EURL-SRM - Analytical Observations Report

Concerning the following…

- **Compound(s)**: Phthalimide, Tetrahydrophthalimide
- **Commodities**: Plant origin
- **Extraction Method(s)**: CEN-QuEChERS
- **Instrumental analysis**: LC-MS/MS

Analysis of the folpet degradant phthalimide and the captan degradant tetrahydrophthalimide by QuEChERS and LC-MS/MS

Version 1 (January 2022)

Background information

In 2016, the legal residue definitions for captan and folpet were modified to include their respective degradants tetrahydrophthalimide (THPI) and phthalimide (PI)1,2. This inclusion has lessened the need for labs to take measures to minimize the degradation of captan and folpet during the various stages of analysis (sample comminution, extraction, cleanup, extract storage, see Figure 1), but has also created new challenges for the labs, as GC-analysis of PI and THPI is tricky. This is because the THPI and PI signals obtained in GC originate partly from the THPI and PI amounts originally present in the extracts and partly from those amounts formed from captan and folpet within the hot GC injector. Using ILIS or other suitable calibration techniques, the thermal losses of captan and folpet within the GC-injector are corrected. The degradants formed, however, add up to the existing THPI and PI signals and are thus overestimated. Summing up an already corrected GC-result of the parent compound to the overestimated GC-result of the degradation product (expressed as parent) will lead to overestimated results for the sum.

A procedure, in which the 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[3]. 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 the said document.

LC-MS/MS offers a possibility to circumvent the problems and errors associated with direct GC analysis of PI and THPI. In the case of PI, analysis via LC-MS/MS additionally circumvents the risk of false positives, resulting when compounds other than folpet (e.g. phthalanhydrite) thermally decompose to PI during GC-injection.

[1] COMMISSION REGULATION (EU) 2016/452 of 29 March 2016 (dealing with captan)
[2] COMMISSION REGULATION (EU) 2016/156 of 18 January 2016 (dealing with folpet)
[3] http://www.eurl-pesticides.eu/userfiles/file/EurlSRM/meth_CaptanFolpet_EurlSRM.pdf

In this context, it should be kept in mind, that PI levels, unrelated to folpet, may be also formed during sample processing (mainly drying), for example when phthalates or phthalanhydrite react with nitrogen-containing compounds[4,5]. The LC-MS/MS approach cannot distinguish between the PI levels originating from folpet and those levels of other origin. It is further worthwhile noticing, that THPI is also not fully specific to captan, as it is also formed from captafol, which was, however, internationally banned many years ago and thus unlikely to be used[6].

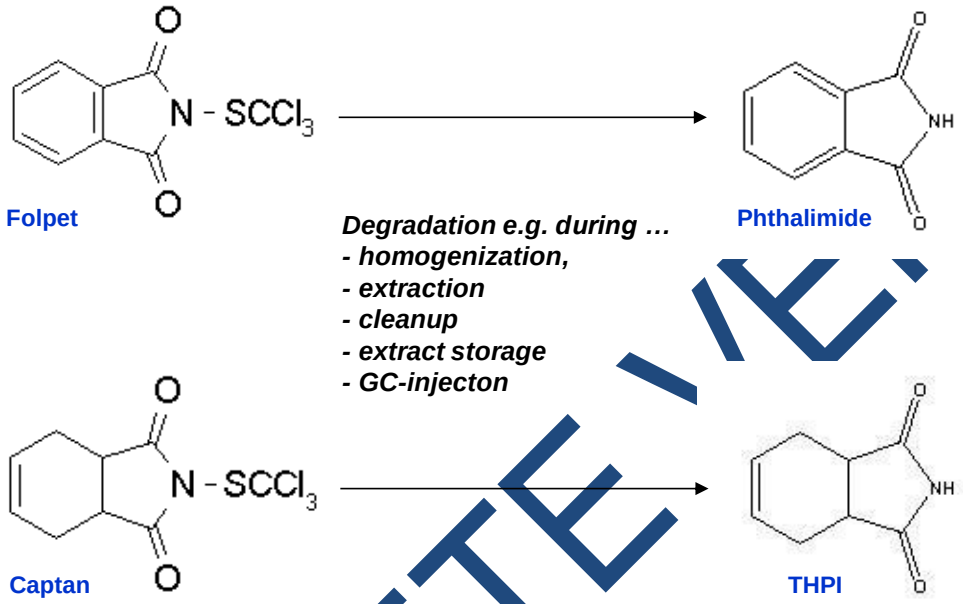


Figure 1: **Degradation of captan and folpet to THPI and PI and compilation of critical steps during analysis**

Brief trials in 2016-19, for direct LC-MS/MS analysis of THPI and PI and of captan and folpet (as such or as in-source fragments) in one single run, 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 at least for the metabolites PI and THPI. A method using QuEChERS and LC-ESI (neg)-MS/MS was therefore published by the EURL[7]. However, a lack of sensitivity during routine analysis was observed for PI and THPI with this approach. Additionally, the method lacked of specifity as for PI just one "real" mass transition was obtained, while the other mass trace was a pseudo-MRM (parent mass/parent mass, m/z 146/146).

[4] Relana (2016/07/22): http://www.relana-online.de/wp-content/uploads/2016/07/PP_16-03_Folpet-PI_vers20160722.pdf

[5] Maximilian Wittig, Julia Biller, Athanasios Nitsopoulos, Albrecht Friedle; Food Chemistry; Volume 374, 16 April 2022, 131544; De novo formation of phthalimide from ubiquitous phthalic acid derivatives during the drying process of tea (Camellia sinensis) and selected herbal infusions

[6] Captafol was identified as a carcinogen and is was withdrawn from the German market in 1986. It is not approved in the EU and according to **https://ntp.niehs.nih.gov/ntp/roc/content/profiles/captafol.pdf** "… *by 2010, no countries were identified that still allowed the use of captafol on food crops.*" Furthermore, captafol is among the compounds the trade of which is regulated by the Rotterdam convention.

[7] https://www.eurl-pesticides.eu/library/docs/srm/EurlSrm_Observation_Captan_Folpet_LC-V1.pdf

A new attempt was thus made to check whether good sensitivity can also be achieved in the ESI positive mode. Both PI and THPI showed better fragmentation patterns in the positive mode, resulting in several useful mass transitions, see Figure 2.

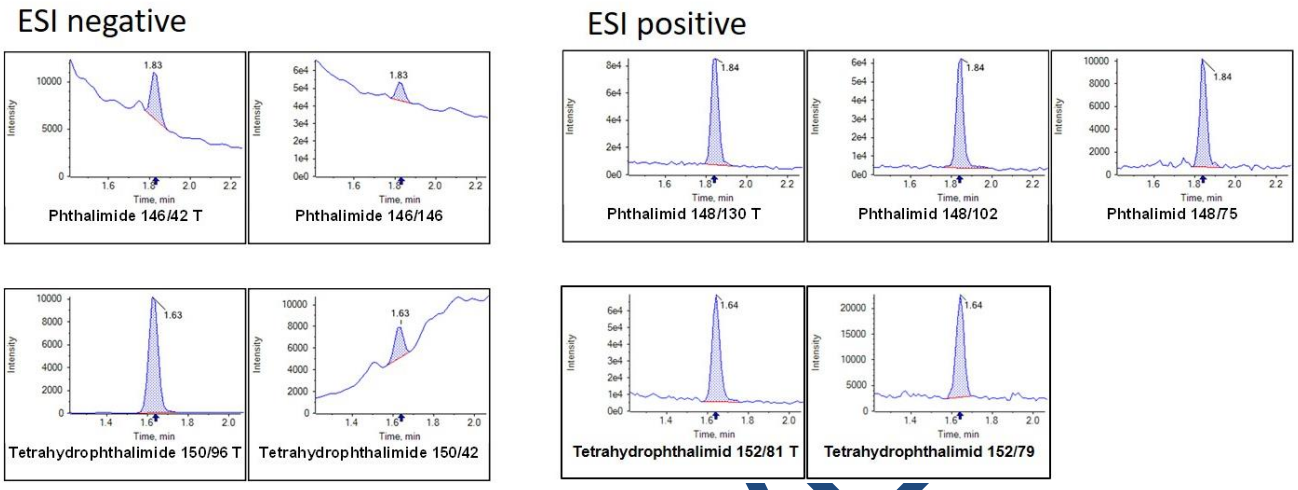


Figure 2: Exemplary comparison of ESI modes using the same eluent conditions for a solvent standard of 0.01 µg/mL in acetonitrile.

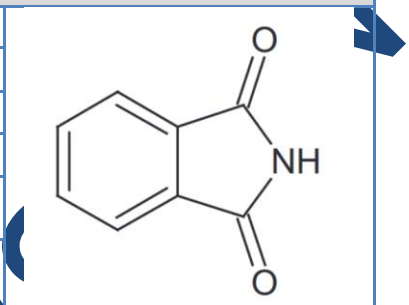
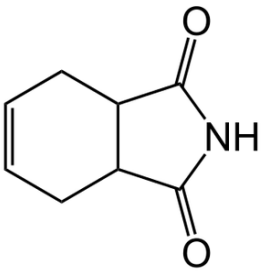
Using this approach, PI and THPI were analyzed by LC-MS/MS in the ESI (pos) mode from QuEChERS extracts. The procedure is straightforward and sensitive, and does not require high-end instrumentation as both PI and THPI are measured sensitively in the ESI positive mode. The procedure has the potential for being incorporated into the multiresidue scheme of labs. If not incorporated it may also run standalone and employed in case of a detection of a marker compound by a routinely employed method (e.g. detection of captan and/or tetrahydrophthalimid by a GC-based method).

Analyte properties

The physicochemical properties and additional information on phthalimid and tetradhydropthalimid are given in Table 1. The data on their respective parent compounds and additional analytical strategies and information can be found in the observations on “Quantification of Residues of Folpet and Captan in QuEChERS Extracts” (Report SRM-07) [8] and “Analysis of Captan, Folpet and their respective metabolites Phthalimide and Tetrahydrophthalimide via LC-MS/MS either directly or following hydrolysis” (Report SRM-42) [5].

[8] https://www.eurl-pesticides.eu/userfiles/file/EurlSRM/meth_CaptanFolpet_EurlSRM.pdf

Table 1: Chemical Properties of PI and THPI

Phthalimide (CAS: 85-41-6) Other names: 1,2-benzenedicarboximide, 1H-isoindole-1,3(2H)-dione		
Parameter	Value/Notes	
Molecular Mass	147.133 g/mol	
Formula	$C_8H_5NO_2$	
Boiling point	366 °C	
pKa	8.4 moderately acidic (computed by chemicalize.com)	
LogP	• Chemicalize.com (computed): ~ 0.68 up to pH 7; drops dramatically from pH 8 onwards; 0 at pH 9; -0.75 at pH 10	
Water solubility	• Chemicalize.com (computed): ~1.8 mg/mL at pH up to 8; increases dramatically from pH 9 onwards • ECHA: 360 mg/l at 25°C	
Stability	Hydrolysis to phthalic acid via phthalamic acid as an intermediate	
Residue definition (EU)	Folpet (sum of folpet and phtalimide, expressed as folpet); Reg. (EU) 2018/832	
Approved in …	**Folpet**: AT, BE, BG, CY, CZ, DE, DK, EL, ES, FR, HR, HU, IE, IT, LU, MT, NL, PL, PT, RO, SE, SI, SK	
Toxicity	Phthalimide itself is not classified according to Reg. 1272/2008 due to its low toxicity in general [9,10]. No ARfD or ADI is set for phthalimide. EFSA (2017): The toxicological reference values of the parent apply to the metabolite phthalimide for the consumer risk assessment.	
Other sources	Possible metabolite from phosmet and ditalimphos; rarely found among a few burning coal fire sites as the natural mineral kladnoite[11]. 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[4,5]. A formation of phthalimide from phthalic anhydride and phthalic acid in the hot GC-injector also takes place.	
Tetrahydropthalimde (CAS: 1469-48-3) Other names: (3aR,7aS)-2,3,3a,4,7,7a-hexahydro-1H-isoindole-1,3-dione, 4-Cyclohexene-1,2-dicarboximide		
Parameter	Value / Notes	
Molecular Mass	151.165 g/mol	
Formula	$C_8H_9NO_2$	
Boiling point	337 °C	
pKa	10.4 slightly acidic (computed by chemicalize.com)	
LogP	• Chemicalize.com (computed): ~ 0.16 up to pH 9; drops dramatically from pH 10 onwards; -0.5 at pH 11	
Water solubility	• Chemicalize.com (computed): ~30 mg/mL at pH up to 9; increases dramatically from pH 10 onwards • ECHA: 12.2 g/l at 20 ± 0.5 °C at pH 3.4	
Stability	Hydrolytically quite stable	
Residue definition EU	Captan (Sum of captan and THPI, expressed as captan); Reg. (EU) 2019/1015	
Approved in …	**Captan**: AT, BE, BG, CY, CZ, DE, EE, EL, ES, FR, HR, HU, IE, IT, LT, LU, LV, NL, PL, PT, RO, SI, SK	
Toxicity	Tetrahydrophthalimide itself is not classified according to Reg. 1272/2008 due to its low toxicity in general [9,10]. No ARfD or ADI is set for tetrahydrophthalimide. "… 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 sources	Possible metabolite of captafol	

[9] Reg. (EC) No 1272/2008 of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Reg. (EC) No 1907/2006, amended by Commission Delegated Regulation (EU) 2021/1962 of 12 August 2021

[10] https://echa.europa.eu/de/registration-dossier/-/registered-dossier/13146/7/11/6

[11] https://www.mindat.org/min-2222.html

Apparatus, Chemicals and Consumables

Chemicals and Materials
The used materials and apparatuses are listed in the QuEChERS standard procedure (EN-15662).

Analytical standards of the analytes
The suppliers of the used analytical standards are shown in Table 2.

Table 2: Sources of Analytical standards (exemplary).

Compounds	Details on standards used		Provider
Phthalimide	Supplier Code / Purity	674338 (99.7 %)	HPC
Tetrahydrophthalimde	Supplier Code / Purity	DRE-C17406500 (99.0 %)	Dr. Ehrenstorfer GmbH

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

Stock solutions of both substances (both e.g. 1 mg/mL) are prepared in acetonitrile, taking the purity of the standard substance into account, and stored in a refrigerator for up to 48 months. Working solutions, e.g. in terms of a mix of both substances, are prepared as necessary in acetonitrile and may be stored in the refrigerator for many months until use.

Sample Preparation

Homogenization:
Samples are homogenized by cryogenic milling using dry ice according to Document Nº SANTE/12682/2019.

Sample preparation:
The samples are extracted according to the QuEChERS-CEN (citrate-buffered) method without applying dSPE-cleanup. As internal standards chlorpyrifos-D_{10} and propyzamide-D_3 (e.g. 100 µL of a mixture in acetonitrile at 10 µg/mL each) may be used. The internal standards are added to the sample portion before extraction. Isotope labelled PI and THPI may also be used to correct for matrix effects even when using a calibration standard based on a different matrix or based on solvent[12].

Measurement:

The extract is directly subjected to LC-MS/MS separation and measurement.

Exemplary LC-MS/MS conditions are given in Table 3.

[12] In this case keep in mind that captan D6 and folpet D4 may also degrade to PI-D4 and THPI-D4 thus influencing the signals.

Table 3: Instrumentation and method details (LC: Agilent 1290 Infinity II; MS: Sciex QTrap 5500+)

Instrument parameters	Conditions						
Column/temperature	Waters Acquity BEH C_{18}, 2.1x100 mm, 1.7 µm; 40 °C						
Pre-column	Van Guard BEH C_{18} 1.7um						
Eluent A	0.01% acetic acid in Water + 5 % acetonitrile						
Eluent B	0.01% acetic acid in acetonitrile						
Gradient	%A	Flow [mL/min]	Time [min]				
	95	0.4	0				
	10	0.4	3.00				
	10	0.4	6.00				
	95	0.4	6.10				
	95	0.4	10.00				
Injection volume	2 µL						
Acquired mass transitions (m/z)	Compound	Mass transitions and their MS-parameters					
		Q 1 (m/z)	Q 3 (m/z)	DP[1] (V)	CE[2] (V)	CXP[3] (V)	
	Phthalimde	148	130	66	23	10	
		148	102	66	35	10	
		148	75	66	37	12	
	Tetrahydrophthalimde	152	81	101	19	10	
		152	79	101	33	12	
	Chlorpyrifos-D_{10} (internal standard)	360	199	95	23	12	
	Propyzamid-D_3 (internal standard)	259	193	61	21	10	
Ionisation mode	ESI Positive						
Ion Source Parameters	Curtain Gas Flow	40 psi					
	Ion Spray Voltage	4500 V					
	Temperature	550 °C					
	Nebulizer Gas Flow	60 psi					
	Heater Gas Flow	70 psi					

1) DP: Declustering Potential
2) CE: Collission Energy
3) CXP: Cell Exit Potential

Validation data:

Validation experiments for phthalimide and tetrahydrophthalimde were conducted using grape homogenate. Both substances were spiked in quintuplicate to 10 g portions of sample homogenate using a mixture of both substances in acetonitrile prepared as described above. The obtained recovery rates and the observed matrix effects are shown in Table 4. Matrix effects were calculated by comparing the respective signal intensities obtained from a standard solution prepared in extract of the respective blank matrix with the signal intensities obtained from an equally concentrated standard solution prepared in acetonitrile. Exemplary chromatograms are shown in Figure 3.

Table 4: Recoveries, relative standard variations (RSD) and the matrix effect for the validation of PI and THPI in grapes at 0.002 mg/kg 0.005 mg/kg and 0.010 mg/kg, each n = 5.

Substance	Matrix	Spiking level (mg/kg)	Mass trace	Calculation using matrix-matched calibration		Matrix effect[1)]
				Mean Rec.	RSD	
PI	grapes	0.002	148/130	103 %	7 %	-62 %
			148/102	106 %	16 %	
			148/75	102 %	15 %	
		0.005	148/130	91 %	10 %	
			148/102	92 %	7 %	
			148/75	85 %	14 %	
		0.010	148/130	88 %	6 %	
			148/102	97 %	5 %	
			148/75	99 %	4 %	
THPI	grapes	0.002	152/81	99 %	14 %	-71 %
			152/79	96 %	14 %	
		0.005	152/81	91 %	5 %	
			152/79	86 %	15 %	
		0.010	152/81	91 %	8 %	
			152/79	89 %	14 %	

1) Calculated from the mean peak areas of the respective matrix-matched calibration vs. the mean peak areas of the solvent calibration (120 % calibration level, each) of the quantifier mass trace of each substance.

Exemplary chromatograms of the validation experiments:

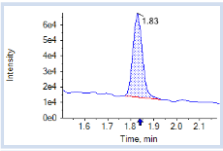
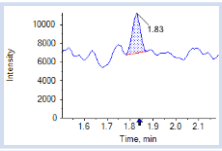
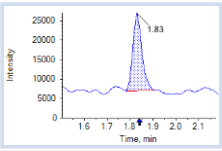
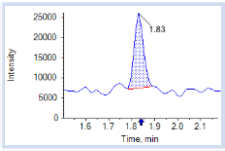
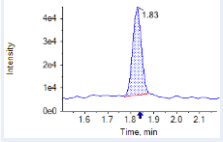
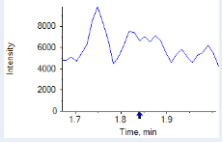
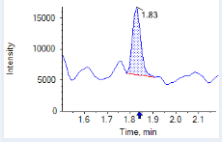
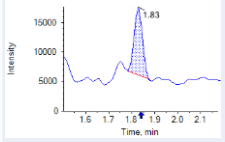
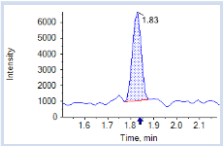
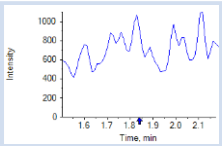
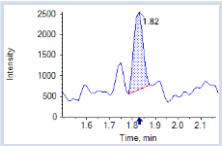
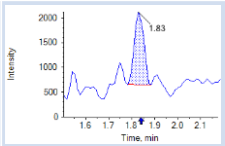
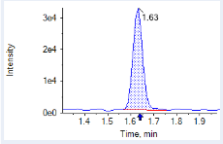
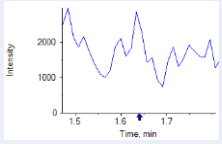
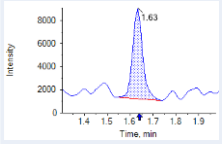
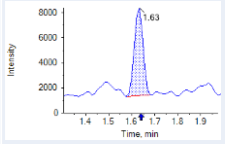
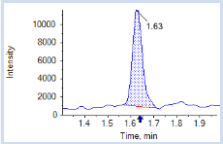
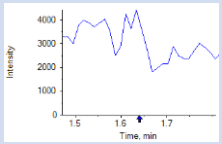
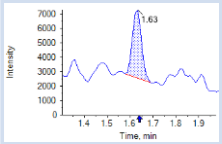
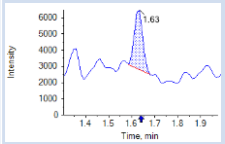
Substance	Matrix	Mass transition	Solvent calibration (120 % level)	Matrix blank	Matrix calibration (120 % level)	Recovery
PI	Grape	148/130				
		148/102				
		148/75				
THPI	Grape	152/81				
		152/79				

Figure 3: Selected chromatograms of the conducted validation of phthalimide and tetrahydrophtalimide in grapes at 0.002 ppm (LOQ).

Intermediate Conclusions and Outlook:

A simple and sensitive method for the analysis of PI and THPI was developed based on QuEChERS extraction. The measurement of both substances involves the determination on a C_{18} column followed by electrospray ionization in the positive ion mode and MS/MS analysis.

Validation of PI and THPI was successful in grapes at 0.002 mg/kg, 0.005 mg/kg and 0.010 mg/kg. This indicates that these compounds may be monitored at very low levels in food of high water content. Further validation experiments on other types of commodities are also planned.

Captan and folpet itself can still be determined with well-established methods using GC. Given the good sensitivity achieved for captan and folpet in GC, and the good sensitivity achieved for THPI and PI by the present procedure, the lowest MRLs for captan (sum) and folpet (sum) in acidic and non-acidic commodities of high water content (at 0.02* and 0.03* mg/kg respectively), are well enforceable. Our further goal is to develop more sensitive LC-MS/MS methods for the parent compounds folpet and captan either by implementing them in a method that covers them and the respective degradants or in another routinely applicable method preferably covering multiple analytes.

The procedure thus has the potential for being incorporated into the multiresidue scheme of labs. If not incorporated the procedure may also run standalone and employed in case of a detection of a marker compound by a routinely employed method (e.g. detection of captan and/or tetrahydrophthalimid by a GC-based method).

History

Action	When	Document Version
Experiments	Q2 2020: Final development of the LC-MS/MS method for the determination of PI and THPI Q3 2020: Validation of PI and THPI on grapes	
Observation document placed online	January 2022	V1