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POSITION PAPER No. 17 - 01

Phthalimid - Part 2:

Unavoidable Artefact !

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Abstract

This position paper is the second publication related to the new residue definition of the fungicide Folpet by regulation (EU) 2016/156 dated 2016/01/18. This regulation is to be applied since 2016/08/26, with the revised residue definition of Folpet:

"**Folpet**: Sum of Folpet and Phthalimid, expressed as Folpet".

Before, Phthalimid - the metabolite of Folpet – was not part of the residue definition. As Phthalimid (hereafter named "PI") is often detected and measured in many food products without the simultaneous presence of Folpet, serious concerns rose up about other sources of PI than of Folpet only.

The first relana® position paper related to Folpet/PI (no. 16- 03 of 22. July 2016) describes and summarises the experiences and data of the relana® laboratory circle dealing with the presence of PI in different kinds of foodstuff. A potential and likely source and the related pathway for the generation of PI is described in this paper.

This new relana® position paper is focussing on the formation of PI as an artefact during the hot injection of sample extracts into any gaschromatografic system (hereafter named "GC-system"). The pre-cursor of PI is the well-known chemical "**phthalic anhydride**" (PSA). Phthalic anhydride as an ubiquitous environmental chemical can easily react under specific conditions (esp. higher temperatures) with primary amino groups (like present in amino acids, peptides, etc.) to form Phthalimid (PI; for details see relana® position paper no. 16- 03).

As a consequence, it is obvious that the common analytical technique for measuring Folpet and PI - the hot injection GC-detection – is a potential source for the generation of false positive levels of PI.

An alternative analytical approach – the LC-MS/MS-technique using atmospheric pressure chemical ionisation (APCI) - is discussed, in order to avoid such false positive results.

Introduction

With regulation (EU) 2016/156 of 18. January 2016 the European Commission has set a new residue definition for Folpet, which now includes the main metabolite "Phthalimid" (PI). The regulation applies since 26 August 2016.

As discussed briefly in the relana® position paper no. 16- 03, PI is often produced within the injection system of the GC. A residue definition of Folpet including PI, which might be produced during the GC injection, bears the risk of relevant over-estimations of the levels of Folpet (as defined by the residue definition) in analytical samples. This aspect is also discussed comprehensively by the working group "pesticides" of the association of German chemists (GDCh). The statement of the working group is already published (but only in German language, see literature).

In order to provide more evidence related to the unavoidable formation of PI by sources/reactions other than of Folpet, the relana®-member Labor Friedle GmbH (Tegernheim, Germany) performed additional investigations and research activities, which will be presented and discussed hereafter.

Test design 1: Formation of Phthalimid (PI) as an artefact of PSA in the hot injectors of GC-systems?

The aim of the first step of the research project was to prove, whether the formation of PI in a hot GC-injector is linked to the presence of PSA (phthalic anhydride). To do so, the following study design was applied:
Fresh parsley was selected as an appropriate commodity. The parsley was dried throughout the night in a laboratory oven at 80° C. The weight of fresh parsley was 274.83 g, while the dried parsley weighed 33,01 g next day. The calculated dry substance of the parsley is 12% corresponding to a water content of 88% of the fresh product.

After that drying process, three different preparations were made:

1. Sample of parsley (dried)
2. Sample of parsley spiked with 50 µl of a PI solution (c = 10 ng/µl)
3. Sample of parsley spiked with 50 µl of a PSA solution (c = 1000 ng/µl).

The sample preparation and clean up was done according to the QuEChERS multi method (which Labor Friedle is accredited for: D-PL-14646-03-00, method ASU L 00.00-115 2014-02):

0,5 g of parsley are weighed and added with 4,5 ml of water prior sample extraction and clean up according to QuEChERS. The final extract (acetonitrile) is re-dissolved in methanol before injection into the GC- resp. LC-systems.

Taking the a.m. parameters into account, the final concentrations of the 3 samples related to the spiked substances are:

1. Sample of parsley (dried)
   PI: not spiked
   PSA: not spiked
2. Sample of parsley spiked with 50 µl of a PI solution (c = 10 ng/µl)
   PI: 1 mg/kg
3. Sample of parsley spiked with 50 µl of a PSA solution (c = 1000 ng/µl)
   PSA: 100 mg/kg. This test is designed in order to identify a possible formation of PI by the presence of relevant levels of PSA.

The analytical results provided are based on 2 different measurement techniques:

a) The common hot injection GC-MS/MS, equipped with a PTV-injector (programmed temperature vaporizer injector). The applied temperature programme is:
   Initial: 60°C; Hold Time: 0,14min;
   Ramp 1: 700°C/min; 280°C ; Hold Time: 15min

b) The LC-MS/MS technique, equipped with an APCI source (atmospheric pressure chemical ionisation), which is a soft ionisation for non-polar compounds. This is essential for the detection of PI.

The results of this first test design are summarised in the following table:

No.	Sample	Phthalimid GC-MS/MS [mg/kg]	Phthalimid LC(APCI)-MS/MS [mg/kg]
1	parsley	0,049	n.d. (<0,1)
2	parsley spiked with 1 mg/kg PI	1,049	1,00
3	parsley spiked with 100 mg/kg PSA	**1,308**	**n.d. (<0,1)**

n.d. = not detected

The related LC(APCI)-MS/MS chromatograms are shown in the graphics below:

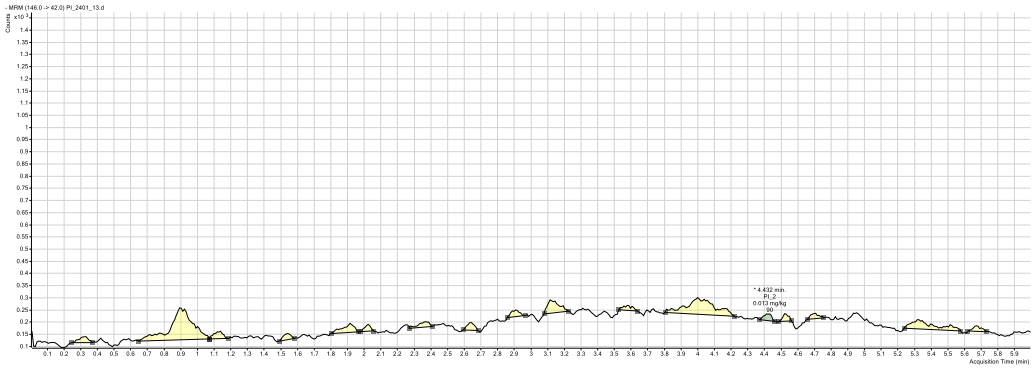

Fig. 1: LC(APCI)-MS/MS chromatogram, sample 1 (parsley without any spike)

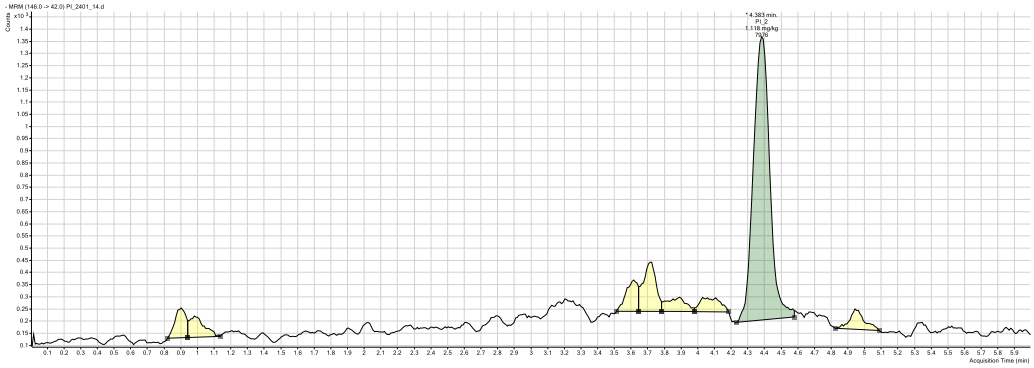

Fig. 2: LC(APCI)-MS/MS chromatogram, sample 2 (parsley spiked with 1 mg/kg of PI)

Discussion of test design 1:
The applied **LC(APCI)-MS/MS** technique provides a reporting limit (RL) of 0,1 mg/kg (related to dry products). The signal (peak) of PI in the spiked sample no. 2 is strong and precise, whereas no signal is present in samples no. 1 (parsley without any spike) and no. 3 (parsley spiked with 100 mg/kg of PSA).
On the other hand, the GC-MS/MS approach provides different results. The parsley itself has a level of 0,049 mg/kg of PI. Consequently, the spiking of PI increases the level in sample 2 up to 1,049 mg/kg. The most interesting result is the increased level of PI in sample no. 3, where 100 mg/kg of PSA were spiked. The level of PI increases to 1,308 mg/kg. This indicates, that a certain amount of PI is formed because of the presence of 100 mg/kg of PSA in the sample (as no PI was spiked to the sample no. 3).

Test design 2: Is there any impact on the formation of PI if PSA is added to the fresh parsley instead to the dried product?

To figure out a possible impact of PSA present in the original, water containing parsley (88% water) is compared with the dried parsley. The test design 1 was repeated while adding one additional test.

Taking the a.m. parameters into account, the final concentrations of the 4 samples related to the spiked substances are:

1. Sample of parsley (dried), PI: not spiked, PSA: not spiked
2. Sample of parsley (dried) spiked with 50 µl of a PI solution (c = 10 ng/µl), ➔ PI: 1 mg/kg
3. Sample of parsley (dried) spiked with 50 µl of a PSA solution, (c = 1000 ng/µl) ➔ PSA: 100 mg/kg.
4. Sample of parsley (**fresh, 50g** = 6g of dried product) spiked with 60 µl of a PSA solution (c = 10 g/l resp. 10.000 ng/µl), dried over night at 80°C; ➔ PSA: 100 mg/kg related to dried product.

No.	Sample	Phthalimid GC-MS/MS [mg/kg]	Phthalimid LC(APCI)-MS/MS [mg/kg]
1	Parsley (dried)	0,053	n.d. (<0,1)
2	parsley (dried)spiked with 1 mg/kg PI	1,053	1,00
3	parsley (**dried**) spiked with 100 mg/kg PSA	**1,328**	**n.d. (<0,1)**
4	parsley (**fresh**) spiked with 100 mg/kg PSA	**1,444**	**n.d. (<0,1)**

(all results are related to dried product)

Discussion of test design 2:
The levels of PI analysed by hot injection GC-MS/MS did not differ significantly depending on the application of PSA on dried resp. fresh parsley (1,3 mg/kg resp. 1,4 mg/kg). Analysing the samples using the LC(APCI)-MS/MS technique, there is still no PI detectable (reporting limit at 0,1 mg/kg).

Test design 3: Is there a correlation between PSA levels in the sample and the formation of PI in the hot injector of a GC-system?

The aim of the second step of the research project was to prove, whether the formation of PI in a hot GC-injector shows a correlation with different levels of PSA in the parsley. To do so, the following study design was applied:
The same dried parsley was used as prepared for test design 1 of the studies. As a first step, three different spiked samples were prepared in order to get a calibration line:

1. Sample of parsley spiked with 25 µl of a PI solution (c = 10 ng/µl)
2. Sample of parsley spiked with 50 µl of a PI solution (c = 10 ng/µl)
3. Sample of parsley spiked with 75 µl of a PI solution (c = 10 ng/µl).

While considering the sample weight and preparations, the final concentrations of the spiked PI are:

1. Sample: 0,5 mg/kg of PI
2. Sample: 1,0 mg/kg of PI
3. Sample: 1,5 mg/kg of PI.

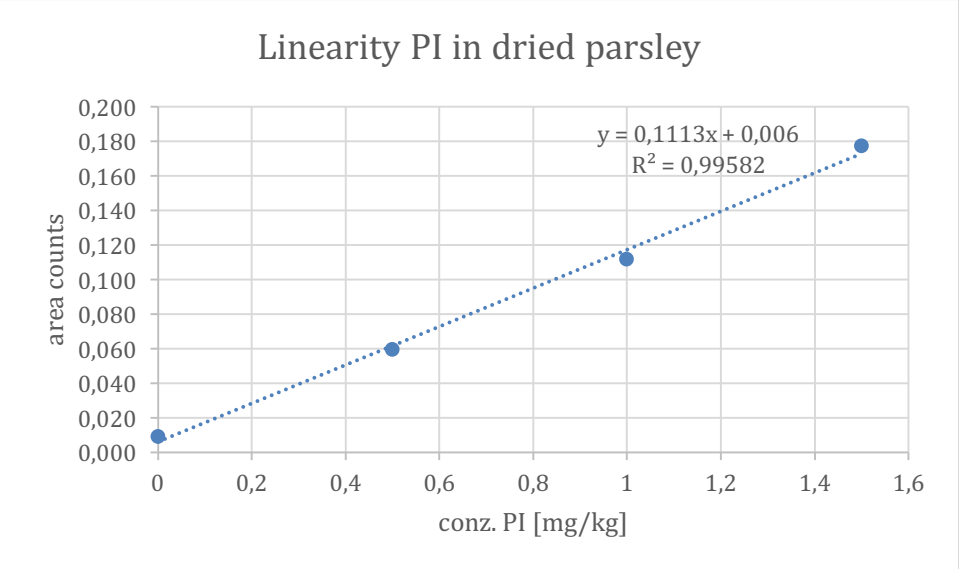

Fig. 3: Calibration line and linearity of PI in dried parsley

From that standard addition calibration, the level of PI in the non-spiked sample is calculated to 0,054 mg/kg which is very close to the result of test design no.1 (0,049 mg/kg calculated using a 1-point-calibration).

After that, the test with different levels of PSA spiking was designed:

1. Sample dried parsley
2. Sample dried parsley spiked with 5µL PSA [1g/L] = 10mg/kg
3. Sample dried parsley spiked with 15µL PSA [1g/L] = 30mg/kg
4. Sample dried parsley spiked with 25µL PSA [1g/L] = 50mg/kg
5. Sample dried parsley spiked with 35µL PSA [1g/L] = 70mg/kg
6. Sample dried parsley spiked with 45µL PSA [1g/L] = 90mg/kg

	conc. PI [mg/kg] GC-MS/MS	conc. PSA [mg/kg]	PI / PSA [%]
sample	0,058	5,757 *	1,008
sample plus PSA (10mg/kg)	0,139	(15,757)	0,880
sample plus PSA (30mg/kg)	0,443	(35,757)	1,239
sample plus PSA (50mg/kg)	0,747	(55,757)	1,340
sample plus PSA (70mg/kg)	1,019	(75,757)	1,346
sample plus PSA (90mg/kg)	1,271	(95,757)	1,327

* level of PSA measured during test design 1
(...) calculated on basis of result "sample", not analysed

The direct proportional correlation is visualised by the following graphic:

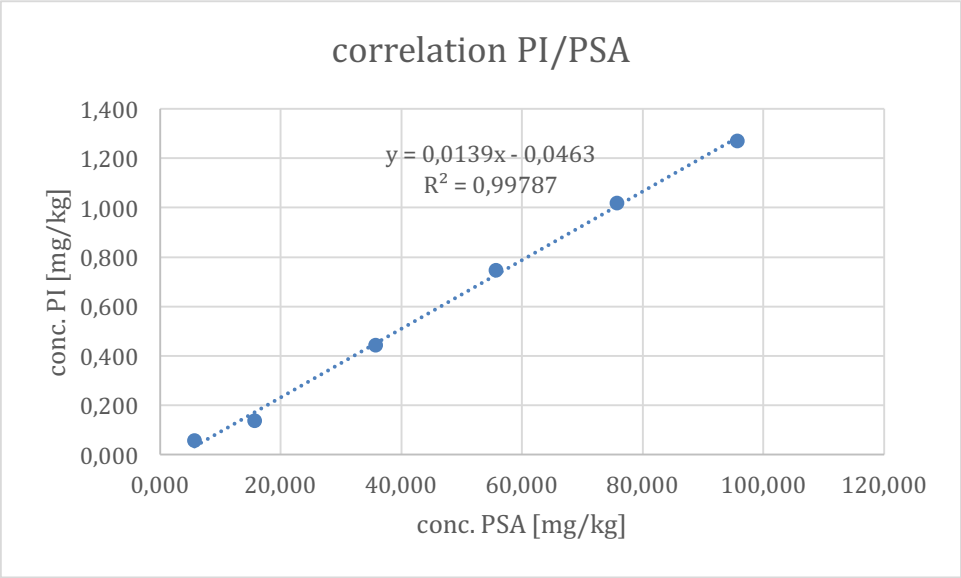

Fig. 4: Correlation line of PI / PSA in dried parsley after hot GC injection

Discussion of test design 3:
It is evident, that there is a direct proportional correlation between the PSA content of a sample and the formation of PI during injection. The mean of the percentage of the ratio PI / PSA is 1,19. The RSD (relative standard deviation) is ±15,2 %.

This correlation was verified and confirmed by repeating the same test design using a different GC-MS/MS system. This system is equipped with a similar PTV injector, running the same temperature program as with instrument 1. The mean of the percentage of the ratio PI / PSA using the second instrument is 1,18, thus confirming the direct proportional correlation.

As a conclusion it is obvious, that ca. 1% of the concentration of PSA in the dried parsley samples is converted into PI during hot GC injection.

Test design 4: Does the drying temperature of the parsley influence the formation of PI?

This test design includes the use of the dried parsley (as of test design 1) and additionally as second test material, a commercially available black tea.

The samples (nos. 3 to 12 and 15) were treated at different temperatures for 30 min always (in closed digestion tubes in a muffle oven). The sample preparation and clean-up was the same as in test design 1 (QuEChERS approach) with subsequent measurement of the final extract by LC(APCI)-MS/MS. This technique was used, as the hot GC injection is not appropriate and would increase the PI levels, as described before. The test design consists of the following samples:

- Sample 1: dried parsley (prepared at 80°C over night, see test design 1)
- Sample 2: dried parsley spiked with 50µL PI [10 ng/µl] = 1mg/kg (used as 1-point-calibration level)
- Samples 3 to 12: dried parsley (30 min at different temperatures, see table below)
- Sample 13: black tea, untreated
- Sample 14: black tea spiked with 50µL PI [10 ng/µl] = 1mg/kg (used as 1-point-calibration level)
- Sample 15: black tea (250°C, 30 min).

Sample No.	Sample	Temperature [°C]	**Phthalimid** LC(APCI)-MS/MS [mg/kg]
1	dried parsley	RT	n.d. (<0,1)
3	dried parsley	80	n.d. (<0,1)
4	dried parsley	100	n.d. (<0,1)
5	dried parsley	150	n.d. (<0,1)
6	dried parsley	175	**0,066**
7	dried parsley	200	**0,595**
8	dried parsley	210	**2,144**
9	dried parsley	220	**2,435**

10	dried parsley	230	**2,769**
11	dried parsley	240	**3,018**
12	dried parsley	250	**3,522**
13	black tea	RT	n.d. (<0,1)
15	black tea	250	**0,282**

RT = room temperature; n.d. = not detected (< reporting limit)

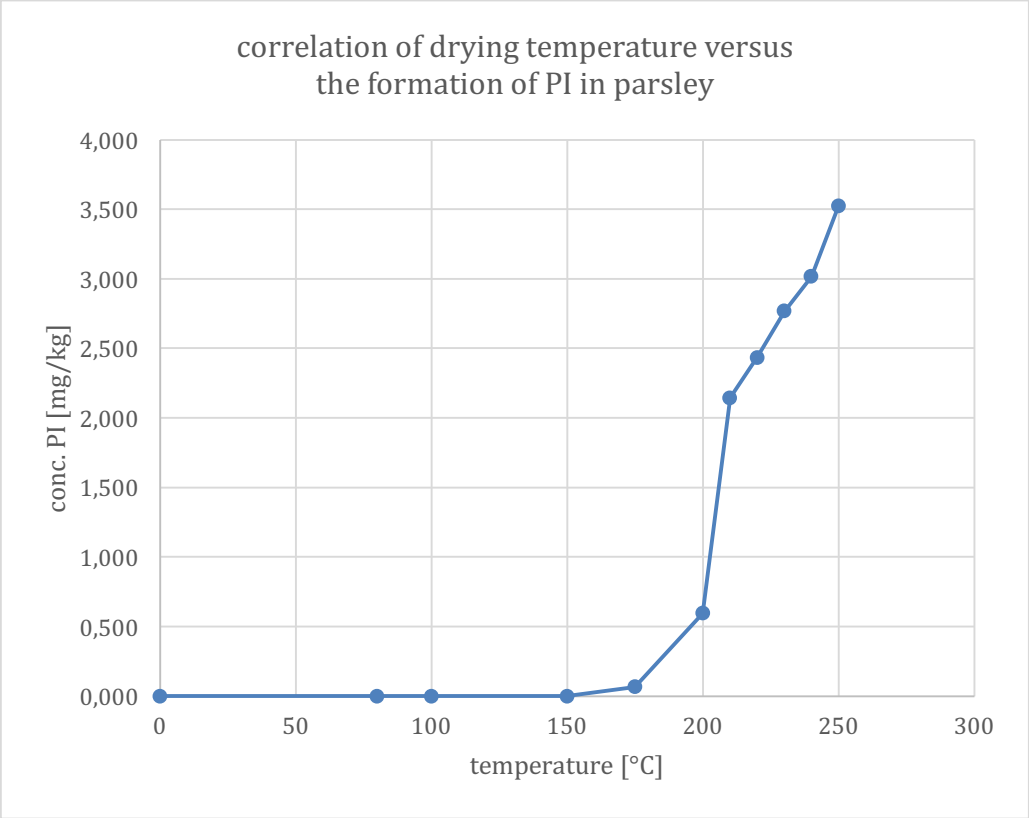

Fig. 5: Formation of PI in dried parsley as a function of temperature during drying

Discussion of test design 4:
Independent of the formation of PI as an artefact of PSA during hot GC injection, products like dried parsley are affected additionally by high processing temperatures. Increasing temperatures facilitate the formation of PI in such products. It is likely, that the pre-cursor molecule is the PSA (as it is present in the used parsley sample at a level of ca. 6 mg/kg). The formation of PI is increasing strongly at temperatures above 200°C.

In black tea, which was investigated during the same test design, a similar increase of the level of PI at a temperature of 250°C was evident.

Conclusions and recommendations

Based on the data provided in this **relana® position paper** and the discussed possible formation processes, considering the ubiquitous presence of phthalic acid and phthalic anhydride, the following conclusions and recommendations resp. demands are defined:

1. It is evident, that Phthalimid is formed as an artefact during analysing sample extracts using the hot injection GC-MS/MS approach. The formation of PI is even more intensive, if levels of PSA are present in the samples.
2. The formation of PI during hot injection GC-MS/MS by the presence of PSA is independent of the texture of the commodity (high water content or dried).
3. As more PSA is present in a food product as higher the level of PI. Approximately, ca. 1 % of the PSA is converted into PI during GC-MS/MS analysis.
4. LC(APCI)-MS/MS is the most appropriate analytical technique to avoid false positive results of PI so far. Unfortunately, the reporting limit of PI is at 0,1 mg/kg related to dried products. Therefore, levels of PI below 0,1 mg/kg cannot be monitored using this technique.
5. The formation of PI as an artefact of PSA (without any Folpet in the sample) is strongly depending on the temperature. At temperatures above 200°C the formation of PI is increasing significantly.
6. As a consequence of the a.m. findings, the residue definition of Folpet – as described and published in reg. (EU) 2016/156: "Folpet = Sum of Folpet and Phthalimid, expressed as Folpet" – is not suitable to supervise the related MRLs in case Folpet itself is not detected in the sample. Moreover, the MRL monitoring is not feasible when making use of GC-MS/MS techniques only.
7. This new residue definition bears a high risk of **false positive results** related to the presence or application of the fungicide Folpet.
8. This risk is also evident if Folpet is detected as it cannot be distinguished, whether the measured levels of Phthalimid are linked to the presence of Folpet or due to the formation from phthalic anhydride. As a consequence, the calculated level of Folpet might be too high and misleading.
9. The most important consequences are related to findings of Phthalimid in organic samples if no Folpet is detected simultaneously. In many samples, the organic guideline value of 0,01 mg/kg will be clearly exceeded when applying the new residue definition of Folpet. Consequently, organic control bodies and authorities, but also the clients of producers and traders of organic products might complain about the compliance of the related food products with the rules of organic farming as published by the regulations (EC) Nos. 834/2007 and 889/2008. This situation would hamper the trading of organic products, although these products would have been produced and processed in compliance with the related organic regulations and rules.

Taking all these facts and aspects into consideration, it is once again highly recommended to re-evaluate the residue definition of Folpet. The detection of Phthalimid only does not indicate a possible non-conformity of organic food products with the related organic regulations.
At least it is highly recommended to re-evaluate the residue definition of Folpet in order to guarantee a verifiable monitoring of the related MRLs. The inclusion of PI into the residue definition of Folpet is not appropriate to get reliable analytical data for monitoring and enforcement activities.

Literature

- Regulation (EU) 2016/156 of 18.01.2016

- Keine zweifelsfreie Überwachung des Rückstandshöchstgehaltes von Folpet möglich; Positionspapier der AG Pestizide
  Lebensmittelchemie 71 (1/2017), pp. 19-20

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