EURL-SRM - Analytical Observations Report

concerning the following…

- **Compound(s)**: Carbofuran, Benfuracarb, Carbosulfan, Furathiocarb, 3-Hydroxy-Carbofuran
- **Commodities**: Various commodities of plant origin
- **Method(s)**: QuEChERS followed by a hydrolysis step
- **Instrumentation**: LC-MS/MS

Analysis of Carbofuran (sum) by applying hydrolysis on QuEChERS extracts

Reported by: EURL-SRM
Version 1 (last update: 20.04.2016)

Background information / Initial Observations:

Carbofuran (CF) is an insecticide, nematicide and acaricide of the carbamate group. Carbosulfan (CS), furathiocarb (FT) and benfuracarb (BF) are pro-pesticides that degrade to CF, which is the active substance. To account for the high acute neurotoxicity of CF very low toxicological reference values were allocated in 2009 (ARfD 0.00015 mg/kg bw)[1] [2]. The metabolite 3-OH-carbofuran (3-OH-CF) was considered exhibiting similar toxicity as the parent.

Originally CS, BF and FT were regulated separately from CF and its metabolite 3-OH-CF. The separate MRLs of CS and BF were, however, difficult to enforce as these two compounds are highly susceptible to degradation during extraction and extract storage, especially where the pH of the matrix is low. For this reason, the QuEChERS protocol (EN-15662) foresees not acidifying the extracts after cleanup by dispersive SPE with PSA, to minimize losses between extraction and measurement. But even then, further losses are expected during sample milling, sample extraction (especially in acidic commodities prior to buffering) or storage of homogenates, especially if pH is low and temperatures high.

In 2012 the MRLs of CF, BF, CS and FT for most products were lowered to their respective consensus LOQ at that time[2]. Depending on the commodity groups, the MRLs were set at 0.02*, 0.05* or 0.1* mg/kg for BF and at 0.01*, 0.02* or 0.05* mg/kg for CF, FT and CS. For CS and CF there were also some cases in which CXLs were taken over in EU legislation (e.g. 0.1 mg/kg for CS and 0.5 mg/kg for CF in oranges, mandarins).

A toxicological re-evaluation by EFSA in 2014[3], however, revealed acute health concerns with these LOQ-based MRLs for CF and with some MRLs of CF, CS and FT. An interim proposal was thus made with MRLs of BF, CS and FT being proposed to be set at the respective consensus LOQs and of CF/3-OH-CF at low levels considering the toxicological reference values established in 2009. The proposals for CF were well below 0.01 mg/kg for food products having high short term consumption (0.001 mg/kg for potatoes, apples and milk and 0.002 mg/kg for

[1] Conclusion on pesticide peer review regarding the risk assessment of the active substance carbofuran (http://www.efsa.europa.eu/en/efsajournal/pub/310r) , published 10 July 2009
[2] Commission Regulation (EU) 2012/899
[3] **http://www.efsa.europa.eu/sites/default/files/scientific_output/files/main_documents/3559.pdf**

stone fruits, grapes, fruiting vegetables, brassica vegetables, salad plants and stem vegetables). All these MRL*s were calculated to be safe based on the toxicological endpoints of BF, FT and CS (see Table 1).

Table 1: ARfD-Calculations using existing / intermediately proposed MRLs:

Commodity	Carbofuran			Carbosulfan		Benfuracarb		Furathiocarb	
	MRL * mg/kg	% ARfD	Note	MRL * mg/kg	% ARfD	MRL * mg/kg	% ARfD	MRL * mg/kg	% ARfD
Oranges	0.01	60	applying a PF for fruit/pulp of 0.1	0.01	27%	0.02	13%	0.01	22%
Apples	0.001	65%		0.01	20%	0.02	10%	0.01	16%
Tomatoes	0.002	78%		0.01	30%	0.02	15%	0.01	25%
Cucumber	0.002	78%		0.01	30%	0.02	15%	0.01	25%
Potatoes	0.001	103%	slight exceedance acceptable as potatoes are processed	0.01	31%	0.02	15%	0.01	26%
Onions	0.002	106%	slight exceedance	0.02	16%	0.05	10%	0.02	13%

Separately regulating BF, CS and FT proved, however, to be of concern not only because of the above-mentioned analytical difficulties but even more so because of acute risks potentially arising following degradation of the 3 pro-pesticides to CF during industrial or household processing. Experiments conducted by the EURL-SRM and shown in this observation report demonstrated that the proposed MRLs for BF, CS and FT are indeed toxicologically rather critical. Finally it was decided to include CS, BF and FT in the new residue definition, for CF in food of plant origin and honey, which was established in 2015[4]. For food products having high short term consumption the MRLs for CF (sum) were set well below 0.01 mg/kg (0.001 mg/kg for potatoes, apples and milk and 0.002 mg/kg for stone fruits, grapes, fruiting vegetables, brassica vegetables, salad plants and stem vegetables). For products of animal origin the current residue definition refers to 3-OH-CF (free and conjugated).

A concern for the laboratories was whether these very low MRLs can be effectively enforced given the 5 components included in the residue definition for CF (sum). A quantitative conversion of CS, BF and FT to CF during analysis was thus considered highly beneficial, as it reduces the number of compounds to be analytically determined from five to only two (CF and 3-OH-CF).

[4] Commission Regulation (EU) 2015/399

The observations described in the following concern the following topics:

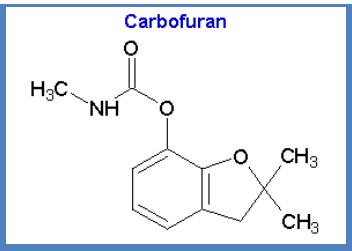
a) Acute risk calculations at different conversion rates of BF, FT and CS to CF
b) Conversion rates of BF, FT and CS to CF in a simulated household processing experiment
c) Recoveries of BF, CS, and FT using EN-15662
d) Stability experiments of BF, FT and CS in QuEChERS extracts of different pH
e) Intentional hydrolysis of BF, FT and CS to CF at various conditions

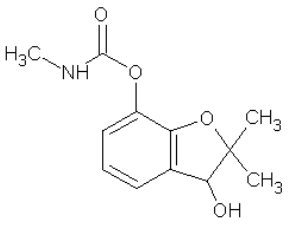
Compound profile:

CF is an insecticide, nematicide and acaricide of the carbamate group. CS, FT and BF are pro-pesticides that degrade to CF, which is the active substance. None of the four compounds is approved in the EU but there are still uses in other countries. Carbofuran acts against soil-dwelling and foliar-feeding insects (including wireworms, white grubs, millipedes, fruit flies, bean seed flies, root flies, flea beetles, weevils, aphids and thrips) and nematodes in vegetables, ornamentals, beet, maize, sorghum, sunflowers, oilseed rape, potatoes, alfalfa, peanuts, soya beans, sugar cane, rice, cotton, coffee, cucurbits, tobacco, lavender, citrus, vines, strawberries, bananas, mushrooms, and other crops. [1] A recent import-tolerance request for mushrooms from China (exposed through rice straw) was rejected following the toxicological re-evaluation of CF.

Facts at a glance:

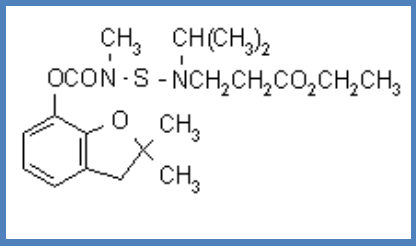
Carbofuran	
Mode of action	Systemic, with predominantly contact and stomach action [1]
LogP	1.8 at 20°C, no effect of pH [2]
Water solubility	315 mg/L at 19.5°C +/- 2.0 °C, no effect of pH [2]
Stability	Unstable in alkaline media. Stable in acidic and neutral media. Decomposes >150 °C [1]
Residue definition EU	**Food of plant origin and honey**: "*Carbofuran (sum of carbofuran (including any carbofuran generated from carbosulfan, benfuracarb or furathiocarb) and 3-OH carbofuran expressed as carbofuran)*" **Food of animal origin:** "*3-OH-carbofuran (free and conjugated) expressed as carbofuran*"
Registration Status	Not approved within the EU
ADI	0.00015 mg/kg bw/day (2009) [2]
ARfD	0.00015 mg/kg bw (2009) [2]
Main metabolite	3-Hydroxycarbofuran



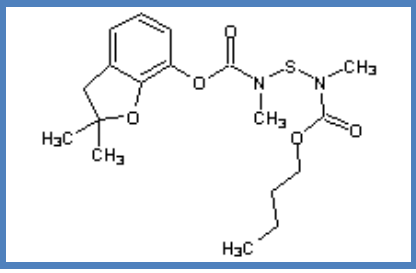


The following pro-pesticides metabolize to carbofuran (the active component):

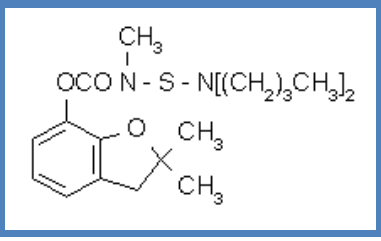
Benfuracarb	
Mode of action	Systemic and contact insecticide with stomach and contact action [1]
LogP	4.22 (25°C) [2]
Water solubility	8 (20°C, pH 4) [2]
Stability	Stable in neutral and weakly basic media, but unstable in acidic and strongly basic media. Stable up to 190 °C [1]
Residue definition EU	**See carbofuran**
Registration Status	Not approved within the EU
ADI	0.01 mg/kg bw/day (2009) [2]
ARfD	0.02 mg/kg bw (2009) [2]



Furathiocarb	
Mode of action	Systemic insecticide with contact and stomach action [1]
LogP	4.6 (25°C) [1]
Water solubility	11 (25°C) [1]
Stability	Stable up to 400 °C [1]
Residue definition EU	**See carbofuran**
Registration Status	Not approved within the EU
ADI	0.0035 mg/kg bw/day (RMS 1999) [3]
ARfD	0.006 mg/kg bw (RMS 1999) [3]



Carbosulfan	
Mode of action	Systemic insecticide with contact and stomach action [1]
LogP	7.42 (25°C, pH-independent) [2]
Water solubility	0.11mg/L (25°C, pH 9) [2]
Stability	Hydrolysed in aqueous media [1]
Residue definition EU	**See carbofuran**
Registration Status	Not approved within the EU [3]
ADI	0.005 mg/kg bw/day (2009) [2]
ARfD	0.005 mg/kg bw (2009) [2]



Experiments conducted and observations:

Materials (exemplary[5]):

Carbofuran (purity 99,5%), purchased from Dr. Ehrenstorfer (Cat #: C11010000)
Benfuracarb (purity 92%), purchased from Dr. Ehrenstorfer (Cat #: CA10475000)
Furathiocarb (purity 99%), purchased from Dr. Ehrenstorfer (Cat #: C13970000)
Carbosulfan (purity 98%), purchased from Dr. Ehrenstorfer (Cat #: CA11030000)
3-Hydroxycarbofuran (purity 98%), purchased from Dr. Ehrenstorfer (Cat #: C 11011000)

Table 2: Instrumentation details:

LC	WATERS Acquity UPLC		
MS/MS	ABSCIEX 5500 Q-Trap, run in ESI positive mode		
MRMs	222.2/122.9; 222.2/165.1		
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	100	0
	0.5	60	40
	7.5	10	90
	11.0	10	90
	11.1	100	0
	14.0	100	0
Flow	0.4 mL min^{-1}		
Injection volume	2 µL, partial loop with needle overfill		
Column temperature	40°C		

Table 3: MS/MS parameters (exemplary ABSciex 5500 Qtrap)

Substance	Intensity	Q 1	Q 3	DP	CE	CXP
Carbofuran	1	222	123	76	29	6
	2		165	76	17	14
Benfuracarb	1	411	190	71	17	10
	2		102	71	45	6
	3		74	71	67	2
Furathiocarb	1	383	195	96	27	10
	2		252	96	19	2
	3		167	96	35	8
Carbosulfan	1	381	118	86	29	20
	2		160	81	21	14
	3		76	76	53	6
3-Hydroxy-carbofuran	1	238	163	76	21	10
	2		181	76	17	10
	3		220	76	11	40

[5] 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

a) Acute risk calculations at different conversion rates of BF, FT and CS to CF:

To start with, a theoretical exercise was conducted to demonstrate that already small conversion rates of BF, CS and FT to CF during food processing can potentially lead to toxicologically critical CF levels, even if the proposed MRLs for the three pro-pesticides were not exceeded.

The theoretical exercise was based on commodities with high portion sizes, namely oranges, cucumbers, tomatoes, apples, potatoes and onions. For these products the proposed MRLs were 0.01 mg/kg for CS and FT and 0.02 mg/kg for BF. Assuming residue levels at the supposedly safe MRLs proposed and considering the molecular weight factors between CF and the three pro-pesticides, it was calculated to which percentage the IESTI for CF was covered/exceeded if the conversion rates to CF were 10%, 20%, 30%, 50% or 100%. This calculation was done for each pro-pesticide commodity combination. Variability factors and the peeling factor in the case of oranges were already considered in the IESTI calculations done by EFSA.

These calculations are shown in Table 4a and 4b. Assuming a **0.01 mg/kg level** (the intermediately proposed MRL for FT and CS in these food products) already a conversion rate of 20% of any of the three pro-pesticides to CF would theoretically lead to an ARfD exceedances of CF in the case of potatoes and onions. The same applies for apples at a conversion rate of 30% and for tomatoes and cucumbers at a conversion rate of 50%. Assuming a **0.02 mg/kg level** (the intermediately proposed MRL for BF) already a conversion rate of 10% of any of the three pro-pesticides to CF would theoretically lead to an ARfD exceedances of CF in the case of potatoes and onions. The same applies for apples at a conversion rate of 20% and for tomatoes and cucumbers at a conversion rate of 30%.

To account molecular weight differences the following conversion factors:

Conversion pair	Factor
CF to BF	1.855
CF to FT	1.73
CF to CS	1.72

Table 4: Theoretical calculation of Acute Risks associated with the conversion of CS, BF or FT to CF during processing assuming different conversion rates. Pesticide/ Commodity/ Conversion rate – combinations where the IESTI (100% ARfD coverage) is exceeded are highlighted in red.

a) Assuming the initial level of CS, BF or FT as being 0.01 mg/kg

Theoretical Conversion rate	Parent	Resulting Carbofuran from 10 ppb parent [ppb]	Oranges	Tomatoes+ Cuccumbers	Apples	Potatoes	Onions
	Carbosulfan	5.81	35	227	378	599	616
100%	Benfuracarb	5.38	32	210	349	554	570
	Furathiocarb	5.78	35	225	376	595	613
	Carbosulfan	2.91	17	113	189	299	308
50%	Benfuracarb	2.69	16	105	175	277	285
	Furathiocarb	2.89	17	113	188	298	306
	Carbosulfan	1.74	10	68	113	180	185
30%	Benfuracarb	1.61	10	63	105	166	171
	Furathiocarb	1.73	10	68	113	179	184
	Carbosulfan	1.16	7	45	76	120	123
20%	Benfuracarb	1.08	6	42	70	111	114
	Furathiocarb	1.16	7	45	75	119	123
	Carbosulfan	0.581	3	23	38	60	62
10%	Benfuracarb	0.538	3	21	35	55	57
	Furathiocarb	0.578	3	23	38	60	61

b) Assuming the initial level of CS, BF or FT as being 0.02 mg/kg

Theoretical Conversion rate	Parent	Resulting Carbofuran from 20 ppb parent [ppb]	Oranges	Tomatoes+ Cuccumbers	Apples	Potatoes	Onions
	Carbosulfan	11.63	70	453	756	1198	1233
100%	Benfuracarb	10.75	65	419	699	1108	1140
	Furathiocarb	11.56	69	451	751	1191	1225
	Carbosulfan	5.81	35	227	378	599	616
50%	Benfuracarb	5.38	32	210	349	554	570
	Furathiocarb	5.78	35	225	376	595	613
	Carbosulfan	3.49	21	136	227	359	370
30%	Benfuracarb	3.23	19	126	210	332	342
	Furathiocarb	3.47	21	135	225	357	368
	Carbosulfan	2.33	14	91	151	240	247
20%	Benfuracarb	2.15	13	84	140	222	228
	Furathiocarb	2.31	14	90	150	238	245
	Carbosulfan	1.163	7	45	76	120	123
10%	Benfuracarb	1.075	6	42	70	111	114
	Furathiocarb	1.156	7	45	75	119	123

b) Conversion rates of BF, FT and CS to CF during simulated household processing

A simple experiment was conducted to get a first idea of the behavior and conversion rates of CS, BF and FT to CF in processed food items. Two different scenarios were chosen:

i. Finely sliced cucumbers left standing for 4h at RT

ii. Grated apple, slightly sprinkled with lemon juice and left standing for 4h at RT

CF, BF and FT were separately spiked at levels representing their proposed MRLs in apples and cucumbers and the CF generated was determined using a procedure that minimizes degradation of CS, BF and FT (QuEChERS with no acidification following dSPE cleanup with PSA sorbent). The conversion rates observed for each compound are shown in Table 4. For BF and CS the CF levels generated within the 4 hours storage clearly exceeded the toxicologically critical threshold. Same applies for CS in the case of apple. Interestingly, despite the higher pH, conversion in cucumbers was more pronounced than in apples. Also conversion rates of FT were higher than of BF and CS, which is in contrast to the observations made during extraction and extract storage, where FT was significantly more stable than BF and CS. This suggests an enzymatically assisted decomposition.

Table 5: Observed conversion rates of CS, BF and FT to CF in two different household processing scenarios;
a) Finely sliced cucumbers left standing for 4h at RT; and
b) Grated apple, slightly acidified with lemon and left standing for 4h at RT
and calculation of the acute risks associated with the released CF in case CS, BF and FT were present at levels corresponding to their MRLs

	Carbosulfan (CS)		Benfuracarb (BF)		Furathiocarb (FT)		MRLs CF	100% ARfD (IESTI)
	Experimentally determined conversion rate to CF	**10 µg/kg*** CS will covert to x µg/kg CF	Experimentally determined conversion rate to CF	**20 µg/kg*** BF will covert to x µg/kg CF	Experimentally determined conversion rate to CF	**10 µg/kg*** FT will covert to x µg/kg CF	µg/kg (ppb)	µg/kg (ppb)
Apple	**33%**	**1.94**	**27%**	**2.88**	**48%**	**2.75**	**1**	**1.54**
Cuccumber	**5%**	**0.27**	**43%**	**4.62**	**76%**	**4.37**	**2**	**2.56**

*Concentrations corresponding to intermediately proposed MRL

c) Recoveries of BF, CS, and FT using EN-15662

Separate recovery experiments of BF, CS and FT in cucumber and orange juice were conducted using the QuEChERS method (EN 15662) with a 15 min first extraction step. The commodities were spiked at 0.1 mg/kg using standard solutions of FT, CS and BF in pure acetonitrile. These were shown to contain some CF as follows: The FT solution contained 0.2% CF (expressed as FT); the CS solution 0.9% CF (expressed as CS); and the BF solution 5% CF (expressed as BF). For the sake of simplicity the deviations in the initial concentration of the parents and CF were disregarded in the following calculations.

The QuEChERS extracts were analyzed either directly or following dSPE with MgSO4/PSA without acidification (as proposed in EN15662).

i. Analysis from raw QuEChERS extracts

By the time of the measurement of the raw orange juice extracts (4h after extraction) BF parent practically disappeared and CS dropped by >90%. By the time of the measurement of the raw cucumber extracts (2h after extraction), BF parent dropped by 85% within 2h and by 95% within 3h whereas CS dropped by 44% within 2h and by 60% within 3h. FT was quite stable with CF corresponding to ca 0.6 % FT being formed in raw cucumber extracts within 3-4 h and to ca. 1% in raw orange juice extracts within 6h.

The decline of BF and CS concentration was, however, not accompanied by a proportional increase of CF. Apparently due to the formation of **intermediate products** the formation of CF was considerably delayed. Interestingly, and although the degradation rates in orange juice extracts were higher than in cucumber extracts the formation rate of CF in cucumber was less delayed than in orange juice extracts. As a result the summed recovery of BF / CS plus CF in the case of orange juice extracts was much lower (ca. 30% for BF and ca. 40% for CS) compared to cucumber extracts (ca. 90% for BF and ca. 95% for CS).

Similar observations were made when injecting standard solutions of BF and CS based on blank raw extracts of orange juice and cucumber. In the solutions based on **orange-juice** BF losses within 3 h after preparation were already >99% with only 30% of the original BF being converted to CF. CS losses were 83% with only 28% being converted to CF. In the solutions based on **cucumber** raw extract BF losses within 3 h were as high as 71% with 50% of the original BF being converted to CF and CS losses were 39% with 26% being converted to CF.

Overall these results indicate that measurement of BF and CS from raw extracts is critical. Losses occur both during extraction and storage of the extracts in vials with the latter factor contributing more, especially if measurement is delayed.

Table 6: Recovery experiments for BF, CS, FT with analysis of CF, BF, CS and FT in QuEChERS RAW Extracts (Calibration in pure acetonitrile)

Spiked at 0.1 mg/kg to the matrix	#	Time elapsed (between extraction and measurement)	Cucumber			Time elapsed (between extraction and measurement	Orange juice		
			% parent left	% CF formed (expr. as parent)	Sum		% parent left	% CF formed (expr. as parent)	Sum
BENFURACARB (BF)	1	2 h	15	70	85	4 h	0.1	30	30
	2	2.5 h	14	73	87	5 h	0.0	30	30
	3	3 h	5	87	93	5.5 h	0.0	33	33
	ø		**11**	**77**	**88**		**0.03**	**31**	**31**
CARBOSULFAN (CS)	1	2 h	56	34	90	4 h	9	33	42
	2	2.5 h	43	53	96	5 h	6	35	41
	3	3 h	40	59	99	5.5 h	5	36	40
	ø		**46**	**49**	**95**		**7**	**35**	**41**
FURATHIOCARB (FT)	1	2.5 h	95	0.5	96	4.5 h	104	0.9	105
	2	3 h	96	0.6	99	5.5 h	101	1	102
	3	3.5 h	99	0.6	100	6 h	103	0.9	104
	ø		**98**	**0.6**	**98**		**103**	**0.9**	**104**

ii. Analysis from QuEChERS extracts following dSPE with $MgSO_4$ /PSA without acidification

The extracts of the recovery experiment described above were thus additionally injected following dSPE cleanup (with $MgSO_4$ /PSA) and no acidification afterwards (in the following abbreviated as CNA extracts = Cleaned-up but Not Acidified). This procedure is recommended in the EN 15662 (QuEChERS standard) for CS, BF and FT (at that time these compounds were separately regulated). Cleanup with PSA causes the pH to rise and reduces degradation. The pH of the extracts in the case of cucumbers was 7.6.

To check the stability of BF, CS and FT during the storage of CNA extracts (following dSPE with PSA, without acidification) standard solutions based on blank CNA-extracts orange juice and cucumber extracts were prepared. In the case of **cucumbers** CNA extracts spiked with CS, the levels of CF increased from ca 1% (level in the acetonitrile working standard) to ca. 3% (expressed as CS) within 8h. In extracts spiked with BF the CF levels increased from 5% to ca 11 % (expressed as BF). In the case of **orange juice** CNA extracts spiked with CS, the levels of CF increased from ca 1% to ca. 2% (expressed as BF) within 11h. In extracts spiked with BF the CF levels increased from 5% to ca 8 % (expressed as BF). In all cases losses of the parents were minor.

This indicates that following cleanup with PSA degradation of BF and CS slows down considerably. This measure will however not compensate the losses during the extraction procedure.

Analysis of CNA-extracts of the recovery studies showed considerably higher losses (see Table 7), indicating that they mostly occurred prior to cleanup with PSA. In the case of **cucumbers** losses of BF ranged between 25-30%, with the CF levels rising to ca. 30% (expressed as BF). Deducting losses of ca. 5% which occurred during the storage of CNA-extracts (over 9-10 hours) the BF losses actually occurring during the extraction step were expectedly in the range of ca. 20-25%. CS losses under the same conditions were in the range of 15 % increasing to ca 25%. In the case of **orange juice** BF losses were in the range of 45-55% with most of them occurring during the extraction step. The CF formed was in the range of 35% (expressed as BF). CS losses ranged around 25% with ca. 20% CF being formed (expressed as BF). CS losses under the same conditions were in the range of 15 % (expressed as CS). Employing the orange juice samples for extraction at room temperature, BF losses increased further by ca. 10% as CS-losses by ca. 7%.

Table 7: Recovery experiments for BF, CS, FT with analysis of CF, BF, CS and FT in QuEChERS Extracts <u>following dSPE-cleanup with PSA and no acidification</u> (Calibration in pure acetonitrile)

Spiked at 0.1 mg/kg to the matrix	#	Time elapsed (between extraction and measurement)	Cucumber			Time elapsed (between extraction and measurement)	Orange juice		
			% parent left	% CF formed (expr. as parent)	Sum		% parent left	% CF formed (expr. as parent)	Sum
BENFURACARB (BF)	1	9 h	75	27	102	11.5 h	46	36	82
	2	9.5 h	69	28	98	12.5 h	53	36	89
	3	10 h	76	34	110	13 h	48	37	85
	ø		**73**	**30**	**103**		**49**	**36**	**85**
CARBOSULFAN (CS)	1	9 h	91	10	100	11.5 h	74	20	94
	2	9.5 h	85	13	97	12.5 h	73	20	93
	3	10 h	82	12	95	13 h	72	19	91
	ø		**86**	**12**	**97**		**73**	**20**	**93**
FURATHIOCARB (FT)	1	9,5 h	100	0.5	101	12	95	1.2	96
	2	10 h	101	0.6	102	13	94	1.2	95
	3	10.5 h	104	0.5	105	13.5	97	1.3	98
	ø		**102**	**0.5**	**103**		**101**	**1.2**	**102**

Overall these results indicate that the analysis of BF and CS using QuEChERS is critical especially in the case of acidic samples. When analyzing raw extracts the main part of the losses occurs during the waiting time between extraction and measurement. Under acidic conditions the formation of CF is delayed leading to reduced summed recoveries. In a routine analysis (e.g. for screening) this could lead to underestimations or

even false negative results. When CNA-extracts are measured losses occurring during the extraction step play the main role. Recovery rates of the parents are acceptable and the conversion rates of BF and CS are increased. Thus, in theory, summed analysis of the CS or BF plus CF is possible. Given the very low MRLs conversion of the parents to CF is however preferred. FT proved to be very stable in both raw extracts experiencing practically no losses.

d) Conversion of BF, FT and CS to CF in QuEChERS extracts of different pH

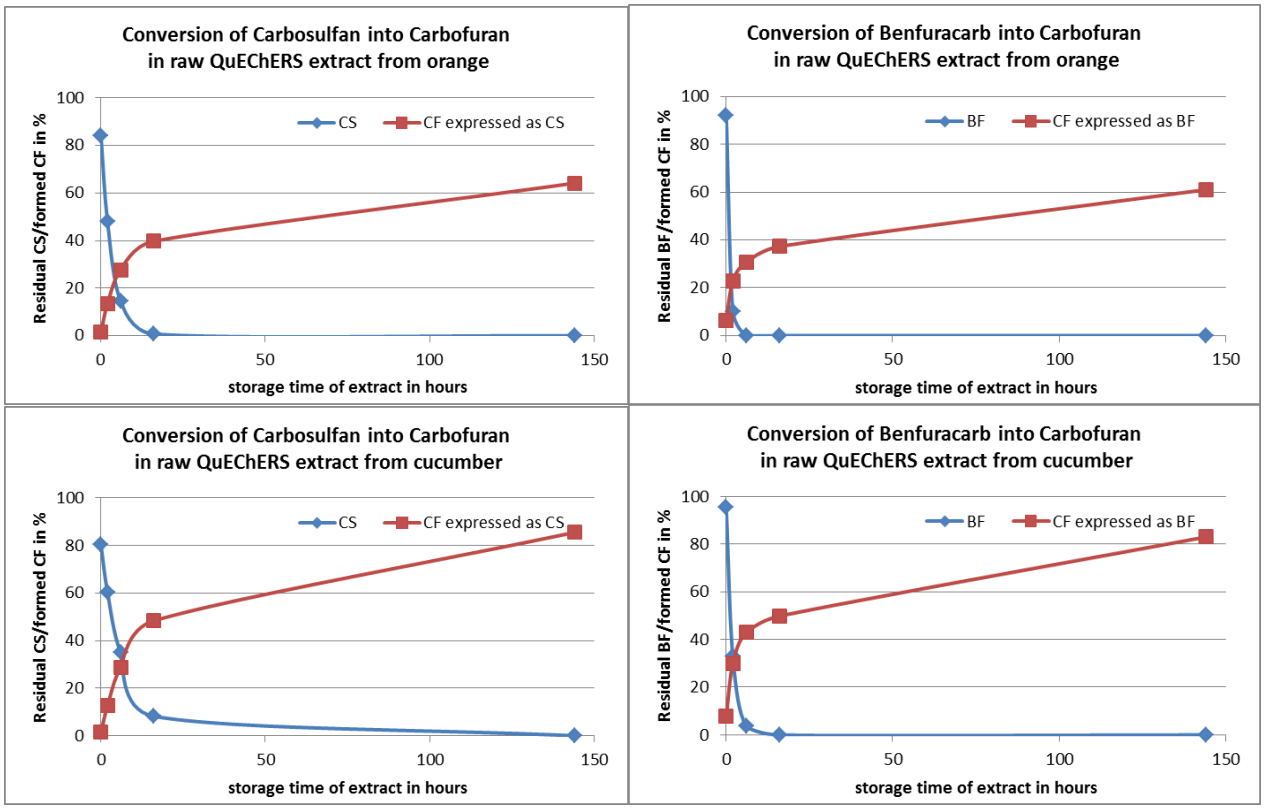
In order to study how CS, BF and FT behave in extracts having different pH, different types of extracts were prepared as follows, spiked and analyzed by LC-MS/MS after certain time intervals.

a) Raw extracts (pH ~ 4 for cucumbers and pH ~ 3.6 for oranges)
b) Raw extracts slightly acidified with 50µL of 5% formic acid in ACN (pH ~3.2 for cucumbers)
c) Raw extracts strongly acidified with 10µL of 99% formic acid (pH ~2.5[6] for cucumbers and oranges)
d) Extracts after cleanup with PSA but not acidified ("CNA extract"; pH 7.6 for cucumbers)
e) "CNA extracts" acidified 10µL of 5% formic acid (10%) (pH ~ 4 for cucumber and ca. 4.4 for orange)
f) "CNA extracts" after adding 10µL of 25% ammonia (pH ~ 9.7 for cucumber and ca. 9.4 for orange)
g) Raw extracts acidified with 10 µL H_2SO_4

a) Raw QuEChERS extracts (pH:~4 for cucumbers and pH:~3.6 for oranges)

Raw QuEChERS extracts of cucumbers (pH ~4.0) and oranges (pH ~3.6) were separately spiked with BF, CS or FT and left standing at room temperature determining at certain time intervals a) the residual parent compounds; and b) the conversion rate to CF. After ensuring that matrix effects do not affect measurement, calibration solutions for CF, BF, FT and CS in pure acetonitrile were used. These were prepared by appropriately diluting the spiking solutions and were found to contain either non-measurable or very insignificant levels of CF. Already within the few minutes (0h) after spiking the extracts, CS losses were in the order of 20%. After 2h CS and BF dropped by 67% and 40% respectively in cucumber extract and by 90% and 52% respectively in the more acidic orange extract. After 18h no BF was left in any of the extracts whereas CS dropped to 8% in cucumber extract and 1% in orange extracts. As in the previous experiment the degradation rate did not correlate with the formation of CF, indicating that certain intermediate products are formed. After 18h, conversion yields of BF to CF in the cucumber extract were only 50% rising to 83% after 6 days. In orange extracts the conversion rates of BF to CF were 37% after 18h and 61% after 6 days. Similar observations were made for CS with conversion rates to CF after 6 days reaching 85% in cucumber and 64% in orange extracts. FT proved to be resistant to hydrolysis in raw QuEChERS extracts, with very minor losses being noticed even after 6 days of extract storage.

[6] pH values as measured with a pH electrode



b) Raw extracts acidified with 10 µL 99% formic acid (pH ~2.5 for cucumbers and oranges)

The strongly acidified raw QuEChERS extracts were separately spiked with BF and CS and measured after 2 hours. In both cucumber and orange extracts BF and CS disappeared after two hours. Conversion rate of BF and CS to CF was quantitative in cucumber but only ca. 60% in oranges for both CS and BF.

c) Raw extracts acidified with 50 µL of 5% formic acid in ACN (pH ~3.2 for cucumbers)

The slightly acidified raw QuEChERS extracts were separately spiked with BF, CS and FT and measured after 6 hours and 5 days. FT remained stable. In both cucumber and orange extracts BF and CS disappeared after 6 hours. After 6 hours the conversion rate to CF was ca 90% in the case of BF and ca. 85% in the case of CS. After 5 days both the conversion rate was quantitative in both cases.

d) Extracts after cleanup with PSA but not acidified ("CNA extract"; pH ~7.6 for cucumbers)

The CNA extracts were separately spiked with BF, CS and FT and measured after 5d. All compounds were stable.

e) "CNA extracts" acidified 10 µL of 5% formic acid (10%) (pH ~ 4 for cucumber and ~ 4.4 for orange)

All three compounds were spiked (FT, BF and CS) and measured after 2 hours, 6 hours and 18 hours. FT remained stable. In cucumber BF dropped by ca 90% after 18 hours with the CF formed reaching only ca. 45% (expressed as BF). CS dropped by ca. 55% after 18 hours with the CF formed reaching only ca. 28% (expressed as CS). In oranges BF dropped by ca 80% after 18 hours with the CF formed reaching only ca. 14% (expressed as BF). CS dropped by ca. 40% after 18 hours with the CF formed reaching only ca. 9% (expressed as CS).

f) "CNA extracts" after adding 10 µL of 25% ammoniac (pH ~ 9.7 for cucumber and ca. 9.4 for orange)

Only FT was spiked and remained stable over 2 days. In conclusion **Carbosulfan and Benfuracarb tend to rapidly degrade in acidic extracts with CF being formed with some delay** (formation of intermediate products). **Furathiocarb is quite stable at acidic as well as at alkaline conditions at room temperature.**

g) Raw extracts acidified with 10 µL 5N H_2SO_4 and heated up

Experiments b) and c) where acidification with formic acid was employed showed that hydrolysis of BF and CF to FT was possible with the formation of CF increasing with time and eventually becoming quantitative after some hours. FT, however proved to be much more resistant to acidic hydrolysis. It was thus decided applying elevated temperature during the acidic conversion in the QuEChERS extract.

When extending hydrolysis time to 6h no significant drop of CF was noticed indicating the CF is quite stable under these hydrolysis conditions.

Hydrolysis Time at 80°C	Conversion rate to CF (expressed as BF or CS or FT)		
	BF	CS	FT
2h	104	96	92
4h	103	96	95
6h	104	102	95

Carbofuran phenol was shown not be formed at any significant levels. The use of Chlorpyrifos-D10 as internal standard is critical as it degrades under these hydrolysis conditions. The formation of 3,5,6-tricloropyridinol (TCPy) was observed. Carbofuran D3 is better suitable as IS.

The final method developed is as follows:

Extraction: Apply the **citrate buffered QuEChERS (EN 15662)**. Weigh 10 g of frozen fruit or vegetable homogenate or 5 g of cereals; adjust water content to 10 mL where necessary, add 10 mL acetonitrile and internal standard (e.g. 100 µL of an appropriately concentrated solution of Carbofuran-D3[7]). Shake 15 min using a mechanical shaker. Add a mixture of 4g $MgSO_4$, 1g NaCl, 1 g trisodium citrate dihydrate and 0.5 g disodium hydrogen citrate sesquihydrate, shake 1 min and centrifuge.

Cleanup: Cleanup via dispersive SPE is optional for fruits and vegetables.

Hydrolysis: Transfer 1 mL of raw extract into vial and add 10 µL 5N H_2SO_4. Nearly quantitative transformation of BF, CS and FT into CF is achieved by heating the vials for 3h at 80°C.

LC-MS/MS analysis: For screening purposes CF, 3-OH-CF as well as BF, FT and CS may be analyzed by LC-MS/MS directly in QuEChERS raw extracts or cleaned-up extracts. In case of positive findings the hydrolysis step can be conducted as described above and LC-MS/MS analysis of CF repeated.

1mL final extract will represent approximately 1 g matrix.

For measurement conditions and recovery figures see EURL-SRM - Analytical Method Report (Analysis of Residues of Carbofuran (sum) using QuEChERS method).

References

[1] e-Pesticide Manual V5.0, Author: C. D. S. Tomlin; 15th Edition, Version 5.0, 2009
Publisher/Contact: The British Crop Protection Council
[2] EFSA
[3] EU Pesticides Database

Document History

Date	Action	Changes
Apr. 2016	Publication of V1	

[7] Before using an internal standard, check if it is stable under the hydrolysis conditions