Observations concerning…

☑ ***a compound*** ☑ ***a matrix*** ☑ ***a method*** ☐ ***other***

Analysis of Pyridate and its metabolite Pyridafol by QuEChERS and LC-MS/MS

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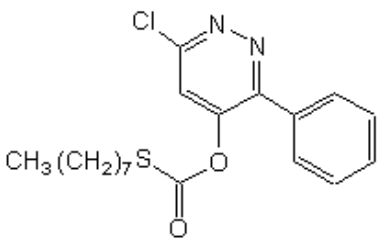
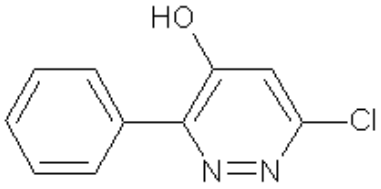
Brief description of problem/observation/solution:

The residue definition of pyridate includes pyridate itself and its degradant pyridafol (which is the actual active component) as well as the conjugates of the latter. In recovery experiments using QuEChERS it was observed that pyridate partially degrades and that the acidic pyridafol experiences losses during dSPE cleanup when PSA sorbent is used. Skipping dSPE with PSA practically quantitative recoveries of pyridate parent and pyridafol are achieved. Simultaneous analysis of parent and metabolite is possible but analysis of the metabolite alone is preferable as degradation of pyridate to pyridafol in calibration solutions leads to biased results and increases analytical uncertainty. In addition the residue definition requires the release of conjugated pyridafol thus suggesting total conversion of pyridate and pyridafol-conjugates to pyridafol. Unfortunately the behavior of the conjugates could not be studied as no samples with incurred pyridate were available. All experiments thus focused on the parent and the free metabolite. Several variations of the QuEChERS method were tested with two main aims: a) to find conditions where the recoveries of pyridate are quantitative, and b) to find conditions where pyridate is fully converted to pyridafol. In-source fragmentation of pyridate to pyridafol was observed in LC-MS/MS analysis indicating that chromatographic separation of the two is needed.

Compound profile:

Pyridate is a post-emergence herbicide that is used for broadleaved weed and grass control in cereals (e.g. maize, rice), brassica crops (e.g. cauliflowers, kohlrabi, broccoli, Brussels sprouts, kale), allium crops (onions, shallots, chives, leek) and other crops (salsify, asparagus, fodder beet, lupins, herbs, rape). The compound is systemic and hydrolyzes within the plant to the active component pyridafol (3-phenyl-4-hydroxy-6-chloropyridazine), which acts as a photosynthesis inhibitor.

Table 1: Properties of pyridate and pyridafol at a glance

Parameter	Value	Notes	
Pka	-		**Pyridate** 
LogP	6.61	calcutated	
Water solubility	1.49	(20°C; pH 7)	
Hydrolytic behavior	pH and temperature sensitive: **DT50:** • 4.9 d @ pH 4 / 25 °C, • 0.44 d @ pH 4 / 50 °C , • 0.28 d @ pH 9 / 25 °C , • 0.3 h @ pH 9 / 50 °C		
ISO common name	O-6-chloro-3-phenylpyridazin-4-yl S-octyl thiocarbonate		
Residue definition EU	**Plant Origin:** Pyridate (sum of pyridate, its hydrolysis product CL 9673 (6-chloro-4-hydroxy-3-phenylpyridazin) and hydrolysable conjugates of CL 9673 expressed as pyridate) **Animal Origin:** CL 9673 expressed as pyridate[1]		
Approved in…	BE, CY, CZ, DE, EE, FI, FR, HU, IE, IT, LT, LU, LV, MT, NL, PL, SI, SK, UK, PT in progess		
Main metabolite	**Pyridafol = CL 9673 = 6-chloro-3-phenylpyridazin-4-ol (ISO common name)**		
	Pka = 6.7 (weak acid) LogP = pH dependent • 2.0 @ pH 4 • 0.56 @ pH 7 • -0.52 @ pH 9		**Pyridafol** 

Experiments conducted and observations:

Standards:

- Pyridate (purity 94%), purchased from Dr. Ehrenstorfer (Cat #: 16640000)
- Pyridafol (purity 98%) purchased from Dr. Ehrenstorfer (Cat #: 11417500)

Note: Stock solutions were prepared considering purity indications of the provider

[1] Following feeding studies pyridate was never encountered. Pyridafol was the main fraction, e.g. 70% of TRR in milk (**http://www.efsa.europa.eu/en/efsajournal/doc/2687.pdf**)

Extraction method:

In order to explore the degradation behavior of pyridate during QuEChERS sample preparation a number of recovery experiments using variations of the QuEChERS method were conducted. Pyridate and pyridafol were spiked into separate sample portions and analysis was conducted by LC-MS/MS. Samples spiked with pyridate were additionaly analyzed for pyridafol to check whether the share of pyridate that is degraded during sample preparation is quantitatively transformed to pyridafol. The results of this experiment are summarized in Table 4.

Measurement:

Table 2: Instrumentation details for Pyridate and Pyridafol:

LC-MS/MS method:	
LC	WATERS Acquity UPLC
MS/MS	ABSCIEX API 4000 Q-Trap, run in ESI positive mode
MRMs Pyridate	379/207, 379/351, 381/209
MRMs Pyridafol	207/68, 209/104
Column	Acquity BEH C18, 2.1x100 mm, 1.7 µm
Pre-column	Acquity BEH C18, 2.1x5 mm, 1.7 µm
Mobile Phase	A: 5 mmol NH_4formate in purified water + 5% Methanol B: 5 mmol NH_4formate in Methanol
Gradient	Time min / Mobile Phase A % / Mobile Phase B % 0 / 90 / 10 5 / 10 / 90 5.1 / 10 / 90 11 / 90 / 10
Flow	0.4 mL min^{-1}
Injection volume	2 µL, partial loop with needle overfill
Column temperature	40°C
Internal Standard	Chlorpyrifos D10

Tab. 3: LC-MS/MS mass Transitions for Pyridate and Pyridafol (ESI-positive mode):

Compound	Tune Sensitivity	First Mass	Second Mass	DP	EP	CEP	CE	CXP	Mode
Pyridate	1	379	207	41	12	16	25	4	ESI pos
Pyridate	2	379	351	41	12	16	37	4	ESI pos
Pyridate	3	379	209	41	12	16	27	4	ESI pos
Pyridafol	1	207	68	46	6,5	14	29	4	ESI pos
Pyridafol	2	207	77	46	6,5	14	43	4	ESI pos
Pyridafol	3	207	104	46	6,5	14	49	4	ESI pos
Pyridafol	3	209	77	46	6,5	14	49	4	ESI pos

Note: Pyridafol can be also measured in the ESI-neg. mode. Mass transitions: 205>66; 205>35 and 207>66

Table 4: Recovery rates of Pyridate and Pyridafol. In the case of Pyridate recoveries are shown for pyridate as such as well as for the "sum of Pyridate and Pyridafol expressed as Pyridate"

			Spiked compound: PYRIDATE @ 0.1 mg/kg							Spiked compound PYRIDAFOL @ 0.1 mg/kg		
			Recoveries Pyridate [%] (Remaining Pyridate)			Recoveries Pyridate [%] (Pyridafol formed during extraction expr. as Pyridate)			Total recovery (∑ Ø)	Recoveries Pyridafol [%]		
#	Matrix	QuEChERS-Conditions	n_1	n_2	Ø	n_1	n_2	Ø		n_1	n_2	Ø
1	**Raspberry** (acidic)	As CEN 15662, without cleanup	103	103	**103**	2	2	**2**	**105**	**102**	**110**	**106**
2	**Cucumber**	As CEN 15662, without cleanup	102	101	**101**	2	2	**2**	**104**	**95**	**93**	**94**
3	**Cucumber**	As CEN 15662 (standard method) (with dSPE using PSA + **immedi-ate** acidification with formic acid)	72	75	**74**	28	27	**27**	**101**	**83**	**83**	**83**
4	**Cucumber**	As CEN 15662 but acidification w. formic acid **after 1 hour**	64	59	**62**	35	36	**35**	**97**	**82**	**81**	**82**
5	**Cucumber**	As CEN 15662 but acidification w. formic acid **after 7 hours**	33	29	**31**	81	81	**81**	**112**	**83**	**81**	**82**
6	**Cucumber**	QuEChERS (modified) (EURL-Method for Chlorothalonil[2] involving acidification w. sulfuric acid prior to extraction)	103	99	**101**	2	2	**2**	**103**	**90**	**94**	**92**
7	**Cucumber**	QuEChERS (modified) (EURL-method for Nicotine involv-ing add. of NaOH prior to extraction to reach pH ~11) and no cleanup[3]	0	0	**0.0**	109	111	**110**	**110**	**91**	**91**	**91**

In-source fragmentation

Considerable in-source fragmentation of pyridate to pyridafol was noticed in LC-MS/MS resulting in large signals at the mass-traces of pyridafol. For example: injecting a pyridate at ca. 0.1 µg/mL in the ESI pos. mode resulted in a pyridafol signal at the retention time of pyridate corresponding to ca. 0.08 µg/mL pyridate (following mathematic conversion of pyridafol to pyridate), see Figure 1. In-source fragmentation seems to take place reproducibly thus accuracy of measurement is not affected. However, in case the pyridafol signal formed through in-source fragmentation is missallocated to pyridafol as such would result in an excessive overestimation of total pyridate. This should particularly be kept track of where pyridafol is measured in the ESI-neg. mode without having a feedback on the retention time of pyridate.

[2] **http://www.crl-pesticides.eu/library/docs/srm/meth_QuEChERSforChlorothalonil_2010.PDF**
[3] **http://www.crl-pesticides.eu/library/docs/srm/meth_NicotineMushrooms_CrlFvCrlSrm.pdf**

Figure 1: Chromatograms of Pyridate and Pyridafol resulting after a recovery experiment of pyridate at 0.1 mg/kg on cucumber using QuEChERS but without any cleanup step (no PSA), (#2, n1 in Table 4).

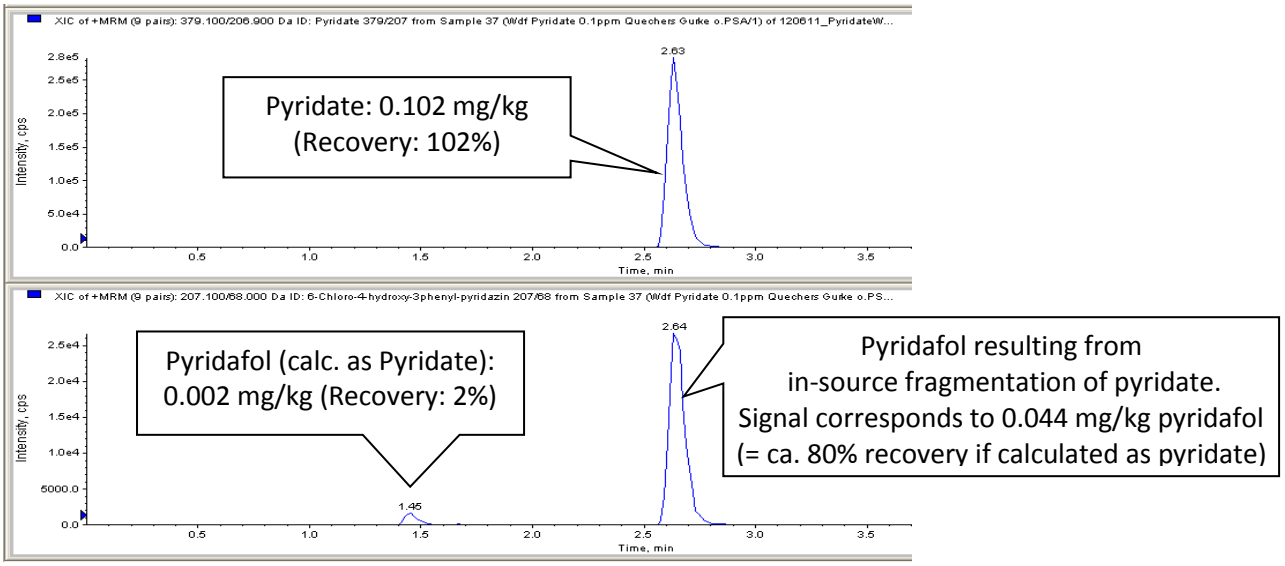


Figure 2: Chromatograms of Pyridate and Pyridafol resulting after a recovery experiment of pyridate at 0.1 mg/kg on cucumber using QuEChERS without dSPE using PSA but with delayed re-acidification (#5, n1 in Table 4).

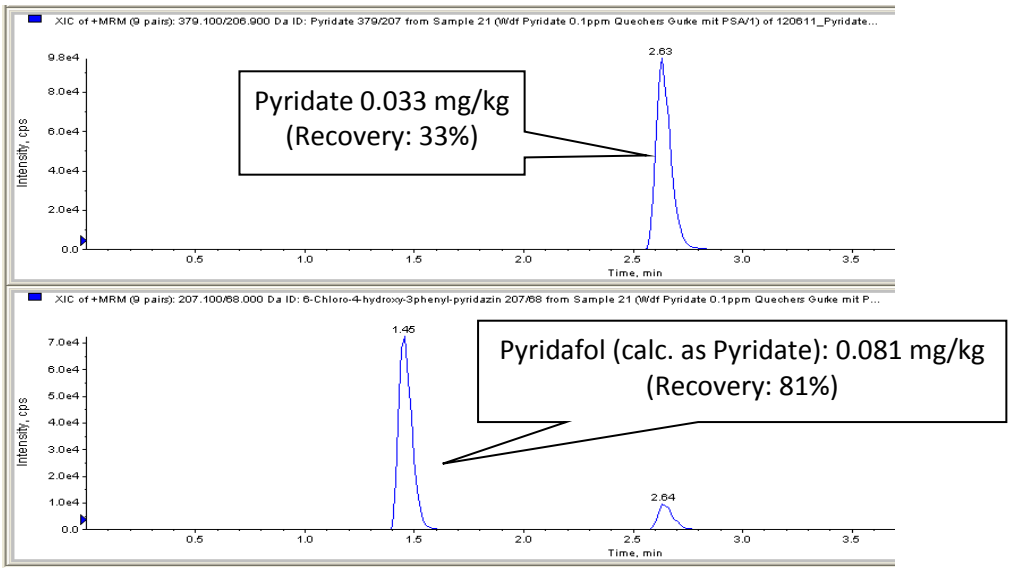
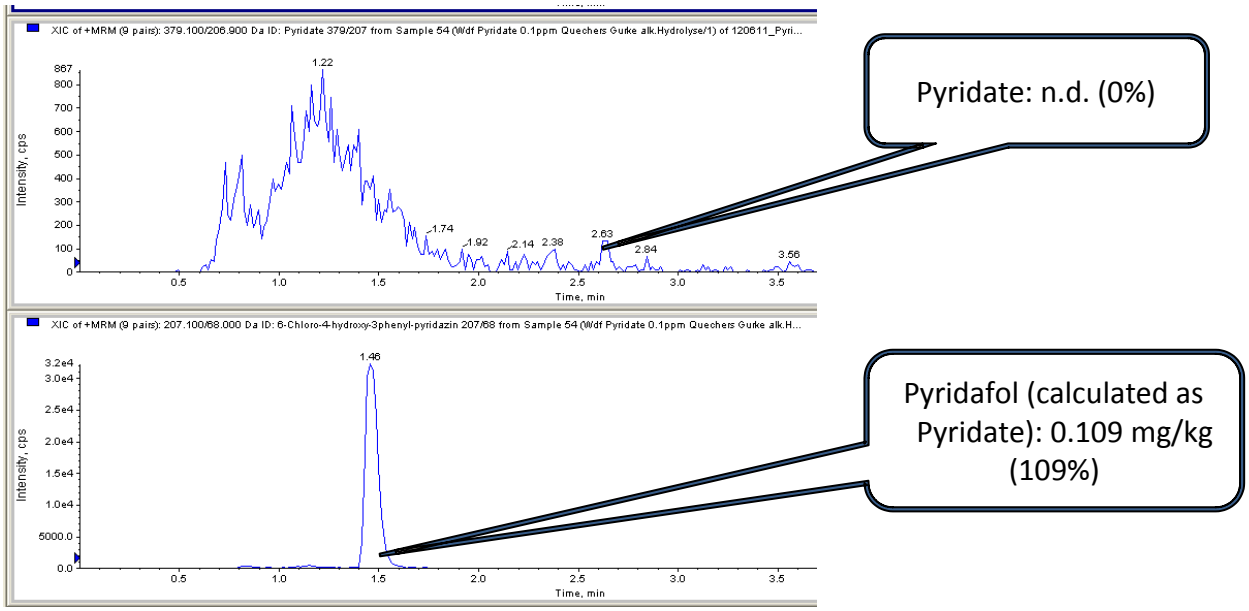


Figure 3: Chromatograms of Pyridate and Pyridafol resulting after a recovery experiment of pyridate at 0.1 mg/kg on cucumber using modified QuEChERS with extraction under alkaline conditions (#7, n1 in Table 4).



Discussion and conclusions:

The results clearly show the following:

a) For both pyridate and pyridafol quantitative recoveries can be achieved using QuEChERS (EN-15662) without cleanup (skipping dSPE-cleanup with PSA),
b) During dSPE-cleanup with PSA pyridate and pyridafol experience losses. Pyridate due to degradation and the acidic pyridafol (pKa = 6.7) due to interaction with PSA.
c) The transformation of pyridate to pyridafol is typically quantitative
d) Analysis of pyridate and pyridafol next to each other is possible but keeping them in separate calibration solutions is indicated to maintain the ability to monitor the degradation of pyridate to pyridafol in solution and avoid errors if this takes place unnoticed.
e) A quantitative conversion of pyridate to pyridafol during analysis is possible by raising the pH either during extraction or of the final extract.
f) Considerable in-source fragmentation of pyridate to pyridafol was noticed in LC-MS/MS but this seems to take place in a reproducible manner and as long as chromatographic separation between pyridate and pyridafol exists this does not affect the accuracy of analysis. This should be particularly kept track of where pyridafol is measured in the ESI-neg. mode without having feedback on the retention time of pyridate.
g) More experiments will follow as soon as commodities with incurred residues and conjugated pyridafol become available.

References:

EFSA Journal 2012;10(4):2687 : Reasoned opinion on the review of the existing maximum residue levels (MRLs) for pyridate according to Article 12 of Regulation (EC) No 396/20051, **http://www.efsa.europa.eu/en/efsajournal/doc/2687.pdf**