extracts are better immediately re-acidified following dSPE clean-up and why dSPE is often skipped when targeting such compounds.

Using standard measurement conditions, captan and folpet exhibit poor sensitivity in LC-MS/MS, so laboratories typically analyze these compounds using GC-techniques. This is quite tricky, as captan and folpet show a tendency to thermally degrade within the hot GC-liner, with tetrahydrophthalimide (THPI) and phthalimide (PI) showing up as additional peaks. This thermal decomposition very much depends on the condition of the GC-system (in particular the surface activity of the liner and the first part of the column), which deteriorates as more and more matrix extracts are injected to the system. The active sites on the liner surface interact with the labile parent compounds, quasi catalyzing their thermal breakdown. At the same time, co-extracted matrix components, which are also present in the extracts, act competitively by masking these active sites, thus reducing decomposition rates. Where sample extracts and calibration standards differ significantly in their protection effect massive quantification errors may occur. These effects are typically addressed by the use of matrix-matched calibrations (including calibrations via standard

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addition and procedural calibrations) or analyte protectants (APs)[1], which act as artificial matrices. Alternatively, isotope-labelled internal standards (ILISs) are employed so far available. Figure 1 summarizes analytical steps within the QuEChERS procedure where special attention is indicated to minimize the degradation losses of captan and folpet.

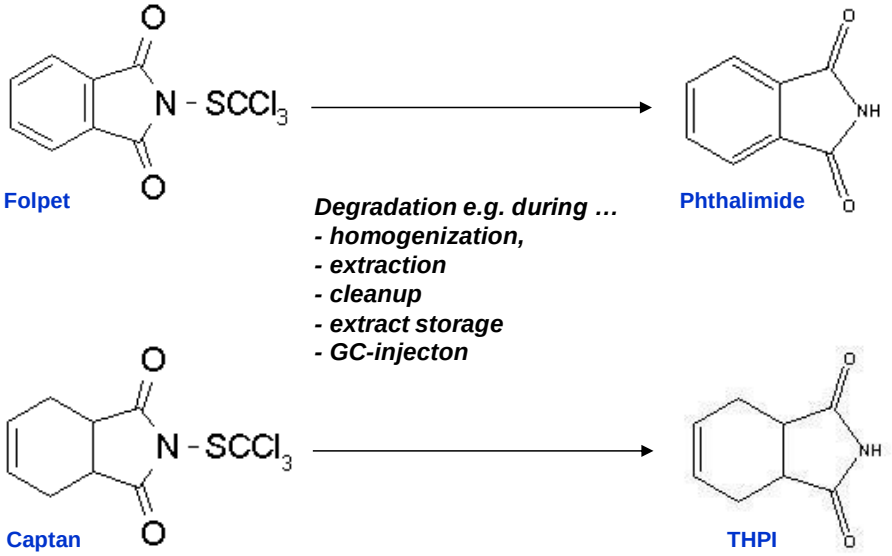


Figure 1: *Degradation of captan and folpet to THPI and PI and compilation of critical steps during analysis*

In 2016, the legal residue definitions for captan and folpet were amended, now additionally entailing their respective metabolites/degradants THPI and PI[2,3], which were identified as important residue markers in primary and processed crops. This regulatory change has solved some problems for the labs (e.g. the concern that captan and folpet are transformed to THPI and PI during sample comminution, extraction and cleanup), but has also put them in front a new analytical challenges when GC-techniques are used for analysis. In the case of the parent compounds accurate GC-quantification can be straightforwardly accomplished by compensating matrix-effects (see above), but the quantification of THPI and PI is more challenging as the signals obtained originate from two different sources. Part of the THPI and PI was originally present in the sample extracts and an additional part is formed through degradation of captan and folpet within the GC system. A simple addition of the already corrected GC-result of the parent and the GC-result of the degradation product (expressed as parent) thus typically leads to overestimated results.

A procedure, in which those parts of THPI and PI formed from parent breakdown during GC-injection are deducted from the respective detected signals, has been elaborated by the EURL-SRM and published in an analytical observations report (**link**). This approach can deliver sufficiently accurate results but it has limitations when it comes to routine applicability. Issues concerning extraction and cleanup of captan and folpet are also discussed in this document.

To circumvent the problems associated with direct GC analysis, the following two alternative procedures were considered worthwhile checking:

a) **Direct analysis of parents and degradation products via LC-MS/MS:** This approach is straightforward and theoretically less prone to errors. Ideally, all four analytes would be

[1] **http://www.eurl-pesticides.eu/library/docs/srm/EURL_Observation-APs.pdf**
[2] COMMISSION REGULATION (EU) 2016/452 of 29 March 2016 (dealing with captan)
[3] COMMISSION REGULATION (EU) 2016/156 of 18 January 2016 (dealing with folpet)

incorporated into multiresidue methods as this is more efficient. If this is not feasible (e.g. due to the need to employ ESI-ion-source settings that do not serve in a multiresidue approach, or due the need to employ APCI, which is typically not used in routine multiresidue analysis) separate methods would need to be employed, that provide the necessary sensitivity. These separate methods should be, however, triggered by a detection of a marker compound by a routinely employed method (e.g. detection of captan by a GC-based method).

b) **Direct analysis of degradants via LC-MS/MS and of parents via GC:** This would be an alternative approach to a) in case captan and folpet cannot be sensitively analyzed via LC-MS/MS. This approach would circumvent the error-prone GC-analysis of THPI and PI, but still measures would need to be taken to ensure accurate GC-quantification of captan and folpet (see above).

c) **Quantitative conversion of parents to THPI and PI followed by LC-MS/MS measurement (using THPI and PI for calibration):** Ideally, the conversion would be triggered by a positive screening detection of a parent compound (captan or folpet) and/or a degradant (THPI or PI). If feasible, the conversion would take place within the QuEChERS extracts (e.g. within autosampler vials) rather than during or prior to the extraction step, as this would obviate the need for a second extraction. An inherent drawback of this conversion-approach is that it does not provide differentiated information on the concentrations of the parents and the degradation products, which would be of use for risk assessment. A separate quantification of the parent and the calculation of the metabolite concentration by deducting the parent concentration from the post-hydrolysis sum would expectedly suffer from increased uncertainty.

   If the degradation products (already present or derived from hydrolysis) cannot be analyzed by LC-MS/MS with the required sensitivity, another **alternative** would be the **measurement by GC-MS/MS following conversion to THPI and PI.** As the parent molecules would not be present any more after hydrolysis, the analysis of the degradants should be expectedly straightforward. The abovementioned limitation in risk assessment, would also apply here. An additional point of concern, inherent to GC-analyses, is the inability to distinguish between PI originally present in the samples and PI formed during GC-injection from other sources (e.g. from phthalic acid and nitrogen containing compounds – see also Relana position paper[4]). Phthalic acid is ubiquitous, but it is also formed during the metabolism of folpet. Irrespective of its origin, phthalic acid is not part of the residue definition and its transformation to PI should thus be minimized. The also ubiquitous phthalates as well as phthalanhydrite[5] would possibly hydrolyze to phthalic acid during the hydrolysis step.

Regarding the above approaches it should be highlighted however, that even if only LC is used, it is still not possible to distinguish between the PI levels originating from folpet and the PI levels originating from elsewhere (e.g. formed as processing artefacts when phthalates or phthalanhydrite react with nitrogen-containing compounds during the drying of food products). It should be also considered, that at least in theory, PI may also originate from phosmet or ditalimphos, which are also reported to metabolize to PI. In this context, it is further worthwhile noticing, that THPI is not specific to captan either, as it is also formed from captafol.

[4] Relana (2016/07/22): **http://www.relana-online.de/wp-content/uploads/2016/07/PP_16-03_Folpet-PI_vers20160722.pdf**
[5] Phthalanhydrite, is also widely distributed but it may be also formed from phthalic acid during food processing (e.g. during they drying of herbs and teas). Phthalic acid is formed from the ubiquitous phthalates.

Brief trials in 2016-17, for direct LC-MS/MS analysis of THPI and PI and of captan and folpet (as such or as in-source fragments), were rather dissatisfying in terms of sensitivity. However, as GC-analysis suffers from difficulties to distinguish between the parts of THPI and PI originally present in the sample and those parts generated within the GC-injector, it was decided to give LC-MS/MS measurement another try in the hope of achieving the required sensitivity. High-end instrumentation and various ionization modes were to be employed in this new attempt.

Compound details:

Table 1: General information on captan

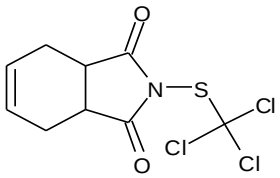
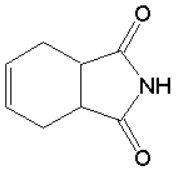
Captan (CAS: 133-06-2), IUPAC: 2-(trichloromethylsulfanyl)-3a,4,7,7a-tetrahydroisoindole-1,3-dione		
Parameter	**Value**	**Notes**
Molecular Mass	300.578 g/mol	
Pka	Does not dissociate	
LogPow	2.57 at pH7	
Water solubility	4.9 mg L-1 at 20 °C	
Stability	A hydrolysis study demonstrated that captan is not stable under the representative processing conditions; captan is almost completely converted into THPI (EFSA Reasoned Opinion 2014). Very sensitive to degradation at high pH and thermally labile (degrades to THPI and further products)	
Hydrolysis rates in water (DT50)	pH4 at 25°C: 12 h; at 40°C: 1.7 h pH7 at 25°C: 2.6 h; at 40°C: 0.5 h pH9 at 25°C or 40°C: too fast to measure	From JMPR Report 2000[6] (referring to Yaron, 1985)
Residue definition EU	Food of plant origin (except wine grapes), honey: Captan (Sum of captan and THPI, expressed as captan); Wine grapes: Captan; Food of animal origin except honey: Sum of THPI, 3-OH THPI and 5-OH THPI, expressed as captan;	
Approved in…	AT, BE, BG, CY, CZ, DE, EE, EL, ES, FR, HR, HU, IE, IT, LT, LU, LV, NL, PL, PT, RO, SI, SK, UK	
ADI / ARfD	0.1 mg/kg bw per d, 0.3 mg/kg bw (EFSA)	

Table 2: General information on tetrahydrophthalimide (THPI)

THPI (CAS: 85-40-5), IUPAC: 1,2,3,6-tetrahydrophthalimide; 3a,4,7,7a-tetrahydro-1H-isoindole-1,3(2H)-dione , $C_8H_9NO_2$			
Parameter	**Value**	**Notes**	
Molecular Mass	151,165 g/mol	Conversion factor from/to parent 0.503 /1.998	
Pka	10.52	slightly acidic	
LogPow	**pH dependent but constant up to pH 9** 0.58 at pH 1-9 (0.46 at pH 10	Calculated by chemicalize.org	
Water solubility	12.2 g/l at 20 ± 0.5 °C at pH 3.4	ECHA	
Stability	Hydrolytically quite stable		
Hydrolysis rates in water (DT50)	150 d pH7 / 20°C (JMPR Report 2000) … hydrolytically stable under conditions representing pasteurisation, backing, boiling/brewing, … slightly unstable under sterilisation conditions (EFSA Reasoned Opinion 2014[7])		
Residue definition EU	See captan		
Approved in…	See captan		
ADI / ARfD	"… THPI, 3-OH THPI and 5-OH THPI were demonstrated to be of lower toxicity compared to captan but data were not sufficient to derive specific reference values … it was concluded that the reference values for captan would also apply…" (EFSA Reasoned Opinion 2014)		
Other Notes	THPI is not specific to captan. It is also a degradant of the pesticide captafol.		

[6] JMPR (2000) : http://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/JMPR/Evaluation00/7CAPTAN.pdf
[7] EFSA Journal 2014;12(4):3663 [55 pp.]. doi: 10.2903/j.efsa.2014.3663

Table 3: General information on folpet

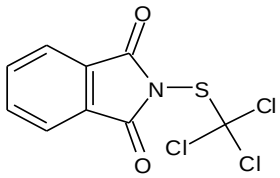
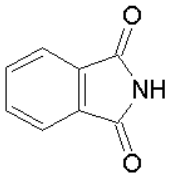
Folpet (CAS: 133-07-3), IUPAC: 2-(trichloromethylsulfanyl)isoindole-1,3-dione			
Parameter	**Value**	**Notes**	
Molecular Mass	296.546 g/mol		
Pka	Does not dissociate		
LogPow	3.02 at pH7 (intermediate to low polarity)		
Water solubility	0.8 mg L-1 at 25 °C		
Stability	Very sensitive to degradation at high pH and thermally labile (degrades to phthalimide and further products)		
Degradation rates	pH 5 at 25°C: 2.6 h pH 7 at 25 °C: 1.1 h pH 9 at 25 °C: 1.1 min	JMPR Report (1999)[8]	
	Comments *from EFSA (2017)*[9]: Folpet degrades predominantly ***to phthalimide,*** (pasteurization 92% AR; baking, brewing/boiling: 58% AR) with levels of **phthalic acid** increasing with temperature and pH (baking, brewing/boiling: 42.2% AR; sterilisation 81% AR)		
Residue definition EU	Folpet (sum of folpet and phtalimide, expressed as folpet) (R)		
Approved in…	AT, BE, BG, CY, CZ, DE, DK, EE, EL, ES, FR, HR, HU, IE, IT, LT, LU, LV, MT, NL, PL, PT, RO, SI, SK, UK		
ADI / ARfD	0.1 mg/kg bw per d, 0.2 mg/kg bw (EFSA)		

Table 4: General information on phthalimide (PI)

Phthalimide (PI) (CAS: 85-41-6), IUPAC: Phthalimide; $C_8H_5NO_2$			
Parameter	**Value**	**Notes**	
Molecular Mass	147,133 g/mol	Conversion factor from/to parent 0.495 /2.02	
Pka	8.4 slightly acidic	All calculated by Chemicalize.org	
LogPow	**pH dependent but constant up to pH 7** pH 1-7: 0.69 pH 9: 0 pH10: -0.75		
Water solubility	370 mg/l at 25°C	ECHA	
Stability	Hydrolysis products were ammonia and phthalic acid, formed via phthalamic acid as an intermediate		
Hydrolysis rates in water (DT_{50})	pH4 / 25°C: 115 d pH7 / 25°C: 56.7 h pH9 / 25°C: 1.1 h	From ECHA report (quoting OECD)	
Residue definition EU	Folpet (sum of folpet and phtalimide, expressed as folpet) (R)		
Approved in…	See folpet		
ADI / ARfD	*EFSA (2017):* The toxicological reference values of the parent apply to the metabolite phthalimide for the consumer risk assessment.		
Other Notes	Phthalimide is not specific to folpet. It is also degradant of the pesticides ditalimphos and phosmet. In presence of compounds with primary amino groups and preferably anhydric conditions it is also formed from phthalic acid and phthalic anhydride. This may explain the high presence of phthalimide in dry products, see also[10]. A formation of phthalimide from phthalic anhydride and phthalic acid in the hot GC-injector also takes place.		

[8] JMPR (1999): http://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/JMPR/Evaluation99/18Folpet.pdf
[9] Modification of the existing maximum residue levels for folpet in apples and pears; EFSA Journal 2017;15(10):5041
[10] Relana (2016/07/22): http://www.relana-online.de/wp-content/uploads/2016/07/PP_16-03_Folpet-PI_vers20160722.pdf

Materials

Table 5: Sources of analytical standards

Substance	Exemplary Sources
Captan	Dr. Ehrenstorfer (LGC Standards)
Folpet	HPC
Tetrahydrophthalimide (cis-1,2,3,6-Terahydrophthalimide)	Dr. Ehrenstorfer (LGC Standards)
Phthalimide	HPC
Captan D_6	Medical Isotopes INC Dr. Ehrenstorfer LGC Standards (solution)
Folpet D_4	Dr. Ehrenstorfer (LGC Standards)
Tetrahydrophthalimide D_6	TRC
Phthalimide D_4	TRC
Propyzamide D_3	CDN Isotopes
Chlorpyrifos D_{10}	Dr. Ehrenstorfer (LGC Standards)
BNPU (Nicarbazin)	Sigma

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

Experimental work

LC-MS/MS experiments were conducted both in the APCI and the ESI mode.

LC-conditions

Given the in-source fragmentation of captan to THPI and of folpet to PI, as well as the in-source fragmentation of THPI (and of captan via THPI) to PI (see below), it is important to ensure a full separation of all 4 components.

A 100 mm BEH C_{18} column (1.7 µm particle size) was used within this study. In a first step, the ESI gradient was optimized having in mind the development of a method involving pos/neg polarity switching (see below). Given the importance of ammonium adducts as quasimolecular precursior ions for captan and folpet an ammonium formate buffer was the first choice. Switching from 5 to 1 mmol ammonium formate had little influence on the signals of captan and folpet (ESI-pos mode) but a considerable increase of the THPI and PI signals (ESI-neg mode) was noticed.

For APCI mode the use of acetic acid proved more suitable than ammonium formate. A flow rate of 0.35 mL/min was considered sufficient to build-up a stable APCI reactant gas, and low enough to avoid problems with overpressure in the system (limit 1000 bar).

LC-instrument details are given in *Table 6*.

Table 6: LC and Ion-source instrumentation settings

LC		Waters Acquity UPLC I-Class		
MS/MS		SCIEX API 5500 Q-Trap		
Column		Waters BEH C_{18} 2.1x 100 mm 1.7 µm		
Pre-column		Waters BEH C_{18} 2.1x 5 mm 1.7 µm		
APCI specific	**Corona needle discharge current** (NC)	-3 µA		
	APCI evaporator probe temperature	450 °C as a generic setting (but varied in some experim.)		
	Mobile Phase	A: 0.01 % acetic acid in water (with 5 % methanol), B: 0.01 % acetic acid in methanol		
ESI specific	**Source temperature**	450 °C as a generic setting (but varied in some experim.)		
	Mobile Phase	A: 1 mmol NH_4-formate water (with 5 % methanol), B: 1 mmol NH_4-formate in methanol		
Gradient		**Time** (min)	**Mobile Phase A** (%)	**Flow (mL/min)**
		0	95	0.35
		3	10	0.35
		6	10	0.35
		6.1	95	0.35
		10	95	0.35
Flow rate		0.35 mL/min		
Injection volume		2 or 5 µL		
Column temperature		40°C		

Employing atmospheric pressure chemical ionization (APCI)

APCI is an LC-MS/MS ionization technique, in which the LC-eluent is nebulized and passed through a heated ceramic tube in order to vaporize completely. A corona discharge needle, which is placed close to the exit of the tube and which has a large potential difference to the curtain plate, induces the ionization of gas molecules. The ionized gas molecules will then react with other gas and solvent molecules resulting in a multitude of charged species within the gas phase. Through collisions with the analyte molecules these are ionized through proton or electron transfer reactions. The analyte ions formed are then guided to the MS. APCI is generally regarded as being a soft ionization technique, where little fragmentation of the ionized molecules takes place within the ion-source. Thermal fragmentation can, however, still occur within the heated ceramic probe. The probe-temperature should be high enough to ensure full evaporation of the eluent, but it can also influence the decomposition of susceptible analytes, especially while these are dissolved in the not yet fully evaporated eluent. Eluent composition and flow-rate, which influence evaporation speed, will thus also influence decomposition.

In flow Injection Analysis (FIA) experiments in the **APCI (pos) mode** no useful precursor ions, neither for THPI and PI nor for captan and folpet could be observed in the scan chromatograms. This is different from what was observed in ESI (pos) mode, where captan and folpet showed useful $[M+NH_4]^+$ and at least captan also useful $[M+H]^+$ ions. It was assumed that this may be related to the extensive degradation of captan and folpet within the hot APCI-interface probe prior to ionization. In the **APCI (neg) mode**, the injection of THPI and PI resulted in the formation of $[THPI-H]^-$ and $[PI-H]^-$ as base-peaks. These two were thus used as precursor ions for MRM mass transitions. $[THPI-H]^-$ and $[PI-H]^-$ were also predominant in FIA experiments of captan and folpet, which suggests an extensive degradation of captan and folpet within the APCI interface. The $[M-H]^-$ ion was observed in the case

of captan at a very low intensity, but not in the case of folpet. This is an indication that captan is slightly more resistant to the harsh conditions within the APCI vaporizer probe than folpet. MRM transitions starting with $[M-H]^-$ were thus only elaborated for captan. The optimized APCI (neg) MS/MS-settings for the various mass-transitions of captan, folpet, THPI, and PI are compiled in *Table 7*.

Table 7: *MRM details for Captan, Folpet, PI, THPI using API 5500 QTrap in the APCI (neg.) mode*

Compound	Ranking	Q 1 (m/z)	Q 3 (m/z)	DP (V)	CE (V)	CXP (V)
Captan	1	150*	96	-70	-28	-9
	2	150*	42	-70	-76	-7
	3	298	35	-65	-42	-5
	3	300	35	-65	-38	-15
	4	298	146	-65	-38	-11
	4	298	148	-65	-20	-9
	4	300	148	-65	-22	-11
Folpet	1	146*	42	-165	-50	-19
	2**	146*	146	-165	-5	-10
	3	178*	146	-115	-24	-11
	4	178*	42	-115	-70	-5
Tetrahydrophthalimide	1	150	96	-70	-28	-9
	2	150	42	-70	-76	-7
Phthalimide	1	146	42	-110	-52	-7
	2**	146	146	-110	-5	-10
BNPU (ISTD)	1	301	137	-65	-16	-7

* In-source fragments of captan or folpet

**In the case of folpet 146/146 was added as there was no other sufficiently intensive MRM-transition of the interface fragment. 146/146 is more intensive than 146/42 but potentially more interfered

Several pre-experiments were conducted to check the impact of various parameters on the ionization behavior of the four compounds. In these experiments, parents and degradation products were injected separately.

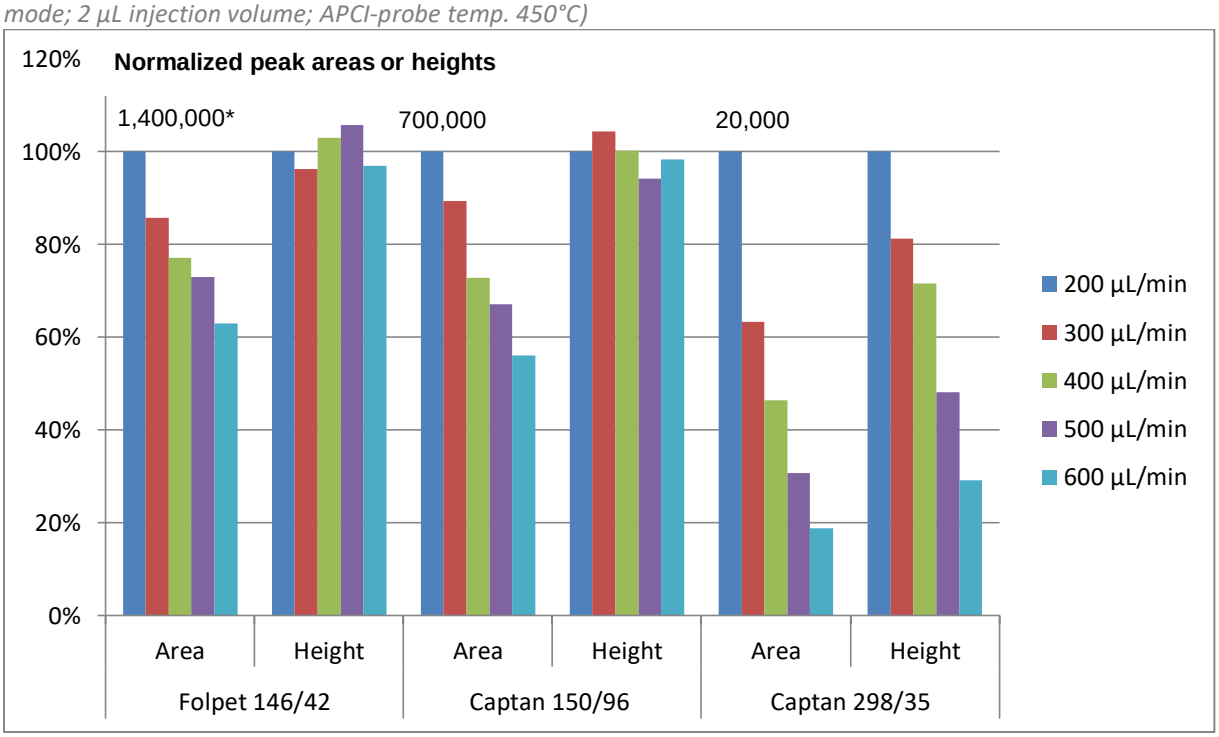
The **influence of NC** was tested at -3 and -5 µA but practically no differences in the signal intensities were observed. The lower value (-3 µA) was thus taken for further experiments.

The **impact of APCI-vaporizer-probe temperatures** was tested at three settings (300°C, 450°C and 550°C), keeping the NC and the eluent flow rate constant (-3 µA / 0.35 ml/min). Between 450°C and 550°C the differences in the signal intensities were marginal, whereas at 300°C all tested transitions showed a signal drop most strongly affecting the MRM transition of the quasimolecular ion (298/35). This seems paradox, as one would expect higher yields of the quasimolecular ion as temperature drops. But this effect may be a due to a delayed eluent vaporization (compared to higher temperatures) and the resulting longer exposition of captan to a still high temperature while still in the liquid phase.

The **impact of the LC-flow rate** on the signal intensities of the various MRM-transitions was tested at 200, 300, 400, 500 and 600 µL/min. As the flow rate increased, the peak areas of the MRM-transitions of the in-source fragments (m/z 150/96 and 146/42) showed a moderate but steady drop (-40% between 200 and 600 µL/min). Interestingly the peak height remained relatively constant, which seems to be due the narrower peak widths at higher flow rates. In contrast, the MRM transition of the captan quasimolecular ion (m/z 298/35), showed a strong drop not only in peak area but also in height. Also here, the effect may be due to a delayed eluent evaporation at higher flow rates, and the prolonged exposition of the parent compounds to higher temperatures, while still in

the liquid phase. In any case, the peak area of captan at the MRM-transition 298/35 was very weak (ca. 35-fold lower than 150/96).

Figure 2: *Impact of eluent flow rate on peak areas of captan and folpet (Sciex API 5500QTrap, APCI-(neg) mode; 2 µL injection volume; APCI-probe temp. 450°C)*

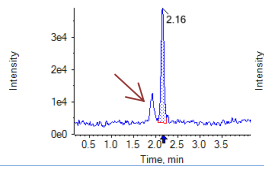
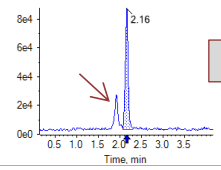
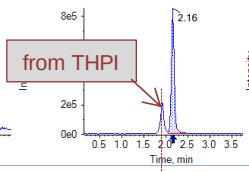
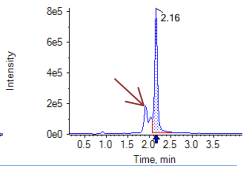
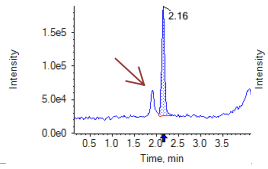
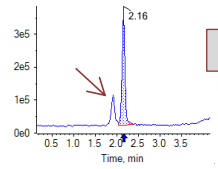
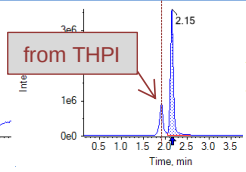
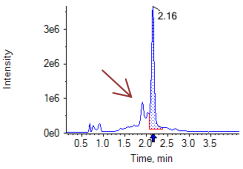
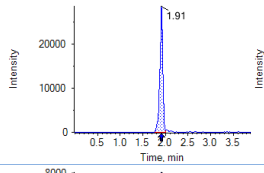
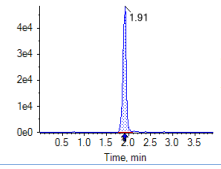
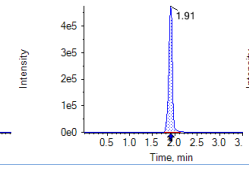
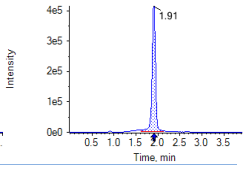
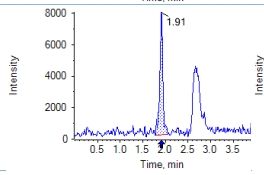
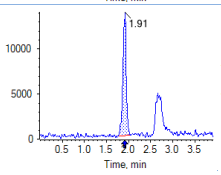
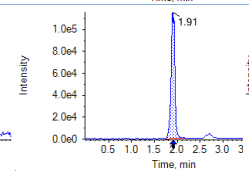
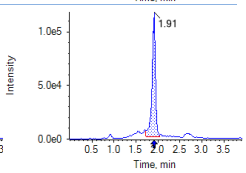


** Absolute peak areas at 200 µL flow rate set at 100%*

Figure 3 and Figure 4 show chromatograms obtained using the following settings: LC-flow rate 0.35 mL/min; APCI-probe temperature 450°C and NC -3 µA.

The chromatograms in Figure 3 refer to the injection of standard mixtures containing **THPI and PI** at 0.005; 0.01 and 0.1 µg/mL in solvent and at 0.1 µg/mL in apple extract. These concentrations have to be roughly doubled if expressed as captan or folpet equivalents. Interestingly, all MRM signal traces chosen for PI (and for folpet following in-source fragmentation) show an additional signal at the RT of THPI, which suggests that **within the APCI-ion source THPI is to some extend dehydrogenated to the aromatic and thus energetically more favorable phthalimide**. This behavior increases the **risk of false positive results** as THPI and PI elute close to each other. In previous experiments involving exposure of captan and THPI to high temperatures (e.g. hydrolysis reactions in GC-vials, GC-analyses as well as LC-MS/MS analyses in the ESI (neg) mode), this dehydrogenation reaction has not been observed. It seems thus more likely that this reaction (which possibly involves electron transfers and hydrogen eliminations) takes place within the APCI ion-source rather than in the heated probe.

Figure 3: Chromatograms of PI and THPI obtained from the injection of differently concentrated standard mixtures (Sciex API 5500QTrap, APCI-neg, 2 µL injection volume; APCI-probe temp. 450°C)

Mass Transition	Solvent-based Standard* 0,005 µg/mL	Solvent-based Standard* 0,01 µg/mL	Solvent-based Standard* 0,1 µg/mL	Apple-Extract based Standard** 0,1 µg/mL
146/42 Phthalimide [PI-H]⁻ (Also THPI → [THPI-H]⁻ → [PI-H]⁻)				
146/146 Phthalimide [PI-H]⁻ (Also THPI → [THPI-H]⁻ → [PI-H]⁻)				
150/96 THPI [THPI-H]⁻				
150/42 THPI [THPI-H]⁻				

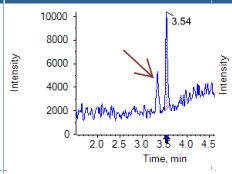
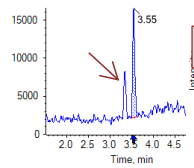
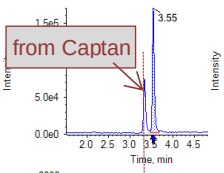
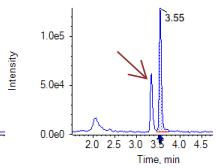
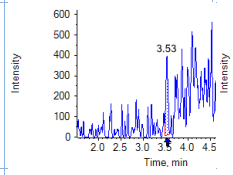
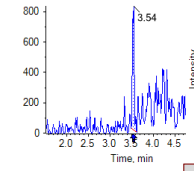
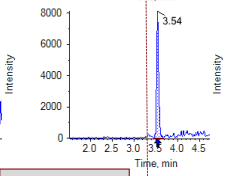
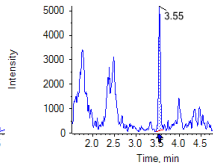
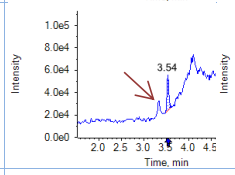
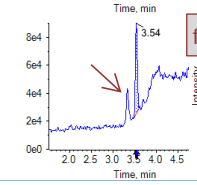
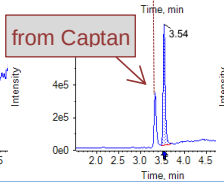
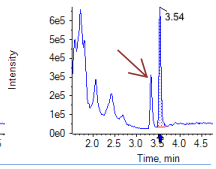
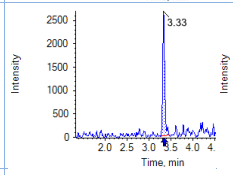
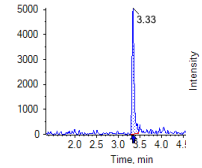
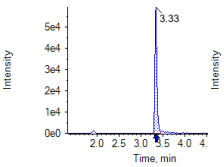
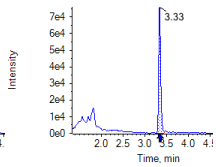
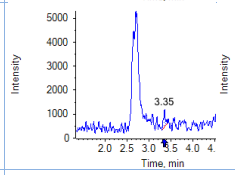
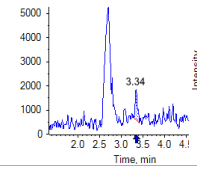
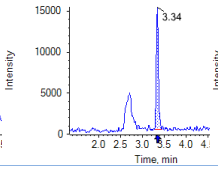
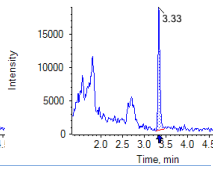
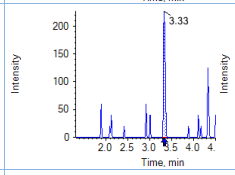
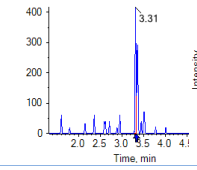
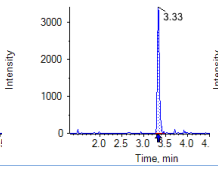
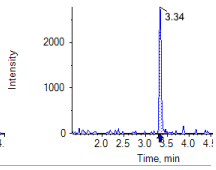
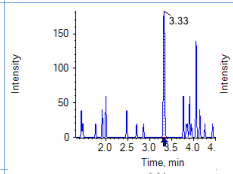
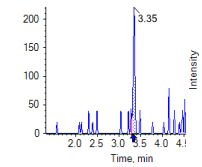
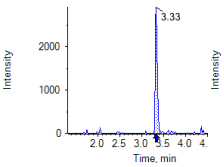
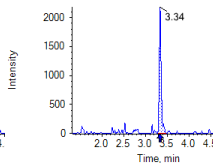
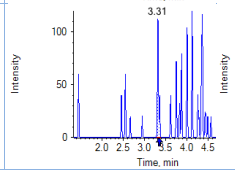
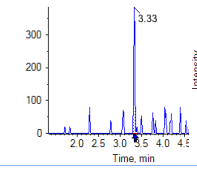
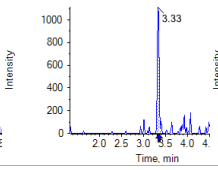
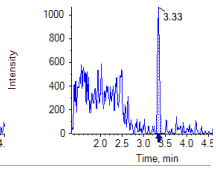
* Solvent= acetonitrile containing 0.4% acetic acid

** QuEChERS raw extract of apple (no dSPE cleanup)

Figure 4 shows chromatograms obtained when standard mixtures of **captan and folpet** were injected at 0.005; 0.01 and 0.1 µg/mL in pure solvent and at 0.1 µg/mL in apple extract. Similar to the FIA experiments, a virtually complete decomposition of captan and folpet to THPI and PI, respectively took place within the interface. Furthermore, THPI partly transformed further to PI, in a reaction that was also observed when injecting THPI itself (see above). Overall, the signals obtained for folpet were more intensive than those obtained with captan, but the chromatograms of folpet showed considerably higher baselines and more background peaks throughout the run.

Under the conditions of this experiment, the most abundant MRM transition of the quasimolecular ion of captan (m/z 298/35) gave a ca. 25-fold less intensive signal than the most intensive MRM transition of captan (m/z 150/96) and a ca. 5-fold less intensive signal than the second most intensive MRM transition (m/z 150/42). Although the signal sensitivity of the MRM transition 298/35 is rather poor, at higher captan levels it can still be used for identification and quantification, as it is more specific and less interfered than the MRM-transitions which are based on the in-source fragment as precursor ion.

Figure 4: *Chromatograms of Folpet and Captan obtained from the injection of differently concentrated standard mixtures (Sciex API 5500QTrap, APCI-neg, 2 µL injection volume; APCI-probe temp. 450°C)*

Mass Transition	Solvent-based Standard* 0,005 µg/mL	Solvent-based Standard* 0,01 µg/mL	Solvent-based Standard* 0,1 µg/mL	Apple-Extract based Standard** 0,1 µg/mL
146/42 **Folpet → PI→ [PI-H]⁻** (Also Captan→THPI → [THPI-H]⁻ → [PI-H]⁻)				
178/146 **Folpet → PI→ [PI-H]⁻**				
146/146 **Folpet → PI→ [PI-H]⁻** (Also Captan→THPI → [THPI-H]⁻ → [PI-H]⁻)				
150/96 **Captan → THPI→ [THPI-H]⁻**				
150/42 **Captan → THPI→ [THPI-H]⁻**				
298/35 **Captan ([M-H]⁻)**				
300/35 **Captan ([M-H]⁻)**				
298/146 **Captan ([M-H]⁻)**				

* Solvent= acetonitrile containing 0.4% acetic acid

** QuEChERS raw extract of apple (no dSPE cleanup)

In the experiments shown in **Figure 3** and **Figure 4** the signals obtained from the injection of THPI and PI (as such) were ~6-fold more intensive than those obtained, for the same ions, when similarly concentrated captan and folpet solutions were injected. Accounting for the molecular weight factor of ~2, the response factors still differ by a factor of ~3. Based on the S/N ratios obtained for THPI and PI analyzing these compounds down to 0.005 mg/kg (0.01 if expressed as parents) would be possible,

if signal suppression is not too extensive. For captan and folpet the S/N ratios at 0.01 µg/mL were, however, not satisfactory. 0.02 µg/mL would be expectedly a well measurable concentration for the parents in matrix extracts showing low matrix effects and interferences.

Employing electrospray ionization (ESI)

The **ESI (pos)** mode is rather unsuitable for THPI and PI as these compounds are acidic in nature. For captan and folpet, however, ESI (pos) proved very useful with ammonium adducts ($[M+NH_4]^+$) predominating the FIA MS-spectra (when using an aqueous-methanolic eluent containing ammonium formate). All elaborated MRM-transitions for captan and most for folpet were thus based on the ammonium adducts as precursor ions. In the case of captan, the signal of the protonated molecules ($[M+H]^+$), was also intensive enough to elaborate MRM transitions.

FIA analyses of **THPI and PI** in the **ESI (neg)** mode showed $[THPI-H]^-$ and $[PI-H]^-$ respectively, as the most abundant peaks in the mass-spectra. The same mass transitions and MS/MS voltage settings as in APCI (neg) were used. In contrast to APCI (neg), no dehydrogenation of $[THPI-H]^-$ to $[PI-H]^-$ was observed in ESI (neg) mode. Captan and folpet did not show useful ions to elaborate mass transitions. The fragmentation of **captan and folpet** to THPI and PI, which was very predominant in APCI (neg) was also not observed.

The MRM-transitions and MS-settings elaborated for the four analytes the ESI-mode are shown in Table 8.

Table 8: *MRM details for Captan, Folpet, PI, THPI using API 5500 QTrap in the ESI (neg.) and ESI (pos.) mode*

Compound	Ranking	Q 1	Q 3	DP	CE	CXP	Mode
Captan	1	300	264	41	11	22	Pos
	2	302	266	41	11	18	
	1	317*	264	41	17	14	
	2	319*	266	41	17	16	
	(3)**	*317**	*300*	*41*	*11*	*16*	
	(4)**	*319**	*302*	*41*	*11*	*14*	
Folpet	1	313*	130	36	39	8	
	1	315*	130	36	37	8	
	2	313*	260	36	17	20	
	2	315*	262	36	17	26	
Chlorpyrifos D_{10} (IS for ESI pos)		360	199	86	23	12	Pos
Tetrahydrophthalimide	1	150	96	-70	-28	-9	Neg
	2	150	42	-70	-76	-7	
Phthalimide	1	146	42	-110	-52	-7	
	2	146	146	-110	-5	-10	
BNPH (IS for ESI neg)		301	137	-65	-16	-7	Neg

* NH4-adduct;

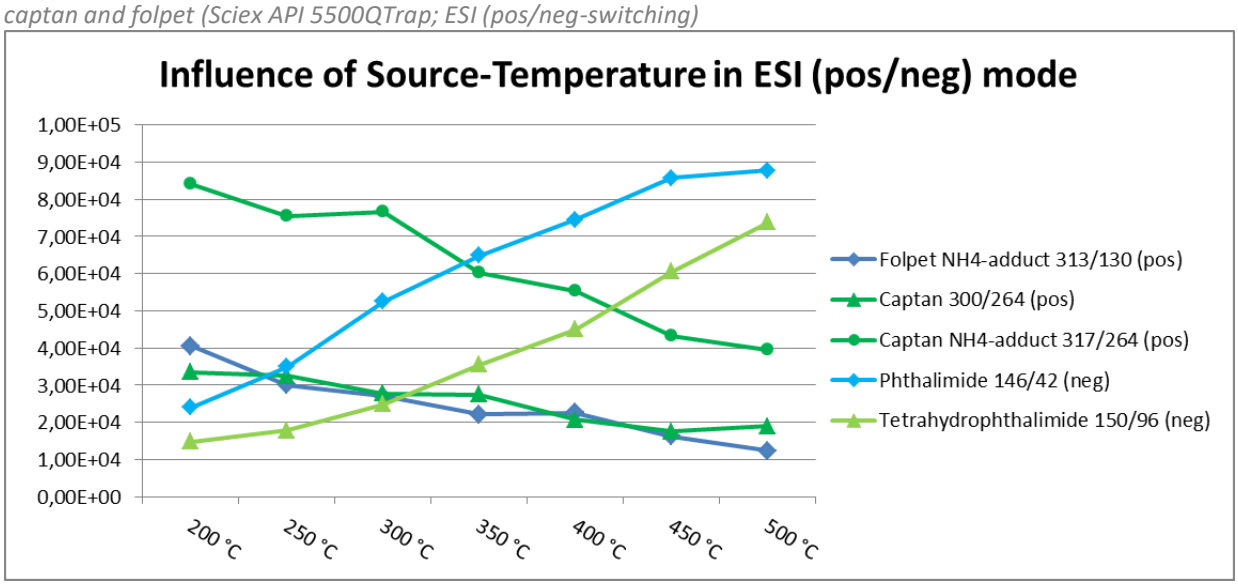
**Mass-transition involves NH3-loss from the NH4-adduct (limited specificity)

The influence of the ESI-source temperature on the signal intensity of the four analytes was studied in the range between 200 and 500 °C with 50°C increments. An LC-MS/MS method in the ESI mode was used entailing continuous pos/neg polarity switching. The parent molecules were measured in the ESI (pos) and the two metabolites in the ESI (neg) mode. As can be seen in Figure 5, increasing the source temperature leads to a decrease in the yields of the quasimolecular ions of captan and folpet. This could be an indication of an increased fragmentation, but not necessarily. As all MRM-

transitions of the above table were measured throughout the run it was possible to check whether $[THPI-H]^-$ to $[PI-H]^-$ show up at the retention times of captan and folpet. This was, however, not the case. Already in the FIA experiments in the ESI (neg) mode (see above), it could be seen that captan and folpet do not produce any intensive precursor ions that would further fragment into $[THPI-H]^-$ to $[PI-H]^-$ within the ESI-source.

The ions $[THPI-H]^-$ and $[PI-H]^-$ of course showed up at the retention times of THPI and PI respectively. Interestingly, their signal intensities increased with increasing ESI-source temperatures and it seems that some further increase can be expected when temperatures rise further. This effect is surely related to the high water content of the eluent (close to 50%), at the time where THPI and PI elute from the column (2 and 2.3 min respectively).

Figure 5: *Influence of ESI-source temperature on the intensities of the various mass-transitions of PI, THPI, captan and folpet (Sciex API 5500QTrap; ESI (pos/neg-switching)*



Possibilities for routine analysis

Considering that pesticide residue laboratories almost exclusively employ the ESI technology in routine LC-MS applications, priority was set in exploring the possibility of analysing the four compounds by LC-MS/MS method(s) employing ESI. From the efficiency point of view, the compounds would be ideally covered by LC-MS/MS methods already routinely employed for multiresidue analysis, or other methods allowing routine multiresidue analysis of pesticides. If this is not feasible, specialized methods would be needed. Either a single specialized method covering all four compounds in one go, or, if the required sensitivity cannot be achieved, two separate specialized LC-MS/MS methods, one for THPI/PI and one for captan/folpet.
Based on the results of the pre-experiments, an LC-ESI-MS/MS method covering all four compounds in one go would require an intermediate ESI-source temperature as a compromise. The method would further need to include negative and positive ionization, either continuously or starting with ESI (neg) for THPI and PI and then switching to ESI (pos) for captan and folpet.

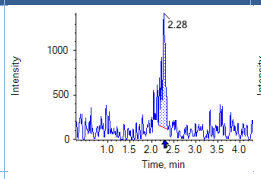
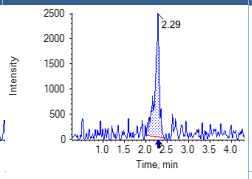
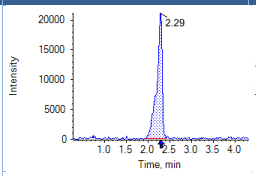
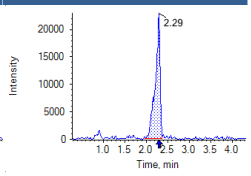
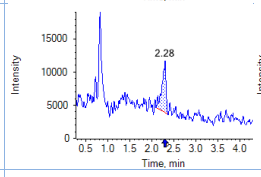
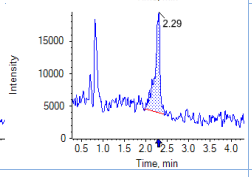
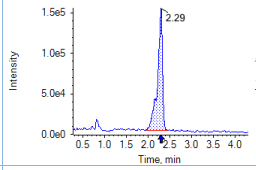
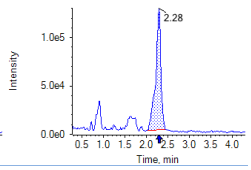
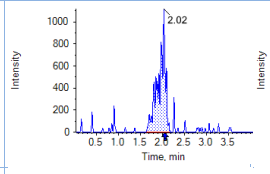
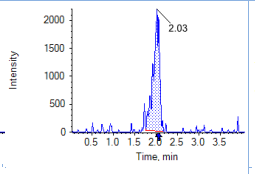
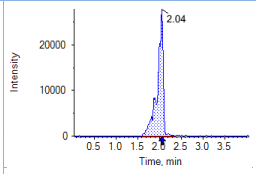
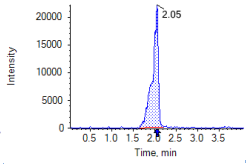
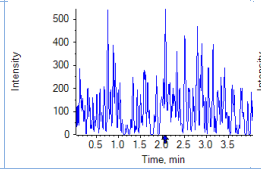
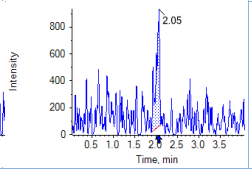
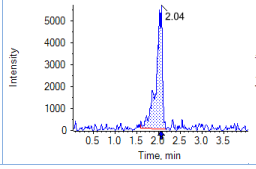
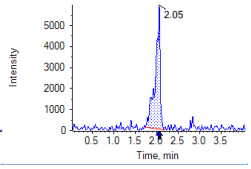
LC-ESI(pos/neg)-MS/MS method using an intermediate ESI-source temperature of 450°C

A mixed-mode method, involving continuous polarity-switching between the positive and negative mode, was set up. Instrument details are given in Table 6. To start with, a temperature of 450°C was chosen as this was considered useful for the early eluting degradants.

Figure 6 shows chromatograms of THPI and PI generated by this method. THPI and PI were detected in the ESI (neg) mode using MRM transitions of the deprotonated molecules. The THPI and PI signals showed a sufficient S/N ratio only at a level of 0.01 µg/mL or higher in contrast to the APCI measurements where satisfactory signals were already obtained at the 0.005 µg/mL level. A lower ESI-source temperature would lower the sensitivity further. It should be noted that in this experiment the injection volume was set at 5 µL to enhance the sensitivity for folpet and captan, but this also caused a pronounced peak-fronting of the two metabolite peaks.

Figure 7 shows chromatograms of folpet and captan obtained by the same method in the ESI (pos) mode. The chromatograms are based on MRM-transitions of the ammonium adducts (folpet and captan) or the protonated molecules (captan). Judging on Figure 5, the high ESI-source temperature of 450°C in this experiment was not optimal for the generation of the quasimolecular ions of captan and folpet. A signal increase by a factor of ca. 2 would be expected if the source temperature was lowered further to e.g. 200 °C, but this might be too low for THPI and PI. Still, captan and folpet showed satisfactory signals with well-defined peaks down to 0.005 µg/mL. For comparison, in the APCI mode satisfactory signals could only be obtained down to the 0.01 µg/mL level.

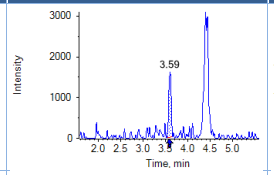
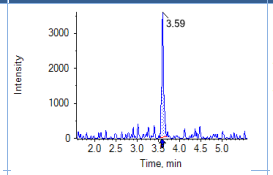
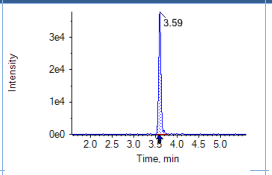
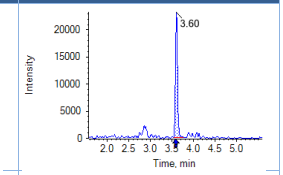
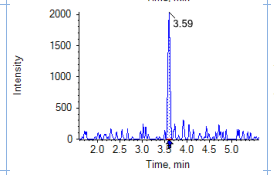
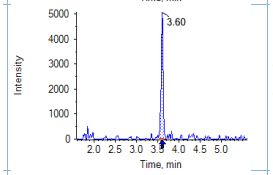
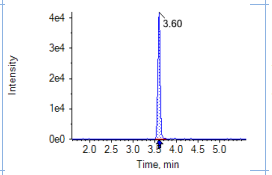
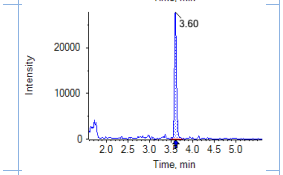
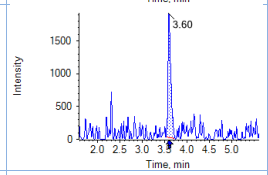
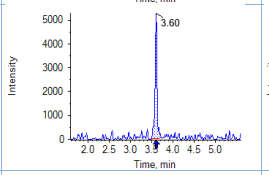
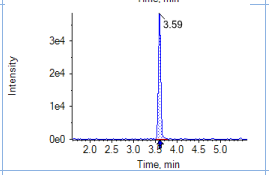
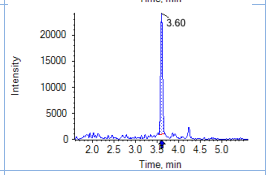
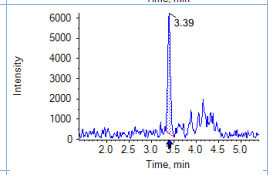
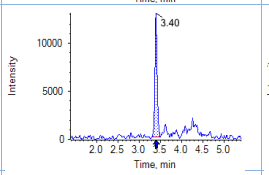
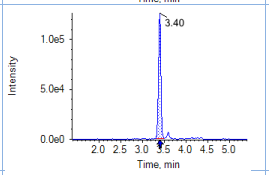
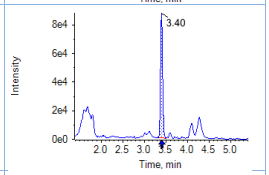
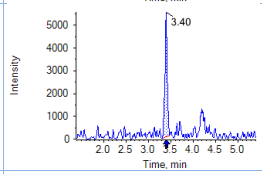
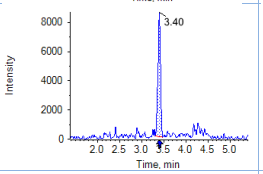
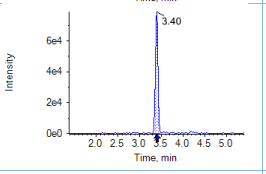
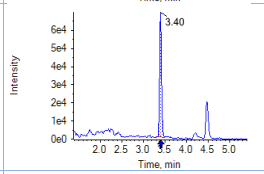
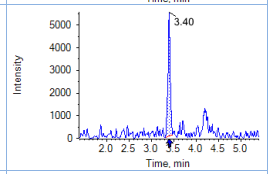
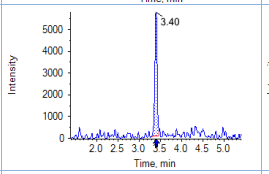
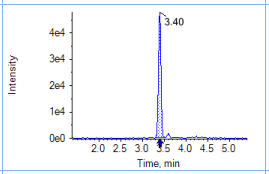
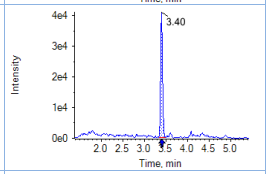
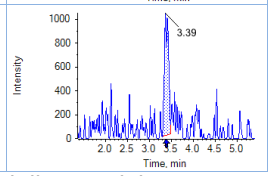
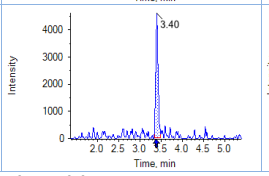
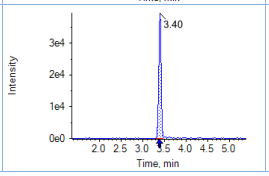
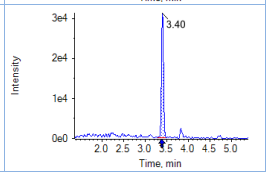
Figure 6: *Chromatograms of PI and THPI obtained from the injection of differently concentrated standard mixtures (Sciex API 5500QTrap; ESI-Source temp. 450°C, ESI (pos/neg-switch); 5 µL injection volume)*

Mass Transition	Solvent-based Standard* 0,005 µg/mL	Solvent-based Standard* 0,01 µg/mL	Solvent-based Standard* 0,1 µg/mL	Apple-Extract based Standard** 0,1 µg/mL
146/42 Phthalimide [PI-H]⁻				
146/146 Phthalimide [PI-H]⁻				
150/96 THPI [THPI-H]⁻				
150/42 THPI [THPI-H]⁻				

* Solvent= acetonitrile containing 0.4% acetic acid

** QuEChERS raw extract of appl (no dSPE cleanup)

Figure 7: *Chromatograms of Folpet and Captan obtained in* ***the ESI (pos) mode*** *from the injection of differently concentrated standard mixtures (Sciex API 5500QTrap; ESI-Source temp. 450°C, Method involved an ESI (pos/neg) polarity switch; 5 µL injection volume)*

Mass Transition	Solvent-based Standard* 0,005 µg/mL	Solvent-based Standard* 0,01 µg/mL	Solvent-based Standard* 0,1 µg/mL	Apple-Extract based Standard** 0,1 µg/mL
313/130 **Folpet** $[M+NH_4]^+$				
315/130 **Folpet** $[M+NH_4]^+$				
313/260 **Folpet** $[M+NH_4]^+$				
317/264 **Folpet** $[M+NH_4]^+$				
319/266 **Captan** $[M+NH_4]^+$				
300/264 **Captan** $[M+H]^+$				
302/266 **Captan** $[M+H]^+$				

* Solvent= acetonitrile containing 0.4% acetic acid

** QuEChERS raw extract of appl (no dSPE cleanup)

Recovery experiments using CEN-QuEChERS

To check the method performance, replicate recovery experiments were performed on apple homogenates (spiking level 0.02 mg/kg), and on wheat flour (spiking level 0.04 mg/kg). The samples were extracted in quintuplicate using the QuEChERS-CEN (citrate-buffered) method without applying dSPE-cleanup. The shaking time during the first extraction step was 15 min. Matrix-matched calibration standards, prepared using extracts of blank (non-spiked) sample portions, were used for quantification. Calibration levels at 60% and 120% of the spiked concentration were chosen. The following internal standards (IS) were used for calculations:

i. Chlorpyrifos D_{10} and propyzamide D_3 (data not shown) for compounds measured in ESI (pos)
ii. Propyzamide D_3 for compounds detected in ESI (neg) or APCI (neg)
iii. Isotopically labelled internal standards (ILISs) corresponding to each of the four analytes.

The extracts were measured by two different LC-MS/MS methods: a) An LC-ESI(pos/neg)-MS/MS (ESI-source temp. of 450°C, see above), and b) An LC-APCI (neg)-MS/MS method (APCI-probe temperature 450°C). For more details see **Table 6**.

1. Analysis via LC-ESI(pos/neg)-MS/MS

Table 9 gives the mean recoveries and RSDs for all measured mass transitions, calculated both via the respective ILISs as well as via a generic ISs, i.e. chlorpyrifos D_{10} for ESI (pos) signals and propyzamide D_3 for ESI (neg) signals. Some exemplary chromatograms for captan and folpet are shown in **Figure 8** and for THPI and PI in **Figure 9**.

In the case of **apples** (spiking level 0.02 mg/kg), and using a generic IS for calculation, the mean recovery rates for **folpet** ranged between 97 and 114 % (average 104 %) and the RSDs between 7.6 and 13.9 % (average 10.2 %), depending on the mass-transition. In the case of **captan** the mean recovery rates ranged between 93 % and 100 % (average 97 %) and the RSD between 8.3 and 12.2 (average 10.2 %). For **PI** the average recovery and RSDs based on the acquired MRMs were 92% and 8 % respectively. In the case of **THPI** only one mass transition could be evaluated (m/z 150/96). Based on this signal the average recovery rate and RSD calculated to 88% and 5.3% respectively. The second MRM of THPI (m/z 150/42) was interfered by the added PI-ILIS (PI-D_4), which is isobaric to THPI. In the particular experiment, an injection volume of 5µL was selected, which caused a peak fronting in the case of THPI, PI and their respective ILISs, and an overlap between the peaks of THPI and PI-D_4. In the case of APCI measurements (see below), where 2 µL were injected, this interference did not show up and symmetric, narrow and well separated peaks were obtained. Similarly folpet D_4 appeared as a large peak in all acquired MRM-chromatograms of the isobaric captan but the separation of folpet and captan remained sufficient even at 5 µL injection volume, so the captan peaks could be integrated. In conclusion, if PI-D_4 or folpet-D_4 are added to the sample, a full chromatographic separation between PI and THPI as well as between captan and folpet is needed to ensure lower LOQs and a better identification certainty.

Recovery calculations based on the ILISs resulted in overall similar recovery rates for captan and folpet as with chlorpyrifos D_{10} . In the case of THPI and PI the ILIS-corrected recovery rates were higher and closer to 100% than those calculated against propyzamide D_4. The RSDs, however, did not significantly improve.

Wheat flour extracts showed stronger mass-spectrometric interferences than apple extracts. Thus, despite having the same compound concentrations in the final extracts as the apple extracts, many MRM-transitions could not be properly evaluated. As shown in **Figure 8**, in the case of wheat flour all aquired MRM-transitions of folpet and all but one of those of captan were strongly interference by matrix components. A better chromatographic separation of folpet and captan from the matrix peaks would be needed. In contrast to apple, extensive losses of captan were noticed during the sample preparation of wheat flour. The recovery rate of captan based on the measurable MRM transition (317/264) was 74 %, whereas in the case of folpet no evaluation was possible. Measurements in the APCI (neg) mode confirm the occurrence of losses (see discussion there).

Table 9: Validation data for Folpet / Captan (ESI pos) and Phthalimide / Tetrahydrophthalimide (ESI neg) all measured using Sciex API 5500 QTrap; PI / THPI were spiked separately from captan/folpet.

Matrix	Sample Weight	Spiking Level (mg/kg)	Compound	Parent ion type	MRM-transition	ESI-mode	Calc. using generic internal standards*			Calc. using ILISs		
							n	Mean Rec.	RSD %	n	Mean Rec.	RSD %
Apple (High water content)	10 g	0.02	**Folpet**	$[M+NH_4]^+$	313/130	Pos	5	**97%**	**7.6**	5	95%	12.3
					315/130			**101%**	**13.9**	5	99%	12.0
					313/260			**114%**	**9.1**	5	113%	9.3
			Captan	$[MH]^+$	317/264			**93%**	**5.8**	5	99%	7.8
					319/266			**99%**	**9.1**	5	106%	9.6
					300/264			**100%**	**12.2**		107%	13.7
					302/266			**95%**	**8.3**	5	100%	6.4
	10 g	0.02	**Phthalimide**	$[M-H]^-$	146/42	Neg	5	**89%**	**10.4**	5	103%	10.7
					146/146			**95%**	**5.5**	5	109%	3.7
			THPI	$[M-H]^-$	150/96			**88%**	**5.3**	5	105%	6.4
					150/42			-**	-**	5	-**	-**
Flour (dry)	5 g	0.04	**Folpet**	$[M+NH_4]^+$	313/130	Pos	5	-***	-***	5	-***	-***
					315/130			-***	-***	5	-***	-***
					313/260			-***	-***	5	-***	-***
			Captan	$[MH]^+$	317/264			**74%**	**5.8**	5	110%	6.9
					319/266			-***	-***	5	-***	-***
					300/264			-***	-***	5	-***	-***
					302/266			-***	-***	5	-***	-***
	5 g	0.04	**Phthalimide**	$[M-H]^-$	146/42	Neg	5	**102%**	**15.4**	5	99%	16.6
					146/146			-*	-*	5	-***	-***
			THPI	$[M-H]^-$	150/96			**99%**	**7.8**	5	106%	9.8
					150/42			-**	-**	5	-**	-**

* Chlorpyrifos D_{10} in ESI (pos) mode/ propyzamide D_3 in ESI (neg) mode

** Mass transition of THPI interfered by phthalimide D_4 ILIS (phthalimide 146/42; phthalimide D_4 150/42)

*** Mass Transition interfered by matrix

*Figure 8: Exemplary LC-MS-MS chromatograms of **folpet and captan** obtained in the validation experiments on apple (0.02 mg/kg) and wheat flour (0.04 mg/kg); measured in **ESI (pos)** but using a method involving pos/neg polarity switching,, using Sciex API 5500 QTrap; inj. volume 5 µL*

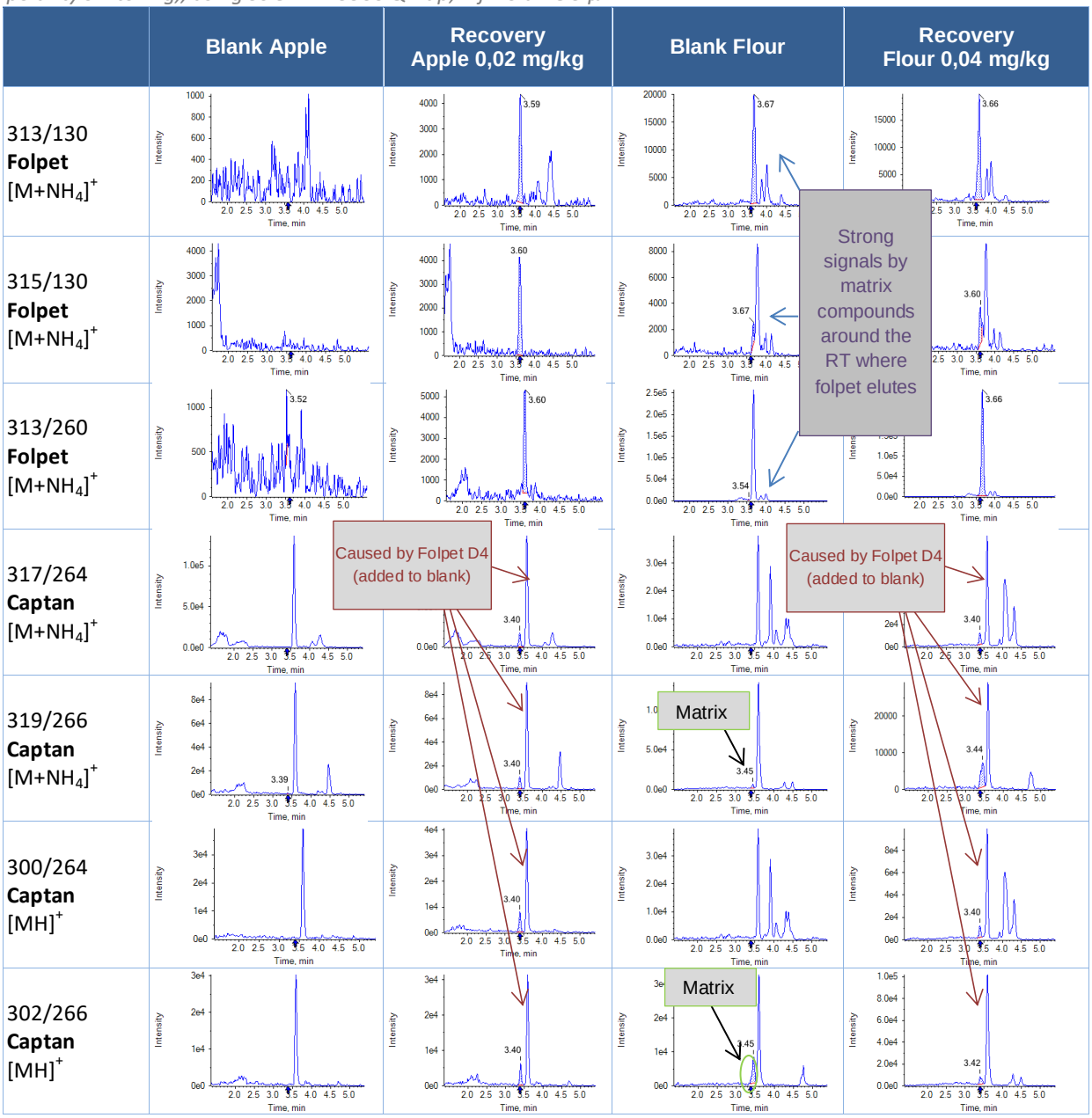
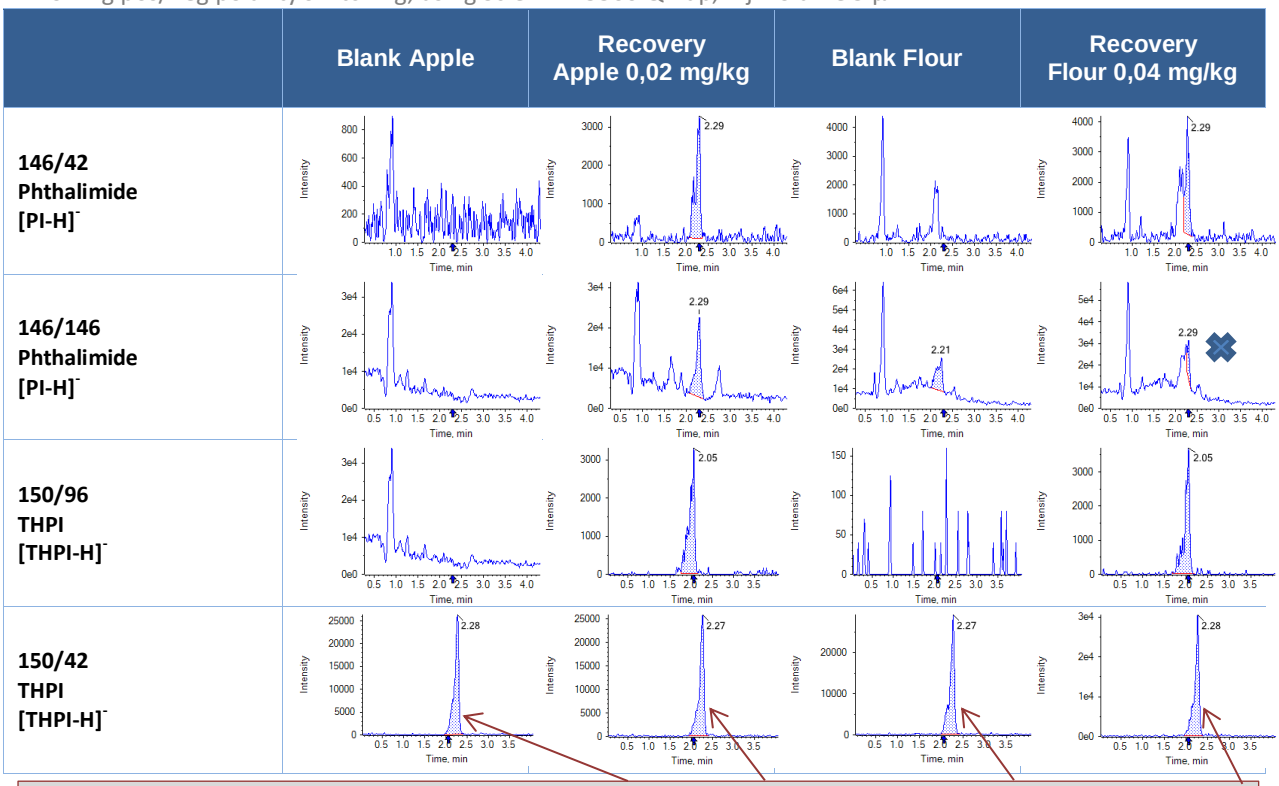


Figure 9: Exemplary LC-MS-MS chromatograms of **phthalimide and THPI** obtained in the validation experiments on apple (0.02 mg/kg) and wheat flour (0.04 mg/kg); measured in **ESI (neg)** but using a method involving pos/neg polarity switching, using Sciex API 5500 QTrap; inj. volume 5 µL



Large peak of phthalimide D_4 with fronting interfering with THPI. Better chromatographic separation would be needed: The injection of a smaller volume reduces fronting (see separation in APCI mode).

2. Analysis via LC-MS/MS in APCI (neg) mode

The extracts of the QuEChERS recovery experiments (see previous chapter) were additionally measured by LC-APCI (neg)-MS/MS. **Table 10** shows the recovery rates and RSDs calculated for captan, folpet, THPI and PI. Some exemplary chromatograms are shown in **Figure 10** for folpet and captan and in **Figure 11** for THPI and PI.

In the case of **apples** the recovery rates of **folpet and captan** ranged between 91 and 109% and the RSDs between 4.9 and 9.1 %. The recovery rate of **PI** was 94% (average of two MRMs), however associated with RSDs close to 20% due to interferences. The recovery rate of **THPI** was 93% with an RSD of 9.1 %.

In the case of **wheat flour** degradation of folpet and captan took place during extraction. The captan recovery rate dropped to 74 % (same figure as in ESI-measurement) and that of folpet to 83 % (average of two MRMs, no figure could be generated using ESI). The recovery rates of THPI and PI ranged between 95 and 97% with satisfactory RSDs between 2.6 and 5.8 %.

The losses of captan and folpet can be explained by the relatively high pH of the extraction solution during the 15 min shaking period, and some minor delays between water addition and extraction as well as between first extraction and addition of buffering salts. The degradation products of folpet (PI) and of captan (THPI), were also measured, and found to be largely matching with the losses of captan and folpet. THPI was quantified at ~0,006 mg/kg which corresponds to to ~0,012 mg/kg

captan (~30 % of spiked level), whereas PI was quantified at ~0,002 mg/kg which corresponds to ~0,004 mg/kg folpet (~10 % of spiked level) An acidification prior to the first extraction would have helped to reduce these losses.

In previous experiments it was shown that the mass transition 150/42 is shared by THPI, captan (following fragmentation to THPI), PI-D_4 and folpet-D_4 (following fragmentation to PI-D_4). These mass-transition overlaps can also be circumvented through chromatographic separation. Two additional, at first sight unexpected, sources of the mass transition 150/42 are THPI-D_6 and captan-D_6., which breaks down to THPI-D_6 in the source. This unusual mass shift of -6 can be attributed to the in-source neutral loss of two HD molecules from $[THPI\text{-}D_6\text{-}H]^-$ transforming it to $[PI\text{-}D_4\text{-}H]^-$, which is isobaric with $[THPI\text{-}H]^-$. This interference occurs at the retention times of THPI and captan and cannot be addressed by chromatographic means, which compromises the analysis of THPI and captan by APCI (neg) when the respective D_6 ILISs are used. Fortunately, an alternative, albeit weaker, mass transition (150/96) can be used for quantification of THPI and captan in such a case. The double HD elimination described here is fully equivalent to the abovementioned neutral loss of two H_2 molecules from $[THPI\text{-}H]^-$ leading to the formation of $[PI\text{-}H]^-$ at the retention times of THPI and captan (mass shift -4). The latter is however not problematic for the analysis of folpet as it can be addressed by a chromatographic separation between THPI and PI or between folpet and captan.

Table 10: *Validation data for folpet, captan, PI and THPI (APCI neg. mode, Propyzamide D_3 as internal standard) all measured using Sciex API 5500 QTrap. PI / THPI were spiked separately from captan/folpet.*

Matrix	Sample Weight	Spiking Level (mg/kg)	Compound	Parent ion type	MRM-transition	ESI-mode	Calc. using generic internal standards*		
							n	Mean Rec. %	RSD %
Apple (High water content)	10 g	0.02	**Folpet**	$[PI\text{-}H]^-$ (interface fragment)	146/42	Neg	5	125	12.5
					146/146			109	9.1
			Captan	$[THPI\text{-}H]^-$ (interface fragment)	150/96			91	4.9
					150/42			-**	-**
	10 g	0.02	**PI**	$[PI\text{-}H]^-$	146/42	Neg	5	86	19.0*** *
					146/146			100	18.0*** *
			THPI	$[THPI\text{-}H]^-$	150/96			93	9.1
					150/42			-***	-***
Wheat Flour (dry)	5 g	0.04	**Folpet**	$[PI\text{-}H]^-$ (interface fragment)	146/42	Neg	5	85	4.3
					146/146			81	5.6
			Captan	$[THPI\text{-}H]^-$ (interface fragment)	150/96			74	4.9
					150/42			-**	-**
	5 g	0.04	**Phthalimide**	$[M\text{-}H]^-$	146/42	Neg	5	100	3.9
					146/146			97	2.6
			THPI	$[M\text{-}H]^-$	150/96			95	5.8
					150/42			-***	-***

* Propyzamide D_3 was used as internal standard for all compounds

** Mass transition 150/42 of captan (or its degradant THPI) was interfered by captan D_6 through the generation of PI-D_4 in a two-step process. First the breakdown of captan D_6 to THPI-D_6 in the interface, and then the generation of PI-D_4, through the elimination of two HD molecules within the ion-source.

*** Mass transition 150/42 of THPI was interfered by THPI-D_6 through the neutral loss of two HD molecules within the ion source and the formation of the isobaric PI-D_4

**** Mass Transition interfered by matrix

*Figure 10: Exemplary LC-MS-MS chromatograms of **folpet and captan** obtained in the validation experiments on apple (0.02 mg/kg) and wheat flour (0.04 mg/kg); measured in **APCI (neg) mode**, using Sciex API 5500 QTrap; inj. volume 2 µL*

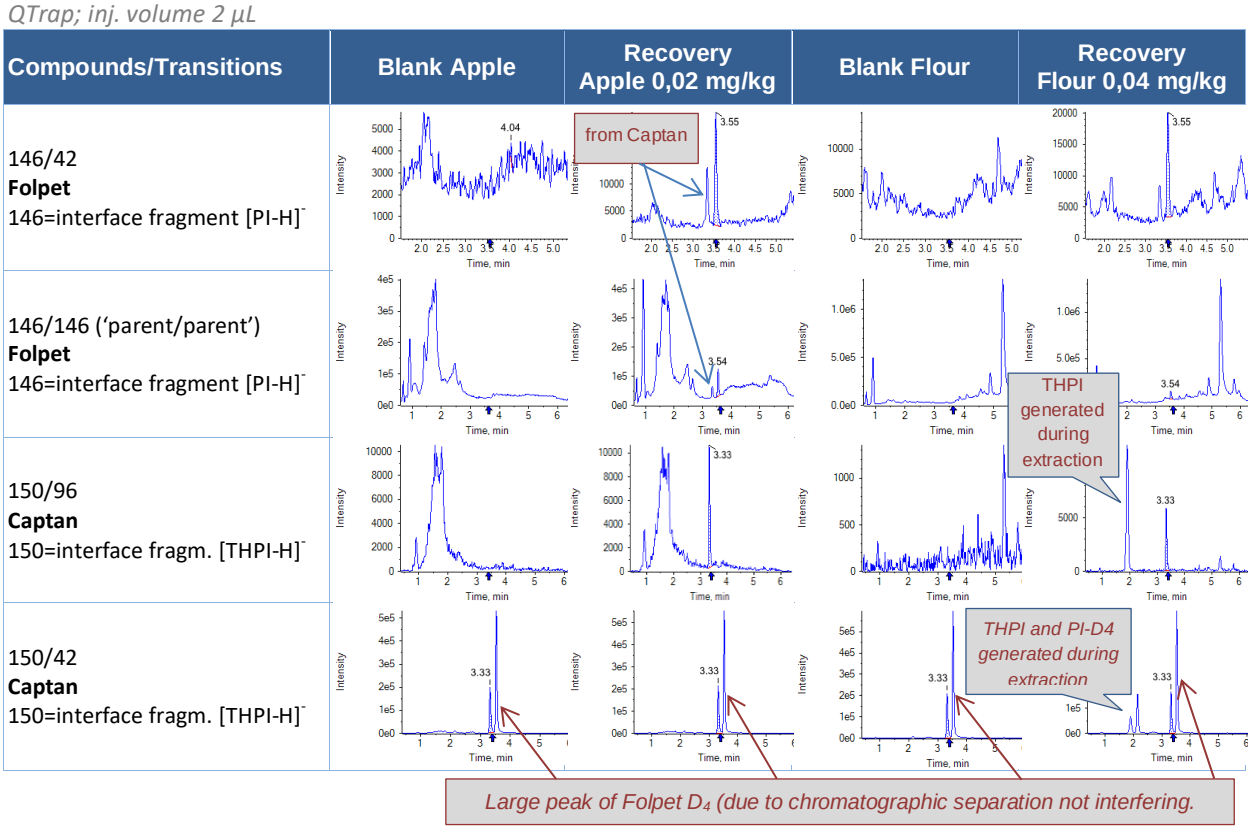
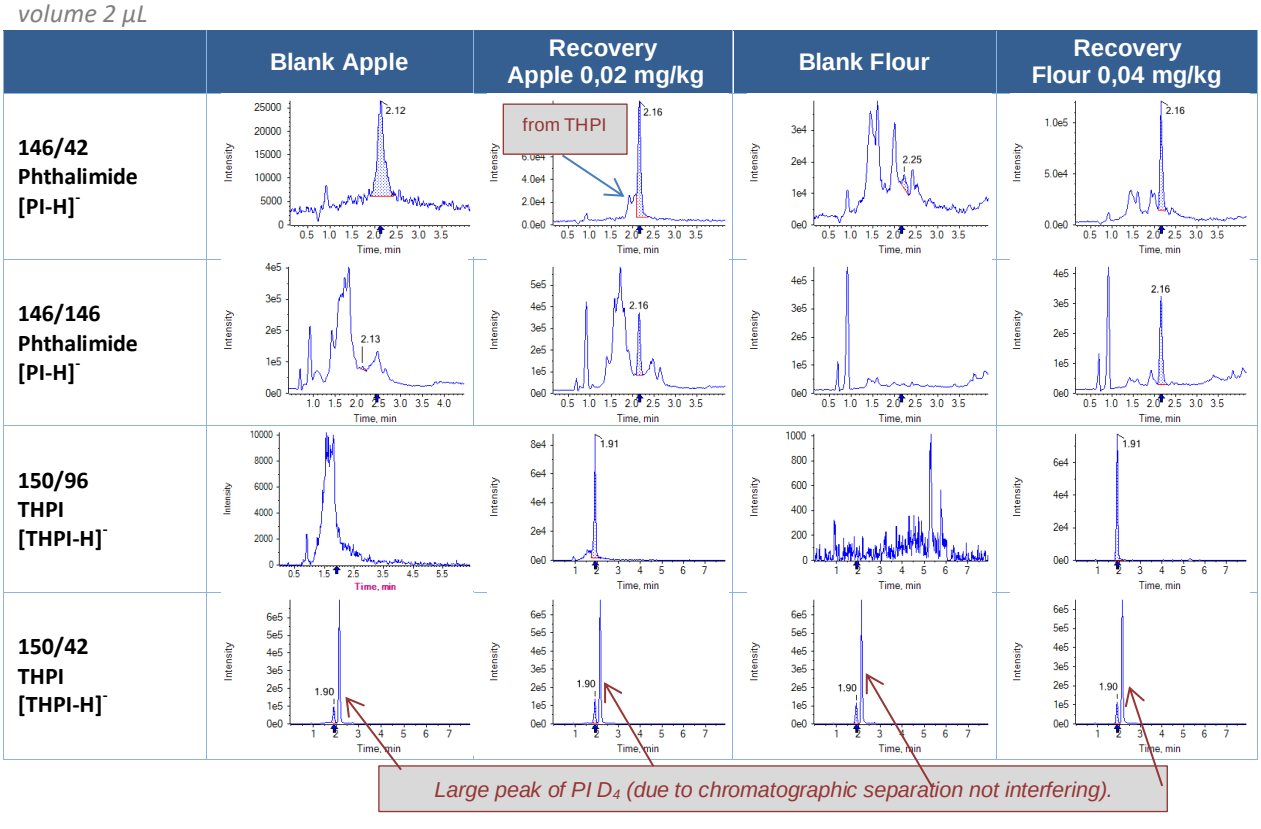


*Figure 11: Exemplary LC-MS-MS chromatograms of **PI and THPI** obtained in validation experiments on apple (0.02 mg/kg) and wheat flour (0.04 mg/kg); measured in **APCI (neg)** mode, using Sciex API 5500 QTrap; inj. volume 2 µL*



Analysis of various market samples using LC-MS/MS APCI negative mode

The first experiments showed, that LC-MS/MS-based analysis of captan, folpet and their metabolites, of routine samples is possible, although still suffering from insufficient sensitivity for some types of samples when compared to the existing MRLs (see **Table 11**). Further efforts to improve measurement sensitivity and to validate the method will follow.

Table 11: *Overview of existing MRLs (extracted 20.03.2019)*

Commodities	MRL* (mg/kg) (set at the LOQ)		Notes
Plant origin (plus honey)	**Captan (Sum of captan and THPI, expressed as captan) (R)**	**Folpet (sum of folpet and phtalimide, expressed as folpet) (R)**	
Fruit and vegetables, fungi (with exceptions)	0.03*	0.03*	
• **Apples, Pears**	10	0.3	
• **Other pome fruit**	10	0.03*	
• **Apricots, Cherries, Peaches**	6	0.03*	
• **Plums**	10	0.03*	
• **Table grapes**	0.03*	6	
• **Wine grapes**	0.02*	20	RD: Captan
• **Srawberries**	1.5	5	
• **Blueberries, currants, gooseberries**	30	0.03*	
• **Raspberries**	20	0.03*	
• **Radishes, salsifies**	0.03*	0.04*	
• **Potatoes**	0.03*	0.06*	
• **Tomatoes**	1	5	
• **Melons**	0.03*	0.4	
• **Fresh herbs**	0.06*	0.06*	
Pulses and cereals, nuts and oily seeds (except wheat and barley)	0.07*	0.07*	
• **Wheat**	0.07*	0.4	
• **Barley**	0.07*	1	
Oily fruits (except olives)	0.07*	0.07*	
• **Olives**	0.03*	0.15*	
Spices, dried herbs and infusions	0.1*	0.1*	
• **Hops**	0.1*	400	
Honey	0.05*	0.05*	
Animal origin (except honey)	**Sum of THPI, 3-OH THPI and 5-OH THPI, expressed as captan**	**Phthalimide**	
Muscle, liver, kidney, fat (of swine, poultry)	0.03*	0.05*	
Muscle, liver, kidney (of ruminants)	0.09	0.05*	
Fat (ruminants)	0.06	0.05*	
Milk	0.03*	0.05*	
Eggs	0.03*	0.05*	

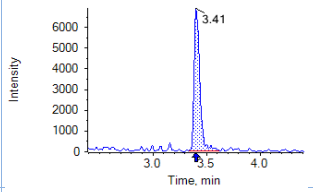
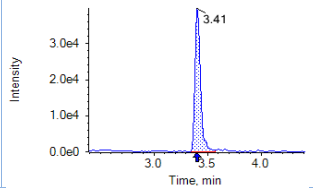
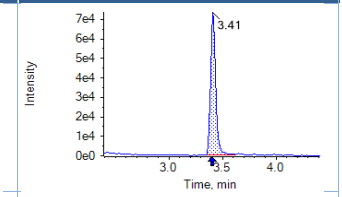
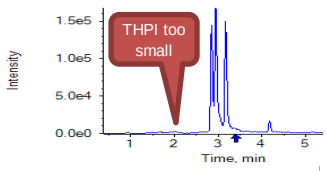
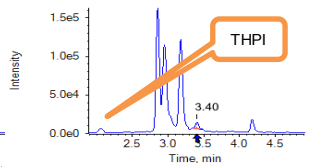
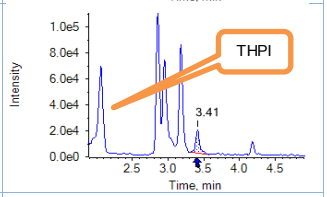
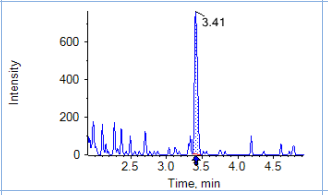
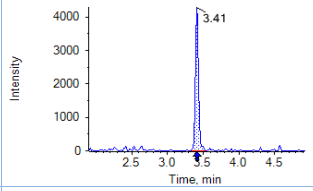
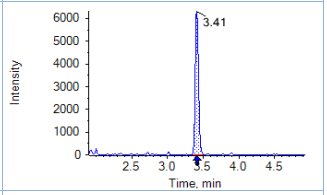
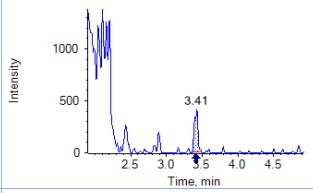
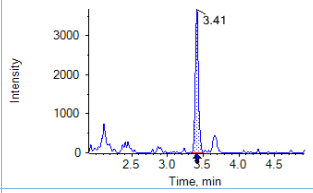
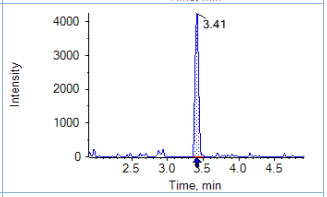
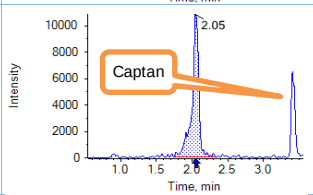
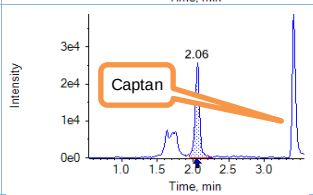
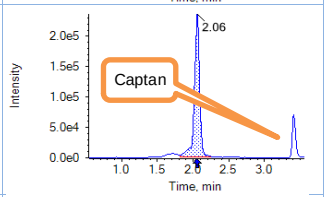
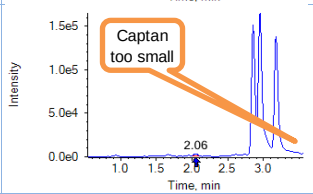
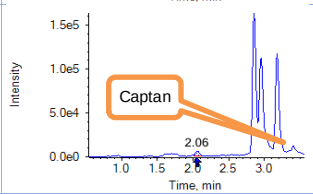
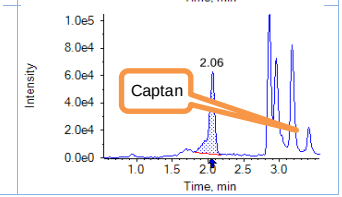
In order to gain experience with the analysis of the four compounds in various matrices at concentration levels as they occur in real samples, it was decided to start a small project in which selected positive samples found to be positive for any of the four compounds, by the CVUAS routine laboratory (using GC-techniques) are re-analyzed by LC-MS/MS. Table 12 gives an overview of a few first results. It should be noted, that in the present table the routine results are shown as generated in routine without any repetition to improve accuracy. It should be also noted, that the GC-results of the degradation products (at the moment only THPI) are most likely overestimated as the parts

generated through thermal decomposition of the parents within the GC-inlet are not deducted. By the conduction of dSPE cleanup with PSA, some breakdown of parents to degradants has surely also occurred but this affected, both GC- and LC-results. The corresponding LC-MS/MS chromatograms are shown in Table 12.

Table 12: *Comparison of captan and THPI levels determined in various positive samples from the market using GC- techniques (routinely) and by LC-MS/MS (within the present study).*

Sample type	GC-MS-MS (calc. with cucumber matched calib + AP)		LC-MS-MS (APCI) (calc. with solvent calib, only ISTD no ILIS)	
	Captan (mg/kg)	THPI (mg/kg)	Captan (mg/kg)	THPI (mg/kg)
Rasperry	0.009	0.006	0.014	0.003
Blueberry	0.094	0.044	0.083	0.004
Apple	0.18	0.083	0.13	0.044

Figure 12: *Exemplary LC-MS-MS chromatograms of* ***captan and THPI*** *obtained from positive samples from the market. Measured in* ***APCI (neg) mode****, using Sciex API 5500 QTrap; injection volume 2 µL*

	Raspberry QuEChERS (following dSPE w. PSA)	**Blueberry QuEChERS (following dSPE w. PSA)**	**Apple QuEChERS (following dSPE w. PSA)**
150/96 Captan (Also THPI → [THPI-H]⁻)			
150/42 Captan (Also THPI → [THPI-H]⁻)			
Captan 298/35			
Captan 300/35			
150/96 THPI Also Captan→THPI → [THPI-H]⁻			
150/42 THPI (Also THPI → [THPI-H]⁻)			

Studies on the hydrolysis of captan and folpet to their degradants before extraction or measurement

In parallel to the development of the LC-MS/MS methods described above, some experiments were performed to examine the possibility of quantitatively hydrolyzing captan and folpet to THPI and PI before extraction or measurement. If this was successful and straightforward from the practical point of view, it would simplify quantification and LOQ setting for captan (sum and folpet (sum), by focusing on a single analyte (the degradant) in each case. The following experiments were conducted:

1. Hydrolysis in matrix at different pH before extraction

Analytical portions of blank cucumber, grape and currant samples were prepared in quadruplicate. Two portions were used as such and the other two first adjusted to pH ~7 (with NaOH). All portions were spiked with captan and folpet at 2 mg/kg and **kept at room temperature for 4 h** before extraction. Extraction was performed using CEN-QuEChERS without cleanup. Table 13 shows the results of this experiment. For captan decomposition was quantitative when pH was adjusted to ≈7 and the transformation yield to THPI was also satisfactory ranging between 84 and 95 %. In the case of folpet dissipation was incomplete in 2 cases and the yields of PI were lower (56 % in the case of grape). The overall impression of this experiment was that conversion yields of folpet and captan do not necessarily correlate, and that pH-adjustment to values close to 7 is cumbersome as it takes a long time for the pH to adjust. Further studies are needed to investigate factors affecting transformation of captan and folpet to THPI and PI.

Table 13: *Conversion rates of captan and folpet to THPI and PI (calculated as parents) when conducting hydrolysis* ***before QuEChERS extraction;*** *spiked commodities: cucumber, grapes and currants*

Commodity	pH Condition	Folpet	PI calc. as Folpet	SUM Folpet		Captan	THPI calc. as Captan	SUM Captan
Cucumber	**pH 5,5 (natural)**	14 %	**64 %**	78 %		0.0 %	**82 %**	82 %
	adjusted to pH 7.2	0.0 %	**80 %**	80 %		0.0 %	**94 %**	94 %
Grape	**pH 3,2 (natural)**	80 %	**5 %**	85 %		70 %	**10 %**	70 %
	adjusted to pH 7.4	1 %	**56 %**	57 %		0.0 %	**84 %**	84 %
Currant	**pH 2,6 (natural)**	80 %	**8 %**	88 %		62 %	**21 %**	83 %
	adjusted to pH 7.4	10 %	**85 %**	95 %		0.0 %	**95 %**	95 %

2. Hydrolysis in matrix before and during extraction at different pH

To further investigate the influence of pH the experiment was repeated with grape as commodity adjusting the pH at 7 or 10 and keeping the hydrolysis time at room temperature constant at 4h. The spiking level was 1 mg/kg. A direct extraction of the pH adjusted samples was also conducted using QuEChERS without cleanup. Shaking time during the first extraction step was 15 min in all cases. Table 13 shows the results of this experiment.

*Table 14: Conversion rates of captan and folpet to THPI and PI (calculated as parents) when conducting hydrolysis **before or during QuEChERS extraction**; spiked commodity: grapes.*

pH adjusted	Delay between pH-adjustment and extraction	Extraction method	Shaking time	THPI	Captan	SUM Captan		PI	Folpet	SUM Folpet
pH 7	No delay	QuEChERS	15 min	**4**	106	**110**		**2**	113	115
pH 10	No delay			**47**	9	**56**		**42**	1	43
pH 7	4h at RT			**78**	5	**83**		**73**	13	86
pH 10	4h at RT			**55**	0.0	**55**		**2**	0.0	2

The highest yields were obtained when letting the samples hydrolyse at a pH of 7 for 4 h, although the hydrolysis was not yet complete in this experiment. At pH 10 and captan and folpet disappeared completely after 4h hydrolysis time, but the transformation yield was poor (55 %) in the case of captan and extremely poor (2 %) in the case if folpet. Alternative reactions must have occurred here.

When extracting for 15 minutes, immediately after pH adjustment, transformation of folpet and captan at pH 7 was minimal, At pH 10 the two compounds hydrolyzed faster with folpet disappearing almost completely and captan declining by ca. 90%. The conversion yields to THPI and PI were, however, not satisfactory being 56% in the case of captan and 42 % in the case of folpet. It seems that at pH 10 folpet conversion is more complete after addition of the extraction solvent rather than in the pH-adjusted sample itself.

In a control experiment it could be shown that PI hydrolysis to phthalamic acid and further to phthalic acid was negligible under these conditions and not the reason for the low conversion yields.

Adjusting pH in different matrices is time consuming due to the inherent buffering of the matrices and would only be practical if an appropriate buffering mixture is used.

3. Hydrolysis by delayed re-acidification after dSPE cleanup with PSA

An additional option for the conversion of captan and folpet tested was the exposition of the two parent compounds to the alkaline conditions following dSPE with PSA. The contact of sample extracts with PSA leads to an increase of the extract pH from values between 4 and 4.5 to values between 7 and 9.

For the test, QuEChERS raw extracts of apples were spiked with captan and folpet (at 0.5 mg/kg), subjected to dSPE cleanup with PSA, and allowed to stand at room temperature for 24 h before re-acidification.

*Table 15: Conversion rates of captan and folpet to THPI and PI (calculated as parents) when conducting hydrolysis **within the PSA-cleaned-up QuEChERS extract of apples***

Sample preparation	THPI	Captan	SUM Captan		PI	Folpet	SUM Folpet
QuEChERS extract of apples spiked, PSA cleanup and re-acidified with a delay of 24h at RT	32 %	66 %	98 %		26 %	55 %	81 %

As can be seen in **Table 15**, decomposition of captan and folpet after 24 h was far from complete with the estimated half-life times being in the range between 30 and 40 h, which means that the

waiting time for a sufficiently complete breakdown of the parents would be in the range of 4-6 days, which is too long for a routine analytical setup. This experiment confirmed that the reaction speed slows down considerably in acetonitrile extracts compared to aqueous samples, and that, due to the occurrence of competitive reactions; the conversion yields of folpet to PI are much lower than those of captan to THPI. Based on the results it can be estimated that if the reaction would be left to finish the yield of PI would be in the range of 50%, which is too low.

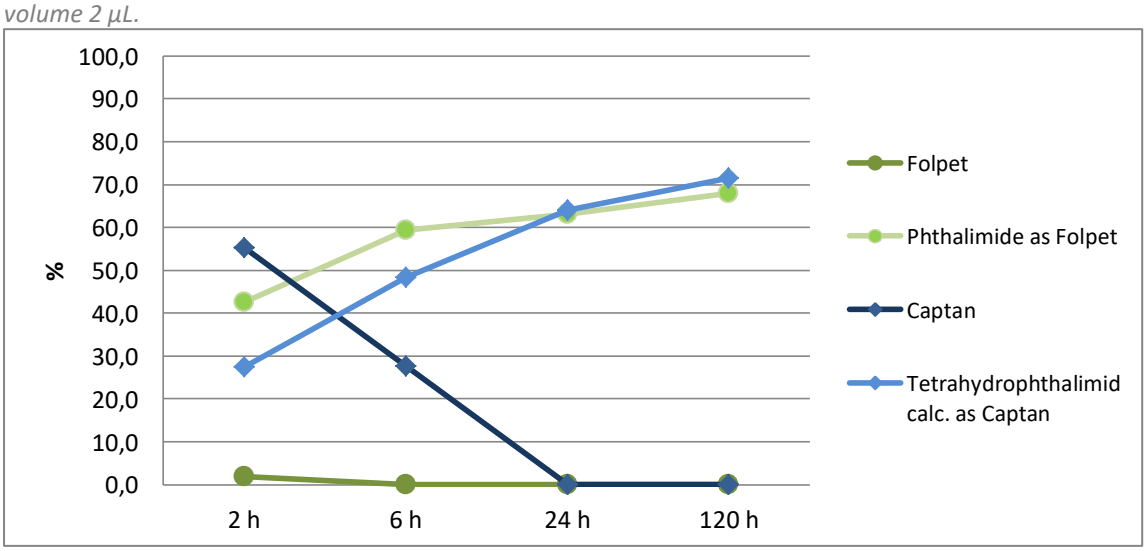
There is, in principle, various options to speed up the reaction including a) addition of catalyzing agents; b) pH increase; and c) temperature increase. The influence of these measures on the conversion yields is difficult to predict and would need to be experimentally studied, with possibly different measures being required to steer the reaction in the preferred direction.

Option a) was estimated being too time consuming in the absence of a hint showing which way to go. Option b) was tested on cleaned-up and raw QuEChERS extracts (see next chapter) and option c) was also briefly checked. In principle heating of reaction mixtures speeds-up reaction kinetics and shortens reaction times. In an experiment the extract was heated to 60°C (ca. 40°C higher than in the initial experiment at RT). By doing this the reaction would be expectedly speed up by ca. 15 fold. Based on the reaction kinetics at room temperature, reaction times of 6 hours for folpet and 10 hours for captan would be expectedly needed for achieving satisfactory breakdown rates at 60°C. An intermediate analysis of the reaction mixtures after a heating time of 2 hours at 60 °C showed a high percentage of parent molecules remaining in the extracts. Due to an accident this experiment could not be completed and will be repeated.

4. Hydrolysis in QuEChERS extracts

In a next step, 50 µL NH_4OH was added to 1 mL QuEChERS raw extract of cucumber cleanup and the reaction progress was monitored over 120 hours at two different temperatures, room temperature and 60°C. The results are shown in **Figure 13** and **Figure 14**.

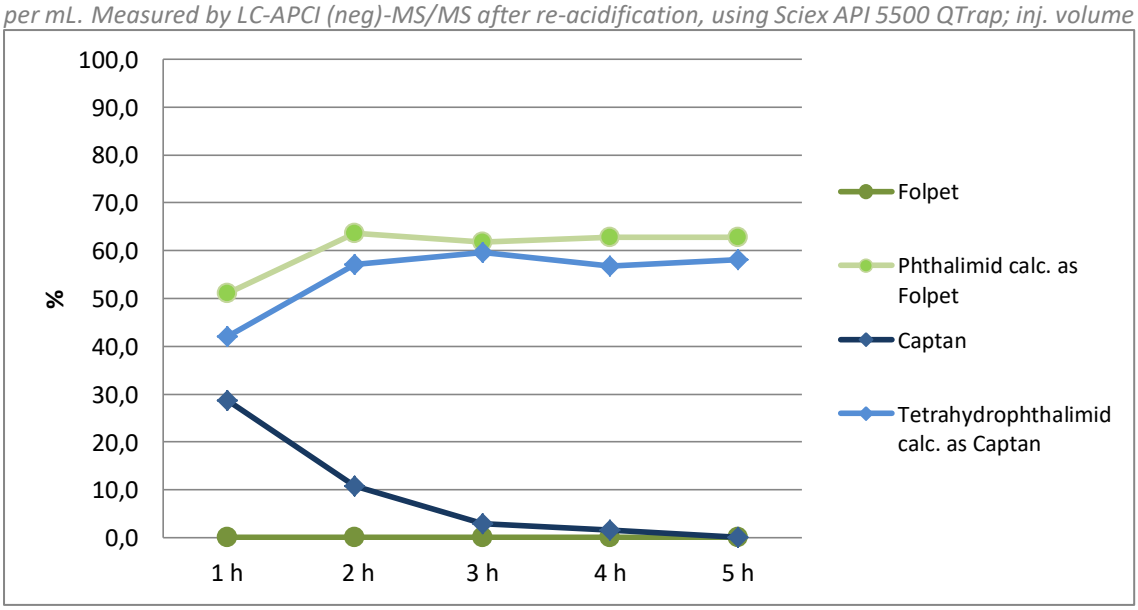
Figure 13: *Hydrolysis of captan and folpet in QuEChERS raw extracts* ***at room temperature,*** *after adding 50 µL NH_4OH (25%) per mL. Measured by LC-APCI (neg)-MS/MS after re-acidification, using Sciex API 5500 QTrap; inj. volume 2 µL.*



At room temperature folpet required ca. 6h reaction time to disappear but phthalimide continued increasing further over many hours, suggesting the formation of intermediates that slowly convert to PI. Captan required a hydrolysis time in the range of ca. 24h to disappear with THPI signals continuing to be increasing even after the entire captan was gone.

At 60°C reaction was clearly faster with captan and folpet practically disappearing after 3-4 hours. In contrast to the experiment at RT, the concentrations of THPI and PI did not increase further after the parents had gone. Still, the absolute yields of THPI and PI (calculated as parents) remained insufficient, indicating that competitive reactions are taking place.

Figure 14: *Hydrolysis of captan and folpet in QuEChERS raw extracts* ***at 60°C*** *after adding 50 µL NH_4OH (25%) per mL. Measured by LC-APCI (neg)-MS/MS after re-acidification, using Sciex API 5500 QTrap; inj. volume 2 µL.*



Discussion and outlook

Lots of effort was put by the EURL-SRM in investigating various possibilities for the accurate determination of captan and folpet together with their respective metabolites (THPI and PI), which recently became legally relevant. Still, analysis within a routine workflow remains challenging. Different possibilities for quantification via GC-MS/MS were summarized in an earlier analytical observations document. Within the present work analysis via LC-MS/MS was thoroughly, but not yet comprehensively studied.

Experience so far indicates that LC-MS/MS is well suited for re-analyzing and accurately quantifying captan (sum) and folpet (sum) in case of GC-MS/MS results suggesting presence of one or more of the four compounds as well as in case of suspected MRL-violation. Considering the low MRLs and the complex residue definition of captan (sum) and folpet (sum), further improvements in sensitivity are needed before this approach becomes fully applicable for routine control of captan and folpet MRLs in food.

Various hydrolysis experiments have been carried out varying matrix, temperature, time, alkalinity and dilution solvents (not all presented here). The relationship of the different parameters for

hydrolysis turned out to be quite complex and the conversion yields were in many cases not satisfying. Further studies are needed in this area as well.

The following investigations would be needed to further improve analysis:

- Improve sensitivity of LC-MS-MS measurement by further optimizing gradient composition
- Optimization of gradient to avoid matrix peaks at retention time of captan / folpet (especially in ESI mode)
- Check further applicability of isotopically labeled standards in analysis
- Measurement of routine samples previously analyzed by GC-MS/MS and result comparison:
    - to gain more experience with the influence of different matrices on analysis
    - to compare the ratio parent / metabolite between LC- and GC- results (and visualize the problem of THPI/PI overestimations during GC-analysis)
- Optimize further hydrolysis conditions (combination of temperature, time, solvent pH) to improve conversion yields and reaction reproducibility.

Literature

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History

Action	When	Document Version
Initial Experiments	On various occasions in 2016 and 2017	V1
Further Experiments	Within 2018 and in Feb-Mar 2019	V1
Observation document placed on-line	June 2019	V1