EURL-SRM – Analytical Observation Report

concerning the following…

- **Compound(s)**: Captan, Folpet, Phthalimide (PI), Tetrahydrophthalimide (THPI)
- **Commodities**: Fruit and vegetables, cereals
- **Extraction Method(s)**: QuEChERS, A-QuEChERS
- **Instrumental analysis**: GC-MS, GC-MS/MS

Quantification of Residues of Folpet and Captan in QuEChERS Extracts

Version 3.1 (last update: 06.04.17)

Short description:

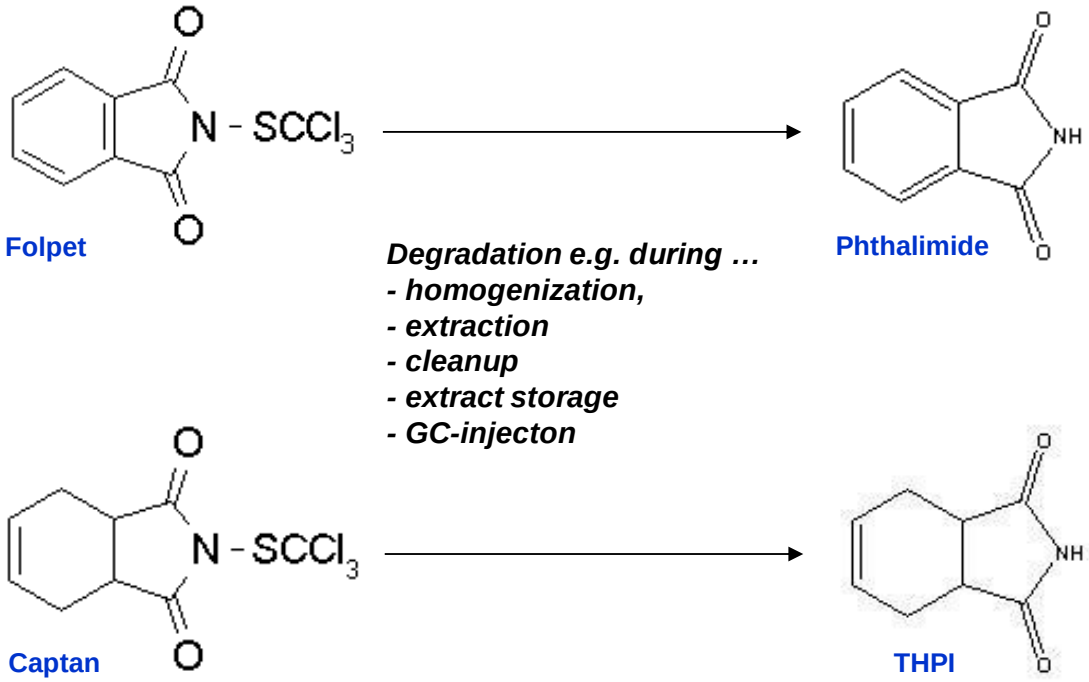
This document describes approaches for the analysis of captan and folpet in QuEChERS extracts via GC-MS or GC-MS/MS. Different approaches for correcting the results of the parent molecules for matrix effects during GC analysis or for losses during the entire procedure are presented and discussed. In addition two approaches for analyzing captan and folpet next to their legally relevant metabolites tetrahydrophthalimide (THPI) and phthalimide are presented and discussed.

Background Information:

Captan and folpet are analytically among the most challenging pesticides due to their non-amenability to LC-MS/MS under standard conditions, and their tendency to degrade both in solution as well as during GC-injection. Special attention is also needed to avoid extensive degradation in standard solutions prepared in e.g. acetonitrile (which can be typically reduced by acidification). Attention is also required to prevent degradation during sample comminution or storage of homogenates, which is especially pronounced, when dealing with commodities of high pH and when working at ambient temperature. When employing the CEN-QuEChERS method, attention is furthermore required during the first extraction step (prior to buffering), especially when dealing with non-acidic commodities such as vegetables or cereals. Particularly critical is the dSPE cleanup step with PSA as sorbent, during which the

pH rises to values in the range between 7 and 9. Quick re-acidification of the cleaned-up extracts with formic acid is recommended here to reduce degradation and ensure good stability of the analytes, when extracts are stored for an extended time prior to measurement. Skipping cleanup with PSA as sorbent is an alternative.

An additional critical point is GC-analysis, as these compounds tend to degrade during injection in the hot GC-liner, which results in two peaks, the parent and the degradant.



This effect very much depends on the condition of the GC-injector (i.e. the activity of the liner-surface). During the lifetime of a GC-liner, non-volatile compounds increasingly accumulate on its surface increasing the number of active sites which interact with analytes, quasi catalyzing their thermal breakdown. Co-extracted matrix components act competitively by masking these active sites thus reducing interactions between liner-surface and analytes and reducing degradation. These matrix effects can lead to massive errors in quantification, if these effects are not sufficiently compensated between sample extracts and calibration standards. Matrix-

matched calibration (including the approaches of standard addition and procedural calibration) are often used for this purpose. Analyte protectants (APs)[1] have a similar effect by acting as an artificial matrix. The use of split injection mode, which reduces the dwell time of the analytes in the injector, has also been shown to reduce degradation and to considerably improve precision[2]. By using isotope labelled internal standards (ILISs) matrix effects can be effectively compensated even when matrix effects between sample and calibration solution are very different as ILISs behave in exactly the same way as the native analytes.

Following reasoned opinions by EFSA on captan[3] and folpet[4] in 2014, the residue definitions of these two compounds have changed in 2016, now entailing both the parent and the main degradation products THPI and phthalimide[5,6]. With this change, the occurrence of THPI and phthalimide as significant metabolites in primary crops, their formation from the parents during food processing, as well as during analysis in the laboratory were taken into account. Nevertheless, this change has also confronted the labs with new analytical challenges related to the fact that upon GC-injection considerable parts of the parent compounds convert to THPI and phthalimide. When analyzing the parents typically the intention is to either largely equalize the degradation in sample and calibration (e.g. via matrix-matching) or to compensate these effects (e.g. via ILIS). In other words the result for the parent is corrected to account for its GC-losses and at the same time the degradation product is formed producing a higher signal than that produced by the degradation product originally present in the extract. A simple summing up the corrected parent result with the result of the degradation product would thus leads to an overestimation. To avoid this, the GC-originated parts of the degradation products need to be deducted from the totally detected THPI and phthalimide.

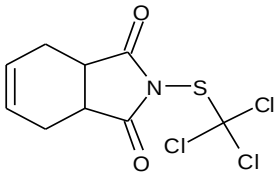
[1] **http://www.eurl-pesticides.eu/library/docs/srm/EURL_Observation-APs.pdf**
[2] http://tools.thermofisher.com/content/sfs/brochures/24-Cochran-Shoot-and-Dilute-GC-MSMS.pdf
[3] EFSA Journal 2014;12(4):3663 [55 pp.]. doi: 10.2903/j.efsa.2014.3663
[4] EFSA Journal 2014;12(5):3700 [55 pp.].doi: 10.2903/j.efsa.2014.3700
[5] COMMISSION REGULATION (EU) 2016/452 of 29 March 2016 (dealing with captan)
[6] COMMISSION REGULATION (EU) 2016/156 of 18 January 2016 (dealing with folpet)

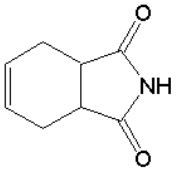
Unfortunately LC-MS/MS, cannot be used to circumvent these problems, as neither the parents nor the two metabolites can be sensitively analysed at least in the ESI-mode. GC-measurement is thus the approach to be followed.

The ubiquitous presence of compounds that may, under certain conditions, transform to phthalimide, such as phthalic acid and phthalic anhydride creates some additional problems with the interpretation of MRL exceedances of dried products and the evaluation of organic products (see Relana position paper[7]). The pesticides phosmet and ditalimphos are also reported to metabolize to phthalimide. The new residue definition of captan is not specific, either as THPI is also formed from captafol.

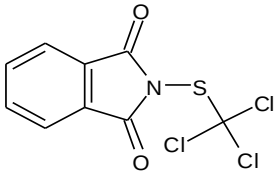
[7] Relana (2016/07/22): **http://www.relana-online.de/wp-content/uploads/2016/07/PP_16-03_Folpet-PI_vers20160722.pdf**

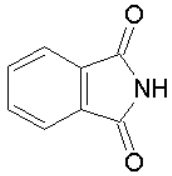
Compound details

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 (intermediate polarity)	
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)	12 h 1.7 h	pH4 25°C pH4 40°C	From JMPR Report 2000[8] (referring to Yaron, 1985)
	2.6 h 0.5 h	pH7 25°C pH7 40°C	
	Too fast to measure	pH9 25°C pH9 40°C	
Residue defin. 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)		

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	0.3 0.58 ca. 0.46	**pH dependent logKow but constant up to pH 9** at 25°C (ECHA, predicted) at pH 1-9 (calculated by Chemicalize.org) 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[9])	
Residue definition EU	Captan including THPI		
Approved in…	See Captan		
ADI / ARfD	… Metabolites 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 for these compounds. … 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.		

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

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)		
Hydrolysis rates in water (DT_{50})	2.6 h	at pH 5 at 25 °C	JMPR Report (1999)[10]
	1.1 h	at pH 9 at 25 °C	
	1.1 min	at pH 9 at 25 °C	
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)		

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 (calculated by Chemicalize), 8.3 (ECHA)		
LogPow	0.69 0 -0.75	**pH dependent logKow but constant up to pH 7** at pH 1-7 (calculated by Chemicalize.org) at pH 9 (calculated by Chemicalize.org) at pH10 (calculated by Chemicalize.org)	
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 (DT50)	115 d	pH4 / 25°C	From ECHA (quoting OECD)
	56.7 h	pH7 / 25°C	
	1.1 h	pH9 / 25°C	
Residue definition EU	Folpet (sum of folpet and phtalimide, expressed as folpet) (R)		
Approved in…	See Folpet		
ADI / ARfD	available studies demonstrate a lower toxicity of phthalimide compared to parent folpet. Phthalimide does not present acute toxicity, its LD50 in mice is above 5 mg/kg bw, it is not mutagenic …. it does not exhibit developmental toxicity; … does not have the potential to induce carcinogenic effects.(EFSA Reasoned Opinion 2014[11])		
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[12]. A formation of phthalimide from phthalic anhydride and possibly also phthalic acid in the hot GC-injector seems possible.		

[10] JMPR (1999): http://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/JMPR/Evaluation99/18Folpet.pdf
[11] EFSA Journal 2014;12(5):3700 [55 pp.].doi: 10.2903/j.efsa.2014.3700
[12] Relana (2016/07/22): http://www.relana-online.de/wp-content/uploads/2016/07/PP_16-03_Folpet-PI_vers20160722.pdf

Materials (exemplary)[13]:

- Captan from Sigma/Aldrich Cat. No. 32054 Sigma-Aldrich
- Folpet from LGC, Cat No. DRE-C13890000 – folpet
- Captan D6 (3,3,4,5,6,6-d6) 98 atom %, Cat No. 805750 Aldrich
- Folpet D4 from LGC, DRE-C13890100
- Formic acid (>96%) was purchased from Sigma-Aldrich
- Acetic acid (>99%) was purchased from Sigma-Aldrich
- Stock solutions of captan, captan D6, folpet and folpet D4 at 1 mg/mL each were prepared by dissolving 15mg of the compounds in 1 mL acetone and filling it up to 15 mL with acetonitrile **containing 0.4 % acetic acid (v/v)** *
- Working solutions were prepared by appropriately diluting stock solutions with acetonitrile **containing 0.4% acetic acid (v/v)***
- All other materials and chemicals used as listed in EN 15662

*** Captan and folpet tend to degrade in acetonitrile standards unless they are acidified**

General considerations regarding extraction, cleanup and GC-analysis:

As mentioned above, both captan and folpet are prone to degradation at various stages of the analytical procedure, with captan mainly transforming to THPI and folpet to phthalimide. Degradation is typically accelerated by high pH and high temperatures and already starts during sample comminution with extensive losses being recorded when non-acidic commodities are milled at ambient temperatures. To some extend degradation also takes place during the first QuEChERS extraction step, with again non-acidic commodities being more problematic. Dispersive SPE cleanup with PSA as sorbent is critical as the extract pH raises considerably causing degradation, which can be minimized if re-acidification (of the isolated extract) is done immediately after the cleanup step. To be on the safe side d-SPE cleanup may be skipped. In view of the new residue definitions, which refer to the sum of parent and degradation product, losses during extraction and cleanup are not critical if the conversion rate to the respective degradation products is very high.

[13] **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

In principle the simplest approach for the **analysis the sum of the parents and their degradation products** would involve full transformation of the parents to the degradation products and analysis of the latter. This transformation could be done at different stages e.g. within sample homogenates, within the extracts or during GC-measurement. A prerequisite for this approach to be successful is that the transformation rates are nearly quantitative. If transformation rates are low, either because of incomplete decomposition of the parent or due to formation of other products the final result might not fulfill the validation criteria in terms of bias. Initial experiments to chemically convert captan and folpet directly in QuEChERS extracts by rising pH (results not shown here) showed conversion rates which were not fully satisfactory. Experiments to achieve satisfactory conversion rates will be continued.

In case of significant parent-degradation at any stage of the procedure, it is very important to keep in mind that if these losses of the parent are corrected (e.g. via ILIS or other approaches), **the result of the summed residue definition cannot be simply derived by summing up the (corrected) parent result and the result of the degradation product (calculated as parent).** This is because the parts of the degradation product formed during analysis are already considered in the corrected result of the parent. **A simple summing up would thus lead to an overestimated result.**

This also applies in cases where the parent-result is corrected only by compensating matrix effects during GC (e.g. via ILIS, matrix-matching or other approaches). The signal of the degradation product will consist of the signal of the degradation product originally present in the sample plus the signal of the degradation product formed in the GC-injector. For quantifying the part of the degradation product that was originally present in the final the parts of the signal formed during GC-injection is required. For this requires knowledge of the original parent concentration in the sample, which can be accurately determined with the help an ILIS, and the knowledge of the transformation rate of Parent to degradation product. Two approaches allowing the parallel determination of both the parents (captan or folpet) and the degradation products (THPI or phthalimide) in the final extracts, is presented in page 21ff.

When it comes to determining only the concentration of the parents (captan and folpet) the situation is simpler. Any losses of captan and folpet can be compensated conveniently **using ILISs** (e.g. captan D6 and folpet D4). If ILISs are added to the homogenate prior to extraction (e.g. 100 µL of a 10 µg/mL solution) their signals can be used to compensate for any losses of the respective native parents occurring during all succeeding steps of the analysis (extraction, cleanup, extract storage, measurement). Losses occurring prior to the addition of the ILIS (e.g. during sample comminution) are, however, not compensated this way. Cryogenic milling and keeping the homogenates frozen until analysis, will minimize such losses. Errors related to deviating concentration of the analytical standard are also not compensated by the use of ILISs. If ILIS is added to extract aliquots it will only compensate for losses during measurement (i.e. matrix effects).

Behaving in exactly the same manner as the analytes, **ILISs can effectively compensate for matrix effects during GC analysis, even when calibration standards are not matrix-matched.** Still some protection of the compounds during sample preparation and measurement is needed to avoid that the final signals become too small to measure. An extensive or even complete degradation of the parent compound in GC will increase analytical uncertainty and may even lead to false negative results (e.g. if the degradation products show poor detection sensitivity). Protection during extraction may include acidification (e.g. use of the acidified QuEChERS approach, see below) and skipping of cleanup with PSA. Protection during measurement may include the addition of **Analyte Protectants** (APs) to both the sample extract and calibration standard(s) (see respective document in the EURL-Website[14]). It is further recommended to **avoid preparing calibration standards in pure solvent** and to maintain the GC-inlet in good operative condition (not too dirty).

Procedural calibration and the approach of **standard addition to sample portions** are alternative approaches to compensate for losses of the parents during the entire procedure, but may not satisfactorily compensate the variability of measurement,

[14] **http://www.eurl-pesticides.eu/library/docs/srm/EURL_Observation-APs.pdf**

which is often quite high for these compounds, even when matrix-matching is used. **The use of ILISs alone or in combination with other approaches, such as procedural calibration, standard addition to sample portions or standard addition to extract aliquots, is thus preferable**.

Experimental

1) Extraction:

Citrate buffered QuEChERS (EN 15662) or the **acidified QuEChERS (A-QuEChERS)** procedure may be applied for these compounds.

acidified QuEChERS procedure (A-QuEChERS) corresponds to the EN 15662 with the only difference being the use of 10 mL acetonitrile containing 1% formic acid instead of pure acetonitrile for extraction and of 4g MgSO4 and 1g NaCl (no citrate buffer salts) for partitioning. Using the EN-15662 method there is higher risk of degradation during various stages of the procedure (e.g. cleanup), especially with dealing with high-pH commodities. Measures for compensating these losses may thus need to be taken (see various options below). A-QuEChERS is more protective for captan and folpet not only during extraction but also during measurement. The extracts, however, typically contain more co-extractives.

2) Measurement:

As both parents and both metabolites cannot be sensitively analyzed by LC-MS/MS (using ESI interface), GC-techniques need to be employed, with all the accompanying problems of thermal degradation in the injector and matrix effects. Analysis via GC-MS with negative chemical ionization (NCI) provides good sensitivity for the chlorine containing parents but very poor sensitivity for the decomposition products, especially THPI.

Below **exemplarily** the measurement conditions used in the experiments presented here:

Analysis of captan and folpet (parents) via GC-MS, NCI:

Results were obtained using the following GC conditions:

- GC: Agilent 6890 GC with autosampler and ChemStation Software
- Injector: HP PTV, solvent vent mode, initial temp. 50°C; initial time: 1 min, vent flow: 20 mL/min; vent time: 0,5min; 720°C/min to 300°C; purge time: 2 min
- Column: HP-5MS, 30 m x 0.25 mm I.D. x 0.25 µm
- Carrier Gas: helium at 2.0 mL/min, constant flow mode
- Oven: 40ºC for 2 min; 30ºC/min to 220ºC, 5ºC/min to 260ºC, 20ºC/min to 280ºC hold for 15 min
- Detector: Agilent 5973 MSD; transfer line at 280º C, NCI,
- Reagent gas: Methane;

Table 1: GC-MS(NCI), SIM Ions monitored

Captan	149,150	**Captan D6:**	155, 156
Folpet	146, 147	**Folpet D4:**	150, 151

Important Note: GC-MS in the NCI negative mode provides a very p**oor sensitivity** for THPI and phthalimide. **Therefore: If THPI and phthalimide are to be analyzed use GC-MS or GC-MS/MS.**

Analysis via GC-MS/MS:

- GC: Thermo Trace 1310 with TriPlusRSH autosampler and Thermo TraceFinder 3.2 Software
- Injector: Gerstel KAS, solvent vent mode, initial temp. 50°C; initial time: 0.8 min, vent flow: 20 mL/min; vent time: 0.5 min; 12°C/s to 300°C; purge time: 1.5 min
- Precolumn: Agilent FS deactivated 0.25 mm x 10 m
- Column: Thermo TG5-SILMS, 30 m x 0.25 mm I.D. x 0.25 µm
- Carrier Gas: helium at 2.0 mL/min, constant flow mode
- Oven: 40ºC for 2 min; 30ºC/min to 220ºC, 5ºC/min to 260ºC, 20ºC/min to 280ºC hold for 15 min;
- Detector: Thermo TSQ8000;
- Transfer line temperature: 300ºC;
- Ion source temperature: 280ºC.

Table 2: GC-MS/MS mass transitions:

Compound	Retention time (min)	Mass transitions [m/z]	Collision energy [ev]
THPI	9.32	151 → 79	10
		151 → 122	10
Phthalimide	7.8	104 → 76	10
		147 → 76	25
		147 → 104	10
Captan	10.6	149 → 70	20
		264 → 79	25
		149 → 79	25
		264 → 105	5
Captan D6	10.6	154 → 84	20
		270 → 84	25
		270 → 154	5
Folpet	10.8	260 → 130	15
		262 → 130	15
Folpet D4	10.8	264 → 134	15
		266 → 134	15
Chlorpyrifos D10	9.8	326 → 262	20
		324 → 260	20
		324 → 292	15

3) Degradation of captan and folpet during homogenization and storage of homogenates

Captan and folpet tend to degrade when milling is done at ambient temperature. Degradation occurs partly during the milling process and mainly during the time homogenates are left standing at ambient temperature until extraction will start. Table 3 shows the observed degradation of captan and folpet in homogenates of cucumber and apple at room temperature while standing for 30 or 180 min. Whereas degradation in the cucumber homogenate is very fast, degradation in the acidic apple homogenate was limited.

It is important to consider that any losses prior to extraction cannot be compensated afterwards by none of the approaches correcting for recovery. Cryogenic milling and keeping the homogenates frozen until analysis will minimize the risk of such losses.

Table 3: Degradation of captan and folpet in homogenates of cucumber and apple, while standing for 30 or 180 min at room temperature.

	Cucumber homogenate		Apples homogenate	
	30 min storage	180 min storage	30 min storage	180 min storage
Compounds	Relative recoveries compared to immediate extraction [%]			
Captan	-63%	-96%	-5%	-15%
Folpet	-51%	-85%	8%	-9%

4) Compensating losses of <u>parents</u> (captan and folpet):

There are various possibilities for compensating losses of parents during the sample preparation procedure (extraction/cleanup) or GC-analysis. Table 4 gives an overview of these options. By combining the use of ILISs with all other approaches both precision and accuracy will be typically improved.

Table 4: Approaches for compensating losses

Approach	Corrects for matrix effects in GC?	Corrects for losses during extraction and cleanup?	Notes
Matrix-matched calibration	**Yes**	No	GC-matrix effects are corrected if calibration standards are prepared from a blank extract of the same type of commodity
Procedural calibration	**Yes**	**Yes**	GC-matrix effects are corrected if the same type of matrix is used as blank
Standard addition to extract aliquots	**Yes**	No	
Standard addition to sample portions	**Yes**	**Yes**	Corrects for losses during extraction and Cleanup if the standard addition is done prior to extraction
Use of ILISs	**Yes**	**Yes**	The use of a blank of the same commodity type is not mandatory.
Use of analyte protectants	**Yes, largely**	No	Will correct for matrix effects if added to both calibration solutions and sample extracts. The use of a blank of the same commodity type is not mandatory.

a) Analysis of <u>parents</u> via standard addition to extract aliquots in combination with ILIS

Via standard addition to extract aliquots matrix effects can in most cases be largely compensated. Very sensitive compounds such as captan, folpet and dicofol, however, tend to show poor signal repeatability due to the complex interplay of many factors including the thermal instability of the compounds (which is quasi catalytically accelerated by the interactions with active sites), the liner surface activity (which increases from injection to injection and which may differ from spot to spot within the liner) and the matrix (which plays a competitive role). The multiple injections involved in standard addition (e.g. 4) reduce the risk of obtaining highly biased results due to spurious errors of this kind. In combination with ILIS precision improves considerably allowing the reduction of the number of standard additions from 3 to just 1 or 2 without compromising accuracy. External calibrations using ILIS are an alternative and do not have to be matrix-matched.

Possible workflow:

Initially a preliminary semi-quanitative analysis of captan or folpet is conducted to estimate their approximate levels. Four 1 mL aliquots of the QuEChERS raw extract (representing 1 g in the case of fruits and vegetables and 0.5 g in the case of cereals) are transferred into GC vials. One of the vials is not spiked whereas the other three are spiked with increasing amounts of the analyte to be quantified. The amounts of analyte to be added in the standard additions should be comparable to the expected amount of analyte in the aliquots (x): e.g.

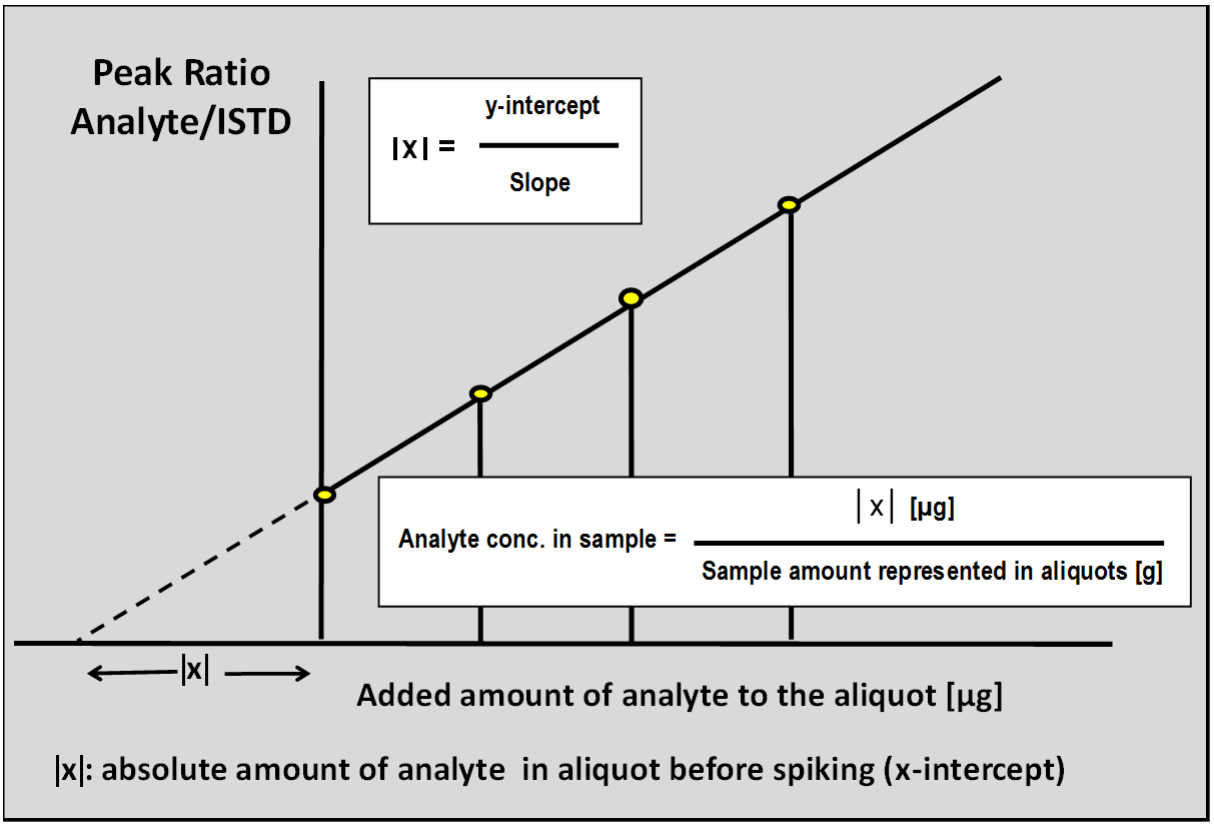
(1) a) No addition, b) + 0.5x, c) +x, d) +1.5x OR

(2) a) no addition, b) + x, c) +2x, d) +3x

Avoid too small additions (which make calibration curve to flat increasing the impact of spurious errors) or too large additions (which make the calibration cure too steep). Important is furthermore that the standard addition curve is linear, as the calculation involves linear extrapolation. To reduce the influence of signal drift avoid injection in the order of increasing or decreasing level, e.g.: 2 → 3 → 1 → 4 or 3 → 2 → 1 → 4 is preferred over 1 → 2 → 3 → 4 or 4 → 3 → 2 → 1

The analyte amount in the original (non-spiked) aliquots is then calculated via linear extrapolation using the regression curve derived by plotting the analyte area (or the area ratio versus an internal standard) against the absolute amount of analyte added to the aliquot. By dividing the derived analyte amount in the non-spiked extract aliquot (in µg) by the amount of sample represented in it (in g) the concentration of analyte in the sample in µg/g (= mg/kg) is obtained.

Figure 1: Schematic standard addition plot



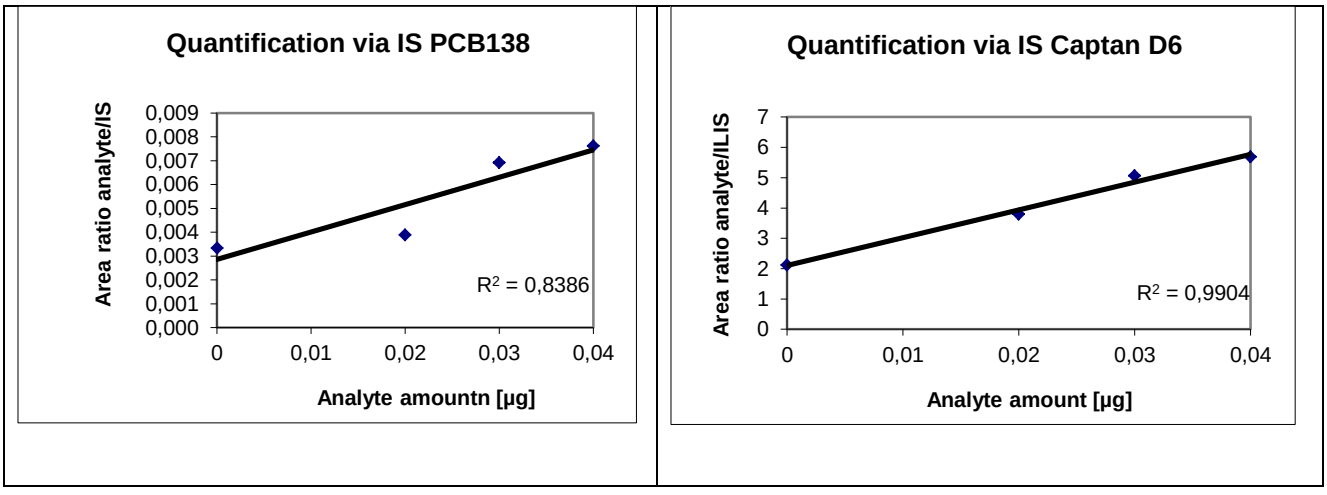
A **guidance document on how to conduct standard addition experiments** is published in the EURL-SRM Website and can be downloaded from here:
http://www.eurl-pesticides.eu/userfiles/file/EurlSRM/StdAdd_Workflow_EurlSRM.pdf

An **excel sheet calculating the analyte concentration based on standard addition to extract aliquots** can be downloaded from here:
http://www.eurl-pesticides.eu/userfiles/file/EurlSRM/StdAdd_to-ExtractAliquots.xlsx .

Exemplary experiment of the standard addition to extract aliquots approach involving the use ILIS:

Peaches containing ca. 0.2 mg/kg of captan (according to preliminary analysis) were analyzed via standard addition procedure. For this captan D6 and PCB138 were spiked to QuEChERS raw extracts. Four 1 mL aliquots of the raw extract were transferred into GC-vials. The first vial was not spiked whereas the other three were spiked with 0,02, 0,03 and 0,04 µg captan (i.e. 40, 60 and 80 µL of a solution containing 0.5 µg captan/mL acetonitrile containing 0.4% acetic acid). Volume correction was conducted to ensure that all aliquots were diluted similarly. Quantification was done once against captan D6 and for comparison purposes also against PCB138 as internal standard. As figures 2 shows, the correlation coefficient of the linear regression curve using PCB138 for quantification is much worse than that of captan D6, which has an impact on the precision.

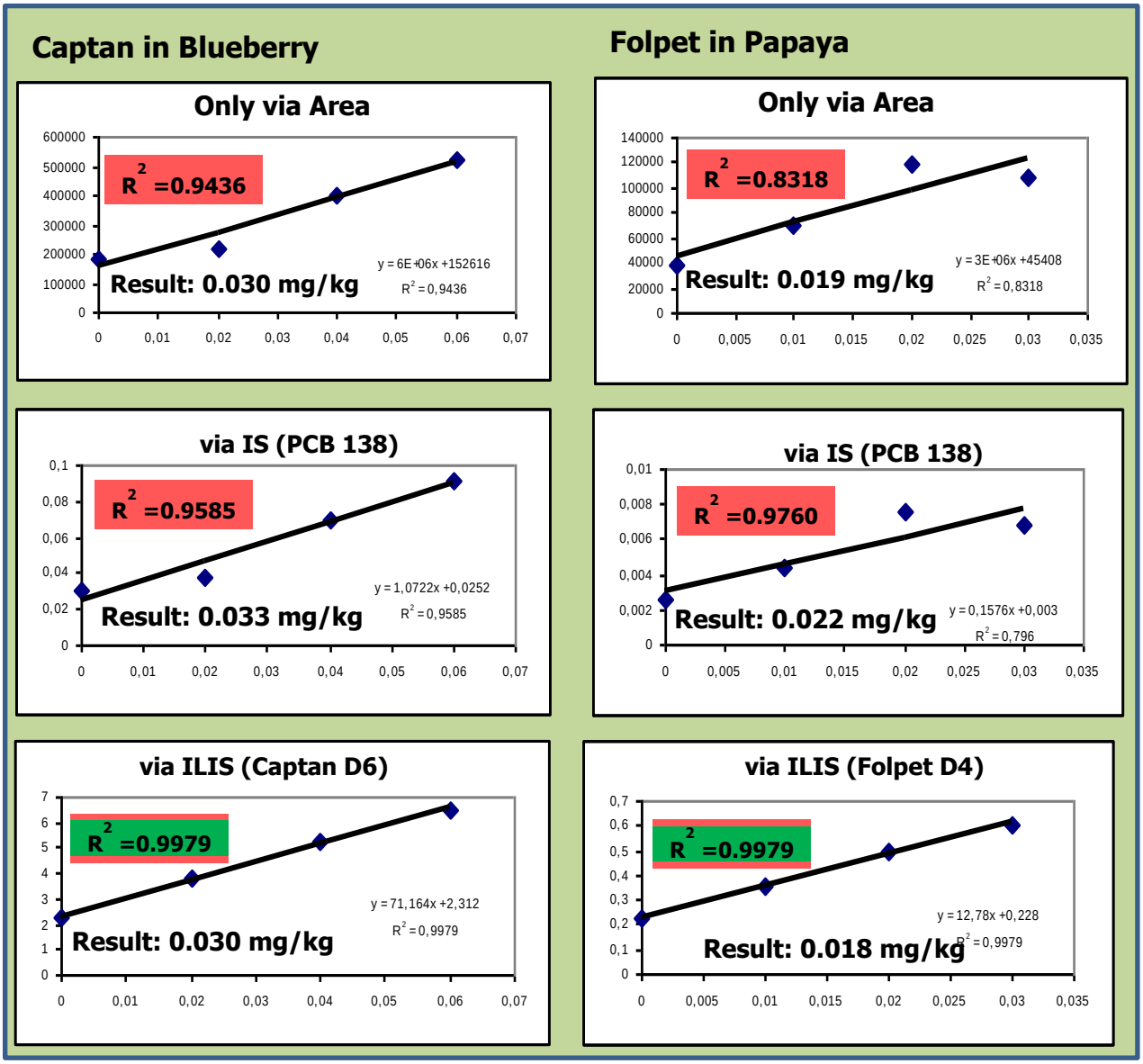
Figure 2: Comparison of linear regression curve of captan in peaches using PCB138 or captan D6 as internal standard for quantification



Similar experiments were also conducted with raw extracts of blueberries containing incurred residues of captan and with papaya containing incurred residues of folpet. In contrast to the ILIS, which had a decisive impact in improving precision, the use of PCP 138 as internal standard had only a very slight impact. In terms of accuracy the results with and without ILIS are still well comparable as the 4 measurements involved in the standard addition sufficiently averaged the slope. Looking at the

coefficients of variation, however, it becomes clear that precision is generally improved when using ILIS.

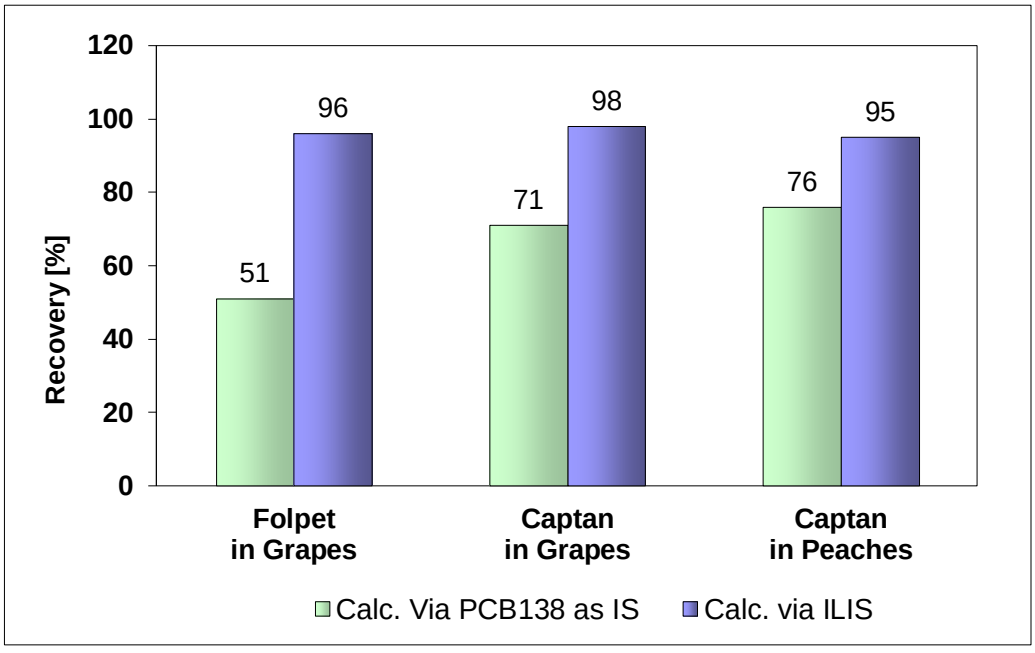
Figure 3: Comparison of linear regression curve of captan in peaches using PCB138 or captan D6 as internal standard for quantification



Correction of captan and folpet losses during extraction/cleanup via ILIS

For the compensation of losses of captan and folpet during extraction and cleanup one of the following three approaches may be used: a) Standard addition to sample portions; b) procedural calibration or c) ILIS (added at the beginning of the procedure). The first two will have similar drawbacks as the standard addition to extract aliquots, as regards problems with measurement variability. The use of ILIS is thus considered the most reliable approach provided that the losses occurred during the procedure are not so extensive to lose the ability to reliably measure the native analyte and/or the ILIS. It should be also kept in mind, that the ILIS will only correct for losses that have occurred after its addition. Measures should thus be taken to minimize any losses during homogenization and during the storage of the homogenate. Figure 4 shows the impact of ILIS in correcting for losses during sample preparation in the case of folpet and captan. These samples were extracted via QuEChERS-CEN and were subjected to a cleanup with PSA, during which losses occurred. These losses were compensated by the ILISs. Both the ILIS and the alternative IS PCB138 were spiked to the sample portions at the same time with the native analytes.

Figure 4: Recoveries of captan and folpet in grapes or peaches calculated via PCB138 or ILIS



5) Simultaneous analysis of captan, folpet and their degradation products THPI and PI

The determination of parents and degradation products at the same time based on GC measurements is quite challenging due to the conversion of the parent to the degradation products within the GC-injector.

If the aim is to determine the original content of both parents and degradation products in the sample, it is indicated to conduct the sample preparation (homogenization, extraction and cleanup) in a way that minimizes the losses of the parents (e.g. cryogenic milling, conduction of acidified QuEChERS, skipping of cleanup with PSA). If only the sum is interesting, losses of the parents are tolerated provided that the transformation rates to the respective degradation products are high, which is typically the case during extraction and cleanup.

In following two procedures are shown, that allow the simultaneous determination of the parents (captan and folpet) and the degradation products (THPI and phthalimide):

a) Procedure involving external calibration and use of parent-ILIS and
b) Procedure involving standard additions and use of parent-ILIS

Both procedures involve the use of parent ILIS to correct the result of the parent.

a) Procedure involving external calibration and use of parent-ILIS

This approach entails the use of parent-ILIS and two additional external calibrations:

- **Sample Extract:** spiked with Parent-ILIS + Second IS (e.g. chlorpyrifos D10)
- **Calibration 1:** containing Parent + Parent-ILIS + Second IS
- **Calibration 2:** degradation product + Second IS.

The first calibration is used to determine the concentration of the parents with the ILIS compensating the losses. Knowing the concentration of the parent, the expected signal of the degradation product that originates from the GC-decomposition is calculated. This signal is deducted from the total signal of the degradation product in order to find out how much degradation product was originally present in the extract.

The concentration of the degradation product in the final extract is then calculated via calibration 2 using the second internal standard. There is nevertheless several aspects to consider when using this approach:

If the **ILIS is added at the beginning of the procedure**, thus correcting for the losses of the parent during the entire procedure, the calculated concentration of the parent in the final extract will be higher than the actual one. With it also the part of the degradation product generated in the injector will be overestimated. As the degradation of the parent during the procedure leads to the formation of degradation product which is measured, the error in the calculation of the sum is reduced but not necessarily fully eliminated, depending extraction rates and conversion yields. To be on the safe side it is thus recommended to conduct the extraction in a way that ensures minimal losses of the parent so that the composition in the final extracts nearly represents the residue situation in the original homogenate.

If the **ILIS is added to an extract aliquot** (assuming that 1 mL extract represents 1 g sample in the case of fruits and vegetables), the above source of error related to losses during extraction and cleanup is eliminated. But still the use of a method that ensures high recoveries of parents and degradation products is advisable to minimize the risk of underestimated results in case poor recovery or parent degradation with low transformation yields to the degradation product.

The use of ILIS largely corrects for the strong matrix effects associated with the decomposition of the parent in the injector. THPI and phthalic acid, if injected as such are typically only moderately affected by matrix effects. This approach can thus, to a certain degree, tolerate differences in matrix effects between sample extract and calibration solution(s) even at larger concentration differences between parent and degradation product. Still, it is recommended to roughly equalize matrix-effects between calibration solutions and standards (e.g. by the use of APs), in order to minimize errors associated with determining the transformation rate of parent to degradation product (which may be variable from injection to injection) via external calibration. In any case when calculating the sum this type of error is partly compensated because a) the concentration of the parent is corrected by the ILIS and

b) an increased or decreased degradation rate of the parent will result in higher or lower signals of the degradation product which is also measured and considered in the sum calculus.

An exemplary pipetting scheme for a possible procedure where the ILIS is given to the final extract and where one-point calibrations are used is presented in Table 5.

Table 5: Exemplary pipetting scheme: Residues of folpet in apples at ~0.5 mg/kg and of phthalimide (PI) at ca. 0.2 mg/kg

Aliquot volume used	1 ml	**Commodity:** Apples **Analytes:** Folpet, PI **Expected Concentrations derived from preliminary experiments**: Captan: ~ 0.5 mg/kg PI: ~ 0.2 mg/kg	
Sample amount represented in the aliquot	1 g		
Expected abs. amount of **folpet** in the aliquot	~ 0.5 µg		
Expected abs. amount of **PI** in the aliquot	~ 0.2 µg		
Conc. of **NATIVE folpet** solution to be used	5 µg/ml		
Conc. of **folpet D4** solution to be used	2 µg/ml		
Conc. of **NATIVE PI** solution to be used	2 µg/ml		
Conc. of **chlorpyrifos D10** solution to be used	2 µg/ml		
	Sample extract	**External Calibration 1**	**External Calibration 2**
Volume of **sample extract aliquot**	**1000 µl**		
Volume of **blank extract** (preferably of same type)		**1000 µl**	**1000 µl**
Added volume of **chlorpyrifos D10** solution *	**100 µL**	**100 µL**	**100 µL**
Mass of **chlorpyrifos D10** added	0.2 µg	0.2 µg	0.2 µg
Added volume of **folpet D4** solution (2 µg/ml)	**100 µL**	**100 µL**	
Mass of **folpet D4** added	0.2 µg	0.2 µg	
Added volume of **NATIVE folpet** solution (5 µg/ml)		**100 µL**	
Mass of **NATIVE folpet** added		0.5 µg	
Added volume of **NATIVE PI** solution (2 µg/ml)			**100 µL**
Mass of **NATIVE PI** added			0.2 µg
Volume of **solvent** to be added	**100 µL**	**-**	**100 µL**
Analyte protectants mixture (see http://www.eurl-pesticides.eu/library/docs/srm/EURL_Observation-APs.pdf)	**30 µL**	**30 µL**	**30 µL**
Final volume	**1330 µL**	**1330 µL**	**1330 µL**

An excel sheet calculating the concentration of parents and degradation products in the sample using this approach can be found in the EURL-SRM website.

Link:**http://www.eurl-pesticides.eu/userfiles/file/EurlSRM/Calculation-captan-folpet-THPI-PI.xlsx**

The conduction of **quality control experiments** using the above described procedure are advisable, to check whether the experimentally determined values of parent and degradation product individually and in the sum are close to the real ones. These experiments can either involve spiking of both parent and degradation product at the beginning of the procedure or spiking of blank extracts.

In a **verifying simulation experiment** it was checked whether captan and THPI can be accurately determined in solvent as well as extracts of apples and cucumbers spiked at known concentrations with folpet (0.2 µg/mL) and phthalimide (0.2 µg/mL). Following the approach described above the calculations were done against fitted (matrix-matched) calibrations. The simulated solutions and the two calibrations were injected 3 times on 3 different days. Folpet as phthalimide (PI) as well as the "sum (expressed as folpet)" were calculated. Using fitted calibrations the deviation of the calculated sum versus the expected one was <5% on average in all cases (see Tables 6-8).

When calculating the simulated apple extract against the solvent based calibrations (as a worst case) the error for the calculated sum increased to 9% but was still moderate (see Table 9). It should be noted however that analyte protectants were added to all above solutions.

Table 6: Simulation experiment - Solvent spiked with known amounts of folpet and PI and calculated against fitted (solvent based) calibrations

In PURE SOLVENT (using solvent-based calibration)	**Folpet**	**PI**	**Sum (calculated as Folpet)**
First analysis			
Real (spiked) [µg/mL]	0.2	0.2	0.603
Determined [µg/mL]	0.194	0.171	0.539
Deviation [%]	**-3%**	**-15%**	**- 11%**
Second analysis			
Real (spiked) [µg/mL]	0.2	0.2	0.603
Determined [µg/mL]	0.186	0.220	0.628
Deviation [%]	**-7%**	**+10%**	**+4%**
Third analysis			
Real (spiked) [µg/mL]	0.2	0.2	0.603
Determined [µg/mL]	0.191	0.182	0.559
Deviation [%]	**-5%**	**-9%**	**-7%**
OVERALL AVERAGE DEVIATION	**-5%**	**-5%**	**-5%**

Table 7: Simulation experiment – Cucumber extract spiked with known amounts of folpet and PI and calculated against fitted (matrix-matched) calibrations

IN CUCUMBER extract (using matrix-matched calibrations)	**Folpet**	**PI**	**Sum (calculated as Folpet)**
First analysis			
Real (spiked) [µg/mL]	0.2	0.2	0.603
Determined [µg/mL]	0.192	0.212	0.620
Deviation [%]	**-4%**	**+6%**	**+ 3%**
Second analysis			
Real (spiked) [µg/mL]	0.2	0.2	0.603
Determined [µg/mL]	0.175	0.220	0.618
Deviation [%]	**-13%**	**+10%**	**+ 3%**
Third analysis			
Real (spiked) [µg/mL]	0.2	0.2	0.603
Determined [µg/mL]	0.201	0.191	0.586
Deviation [%]	**+0.5%**	**-5%**	**- 3%**
OVERALL AVERAGE DEVIATION	**-6%**	**+4%**	**+1%**

Table 8: Simulation experiment – Apple extract spiked with known amounts of folpet and PI and calculated against fitted (matrix-matched) calibrations

In APPLE Extract (using matrix-matched calibrations)	**Folpet**	**PI**	**Sum (calculated as Folpet)**
First analysis			
Real (spiked) [µg/mL]	0.2	0.2	0.603
Determined [µg/mL]	0.212	0.185	0.585
Deviation [%]	**+6%**	**-8%**	**- 3%**
Second analysis			
Real (spiked) [µg/mL]	0.2	0.2	0.603
Determined [µg/mL]	0.189	0.179	0.550
Deviation [%]	**-6%**	**-11%**	**-9%**
Third analysis			
Real (spiked) [µg/mL]	0.2	0.2	0.603
Determined [µg/mL]	0.195	0.225	0.648
Deviation [%]	**-3%**	**+13%**	**+7%**
OVERALL AVERAGE DEVIATION	**-1%**	**-2%**	**-2%**

Table 9: Simulation experiment – Apple extract spiked with known amounts of folpet and PI and calculated against **solvent-based** calibrations

In APPLE Extract (using SOLVENT-BASED calibrations)	**Folpet**	**PI**	**Sum (calculated as Folpet)**
First analysis			
Real (spiked) [µg/mL]	0.2	0.2	0.603
Determined [µg/mL]	0.202	0.243	0.693
Deviation [%]	**+1%**	**+21%**	**+ 15%**
Second analysis			
Real (spiked) [µg/mL]	0.2	0.2	0.603
Determined [µg/mL]	0.179	0.207	0.596
Deviation [%]	**-10%**	**+4%**	**-1%**
Third analysis			
Real (spiked) [µg/mL]	0.2	0.2	0.603
Determined [µg/mL]	0.199	0.240	0.648
Deviation [%]	**-0.5%**	**+20%**	**+13%**
OVERALL AVERAGE DEVIATION	**-3%**	**+15%**	**+9%**

b) Procedure involving standard additions and use of parent-ILIS

This approach also entails the use of the parent-ILISs for the correction of the parent results but calibration standards are prepared via standard additions to extract aliquots:

Sample Extract: spiked with Parent-ILIS + Second IS (e.g. Chlorpyrifos D10)

StAdd1: spiked with Parent-ILIS + Second IS + NATIVE parent

StAdd2:: spiked with Degradation Product + Second IS

Also in this approach it is preferable to extract the sample in a way conserving parent and degradation products, and to spike the ILIS to the extract aliquots used for the standard addition experiments.

The amount of parent and degradation product to be spiked in the standard additions should be determined in preliminary experiments. Following standard addition of the native parent its peak area should be increased by a factor of ca. 1.5-3. Similarly, following standard addition of the degradation product its peak area should be increased by a factor of ca. 1.5-3.

This approach has the advantage that the calibrations are in all cases exactly matrix-matched, which reduces the risk of errors related to varying degradation rate.

Table 10 shows an exemplary pipetting scheme.

Table 10: Exemplary pipetting scheme: Residues of captan and THPI in nectarines

Aliquot volume used	1 ml	**Commodity:** Nectarines **Analytes:** Captan, THPI **Expected Concentrations derived from preliminary experiments**: Captan: ~ 0.5 mg/kg THPI: ~ 0.2 mg/kg	
Sample amount represented in the aliquot	1 g		
Expected abs. amount of **captan** in the aliquot	~ 0.5 µg		
Expected abs. amount of **THPI** in the aliquot	~ 0.2 µg		
Conc. of **NATIVE captan** solution to be used	5 µg/ml		
Conc. of **captan D6** solution to be used	2 µg/ml		
Conc. of **THPI** solution to be used	2 µg/ml		
Conc. of **chlorpyrifos D10** solution to be used	2 µg/ml		
	StAdd 0 (no addition)	**StAdd 1 (+ Captan)**	**StAdd 2 (+ THPI)**
Volume of sample extract aliquots	**1000 µl**	**1000 µl**	**1000 µl**
Added volume of **chlorpyrifos D10** solution	**100 µL**	**100 µL**	**100 µL**
Mass of **chlorpyrifos D10** added	0.2 µg	0.2 µg	0.2 µg
Added volume of **captan D6** solution (2 µg/ml)	**100 µL**	**100 µL**	
Mass of **captan D6** added	0.2 µg	0.2 µg	
Added volume of **NATIVE captan** solution (5 µg/ml)		**100 µL**	
Mass of **NATIVE captan** added		0.5 µg	
Added volume of **NATIVE THPI** solution (2 µg/ml)			**100 µL**
Mass of **NATIVE THPI** added			0.2 µg
Volume of **solvent** to be added	**100 µL**	**-**	**100 µL**
Final volume	**1300 µL**	**1300 µL**	**1300 µL**

Note: If the matrix shows little protection of the parents and the signals are low it is also advisable to **add analyte protectants** (e.g. 30 µL of the mixture described here: **http://www.eurl-pesticides.eu/library/docs/srm/EURL_Observation-APs.pdf**)

The standard addition with native parent is used to calculate the concentration of native standard in the extract (and the sample). Here again the ILIS corrects for any deviations in the GC-decomposition. The signals of the degradation product in all three solutions, the non-spiked one, the parent-spiked one and the one spiked with degradation product are also recorded. The difference between the second and the first signal represents the response of the degradation product when injecting a certain known amount of parent. Using this value and the previously calculated concentration/amount of native compound in the unspiked extract the signal of the degradation product originating from the parent degradation in the injector can be

calculated. This signal is then deducted from the overall signal of the degradation product in the unspiked extract to obtain the signal of the degradation product originally present in the extract. The standard addition with the degradation product is used to calculate the response of the degradation product when injecting a certain known amount of it. By comparing this signal with the calculated signal of the degradation product originally present in the extract, the original amount/concentration of the degradation in the sample extract is calculated.

An excel sheet calculating the concentration of parents and degradation products in the sample using this approach can be found in the EURL-SRM website. Link: **http://www.eurl-pesticides.eu/userfiles/file/EurlSRM/Calculation-captan-folpet-THPI-PI-ViaStadd.xlsx**

Compared to the approach involving external calibration this internal calibration approach has the disadvantage that each extract requires its own set of calibrations. With the external calibration approach one set of calibration solutions can in principle be used for several samples, but matrix-effects should optimally be equalized in case of batches with different sample types (e.g. via addition of APs).

Also here, **quality control experiments** using the above described procedure are advisable, to check whether the experimentally determined values of parent and degradation product individually and in the sum are close to the real ones. These experiments can either involve spiking of both parent and degradation product at the beginning of the procedure or spiking of blank extracts.

In an experiment using simulated extract the accuracy of the method was checked for captan and folpet in cucumber extracts. The deviation of the sum from the spiked value was within the limits in both cases (+12 and +11% respectively). In the case of THPI and PI the deviation was higher (+14% and +16% respectively). Calculating THPI and PI based on their response alone, not taking into account the degradation in the injector would have led to an overestimation of +36% and +46% respectively. Further experiments will follow.

Table 11: Simulation experiment – Cucumber extract spiked with known amounts of folpet and PI using the approach involving standard addition (n=1)

	Folpet	**PI**	**Sum (calc. as Folpet)**
Real (spiked) [µg/mL]	0.50	0.50	1.508
Determined [µg/mL]	0.54	0.57	1.69
Deviation [%]	**+8%**	**+14%**	**+12%**
	Captan	**THPI**	**Sum (calc. as Captan)**
Real (spiked) [µg/mL]	0.50	0.50	1.508
Determined [µg/mL]	0.50	0.58	1.66
Deviation [%]	**-0%**	**+16%**	**+11%**

Document History

Action	When	Version
Experiments for quantification using standard addition	2002-2008	
Experiments using Standard addition and ILIS	2008	
Publication of first document in the web	Oct 2008	V1
Updated version of document with description on how to use APs	June 2010	V2
Experiments for the inclusion of main metabolites	2015-2017	
Publication of new extensively enlarged document including, extended discussion of analytical difficulties and solutions for the analysis of parents and degradation products at the same time	April 2017	V3
• Editorial corrections throughout the text, • Shift of misplaced link of excel file of method 5b), • Introduction of a pipetting scheme for the approach 5a), (new Table 5) • Introduction of first quick validation data of procedure 5b) (new Table 11) • Replacement of two mass transitions for captan	April 2017	V3.1