Observations concerning…

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

Determination of Prochloraz (sum) via its Metabolites

Reported by: EURL-SRM

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Problem and goals:

The current residue definition (RD) of prochloraz (sum) calls for the use of procedures involving a hydrolysis step to release the 2,4,6-trichlorophenol (TCP) moiety. This moiety is contained in prochloraz as well as in several of its known metabolites. Analytical procedures involving hydrolysis are typically cumbersome and in most cases the benefit from conducting them does not justify the effort, thus very few laboratories employ them routinely. An alternative approach to, at least partly, fulfill the current residue definition would be the direct analysis of available MRM-amenable TCP-containing metabolites. In a recent reasoned opinion EFSA proposed a RD, which includes the parent and two MRM-amenable metabolites (BTS 44595 and BTS 44596). We aimed to study the analytical behavior of these two metabolites, and of three additional TCP-containing metabolites (BTS 9608, BTS 40348 and TCP). The occurrence of these metabolites and of prochloraz in real samples was also studied.

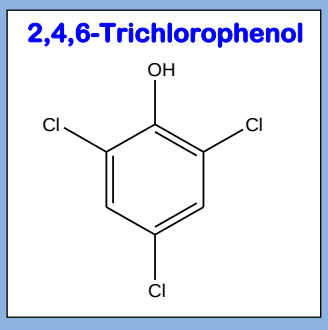
Brief description of observations and conclusions:

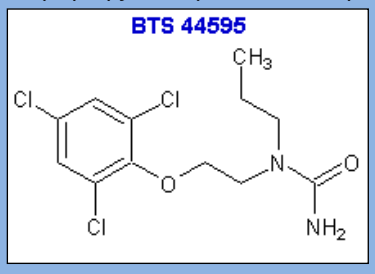
In GC-analysis the 4 studied prochloraz metabolites and prochloraz itself partly converted to TCP. The GC-conversion rates were variable depending on various factors such as instrument condition, matrix type and the presence/absence of analyte protectants. Prochloraz and its metabolites can all be analyzed via LC-MS/MS, though BTS 9608 and free TCP require ESI (neg) mode with the former showing poor detection sensitivity. Furthermore, in-source fragmentation of certain metabolites to other metabolites was noticed in LC-MS/MS requiring good LC-separation of the affected metabolites to avoid quantification errors.
Analysis of various real samples showed that parent prochloraz along with its metabolites BTS 44595, BTS 44596 and BTS 40348 are the major among the studied components. BTS 9608 and free TCP were, if at all, only present at very low levels. Various options to determine prochloraz (sum) according to the current residue definition using the GC and LC results of individual TCP-containing prochloraz metabolites are discussed. Although TCP can be very sensitively analyzed by GC-MS, TCP values derived from GC analysis should not be summed up with any values for prochloraz and other metabolites (derived by LC-MS/MS) as this will lead to overestimations. Due to the uncertainty in the GC analysis for TCP and prochloraz, GC-results should preferably only be used for screening purposes. LC-MS/MS results are more reliable provided that certain metabolites are chromatographically well separated.

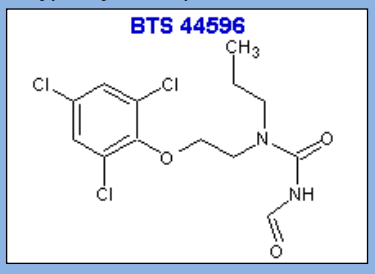
Compound profile:
Prochloraz is a fungicide that is widely used in the production of various crops such as cereals, vegetables and mushrooms. It is also used post-harvest as a dip treatment against storage or transit diseases of citrus fruit, mangos, papayas, pineapples and other tropical fruit. MRLs are set in the Commission Regulation (EU) No 520/2011 of 25 May 2011 with the RD being defined as follows: *Prochloraz (sum of prochloraz and its metabolites containing the 2,4,6-trichlorophenol moiety expressed as prochloraz)*. Some information on prochloraz including physicochemical properties and metabolites is shown in Table 1.

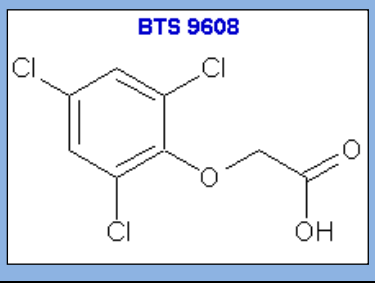
Table 1: Prochloraz and its main metabolites

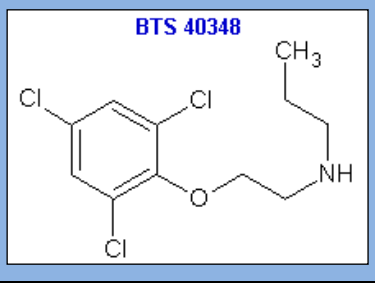
Parameter	Value	Notes	Prochloraz
Pka	3.8	at 20°C	
Log $P_{o/w}$	3.5	at pH 4-8	
Water solubility	26.5 mg/L @ pH 5-9	increases at lower pH	
Hydrolytic behavior	Stable at pH 5-7, DT50@ pH 9 : 80d	at 22°C	
EU Residue definition	Prochloraz (sum of prochloraz and its metabolites containing the 2,4,6-trichlorophenol moiety expressed as prochloraz)		
EFSA Proposal	Sum of prochloraz, BTS 44595 and BTS 44596, expressed as prochloraz		
Authorized in	AT, BE, BG, CY, CZ, DE, EE, EL, ES, FI, FR, HU, IE, IT, LT, LU, LV, NL, PL, PT, RO, SE, SI, SK, UK		
Main metabolites (both in the primary and in rotational crops) [1]	BTS 44595 (1-propyl-1-[2-(2,4,6 trichlorophenoxy)ethyl]urea) BTS 44596 (3-formyl-1-propyl-1-[2-(2,4,6-trichlorophenoxy)ethyl]urea) BTS 45186 (2,4,6-trichlorophenol =TCP) BTS 9608 (2,4,6-trichlorophenoxyacetic acid = 2,4,6-T) BTS 40348 (N-propyl-N-2-(2,4,6-trichlorophenoxy)-ethylamine)		











Experiments conducted and observations:

1. Analysis of prochloraz and its metabolites by GC-MS

When subjected to GC analysis prochloraz and its metabolites partly degrade to 2,4,6-trichlorophenol (TCP). When injecting prochloraz metabolites the only identifiable peak obtained corresponds to TCP. Prochloraz itself gives two peaks, one corresponding to prochloraz and one to TCP. The shape of the chromatograms suggests that these degradations to TCP mainly take place in the injector area.
Table 2 exemplarily shows the degradation rates observed in GC-MS (CI-neg.) when injecting standards in pure solvent and spiked QuEChERS cucumber extracts. Prochloraz and its metabolites were separately spiked at a level of 0.1 to 1 mg/kg. The system was calibrated using matrix-matched TCP standards in each case.

Table 2: Degradation rates of prochloraz and its metabolites to TCP in GC-MS (CI-neg.) depending on the composition of the solution (cucumber extract)

Compound injected	Exemplary conversion rates to TCP in GC-MS			
	Cucumber extract			
	Following dSPE cleanup (w. PSA)		No cleanup	
	with AP	w/o AP	with AP	w/o AP
Prochloraz	11 %	10 %	0 %	20 %
BTS 40348	27 %	52 %	41 %	60 %
BTS 44595	67 %	103 %	63 %	97 %
BTS 44596	38 %	86 %	40 %	57 %
BTS 9608	53 %	99 %	6 %	30 %

Repetitive injections of standards in different matrices and different cleanup procedures resulted in strongly fluctuating conversion rates to TCP. Overall exact quantification of prochloraz and its metabolites by GC is difficult. In general decomposition was less pronounced in presence of Analyte Protectants (APs). Based on these observations prochloraz and its metabolites can be ranked as regards their decomposition tendency to TCP as follows:

BTS 44595 >> BTS 9608 / BTS 44596 / BTS 40348 >> Prochloraz

We have additionally checked whether quantitative conversion of prochloraz and its metabolites to TCP can be achieved in GC as this would comprise an alternative procedure for the analysis of prochloraz (sum). For this we have injected extracts cleaned up by dSPE using PSA sorbent without acidifying to keep the pH high. However complete conversion could not be achieved with prochloraz (parent) being most resistant.

Possible errors to avoid in practice when using GC for the analysis of prochloraz: Being unaware that in GC prochloraz and its metabolites convert to TCP leads to the assump-

tion that the TCP signals obtained originate purely from TCP contained in the samples. This may lead to erroneous approaches for determining prochloraz (sum), e.g.:

a) Quantification of prochloraz and TCP via GC and calculation of prochloraz (sum). This approach will lead to an underestimation of prochloraz (sum) as the GC-conversion of the metabolites into TCP is typically not quantitative. *Note: This approach could potentially lead to correct results if the conversion rates of the various components to TCP were nearly quantitative and reproducible. In principle one could try to modify the extracts in such a way that conversion in the GC-injector is strongly promoted to become quasi quantitative. This aspect will have to be tested further at a later stage.*

b) Quantification of prochloraz and TCP via GC and separate quantification of one or more metabolites of prochloraz via LC-MS/MS. Then calculation of prochloraz (sum) based on these results. This approach will most probably lead to overestimated results of prochloraz (sum) as components decomposing to TPC in GC will be to some degree counted double.

c) Quantification TCP only via GC and separate quantification of prochloraz or prochloraz and one or more of its metabolites via LC-MS/MS. Then calculation of prochloraz (sum) based on these results. As in b) this approach will most probably lead to overestimated results of prochloraz (sum).

d) 2,4,5-T and 2,4,5-TP degrade in the GC injection system to 2,4,5-TCP, which has a similar retention time as 2,4,6-TCP and as the mass spectra are similar there is a potential for misidentification.

2. Analysis of prochloraz and its metabolites by LC-MS/MS

Prochloraz, BTS 44595, 44596 and 40348 can be analyzed via LC_MS/MS in the ESI positive mode. BTS 9608 and 2,4,6-TCP, both having acidic groups, are analyzed in the ESI negative mode:

Table 3: LC-MS/MS mode required for prochloraz and its metabolites

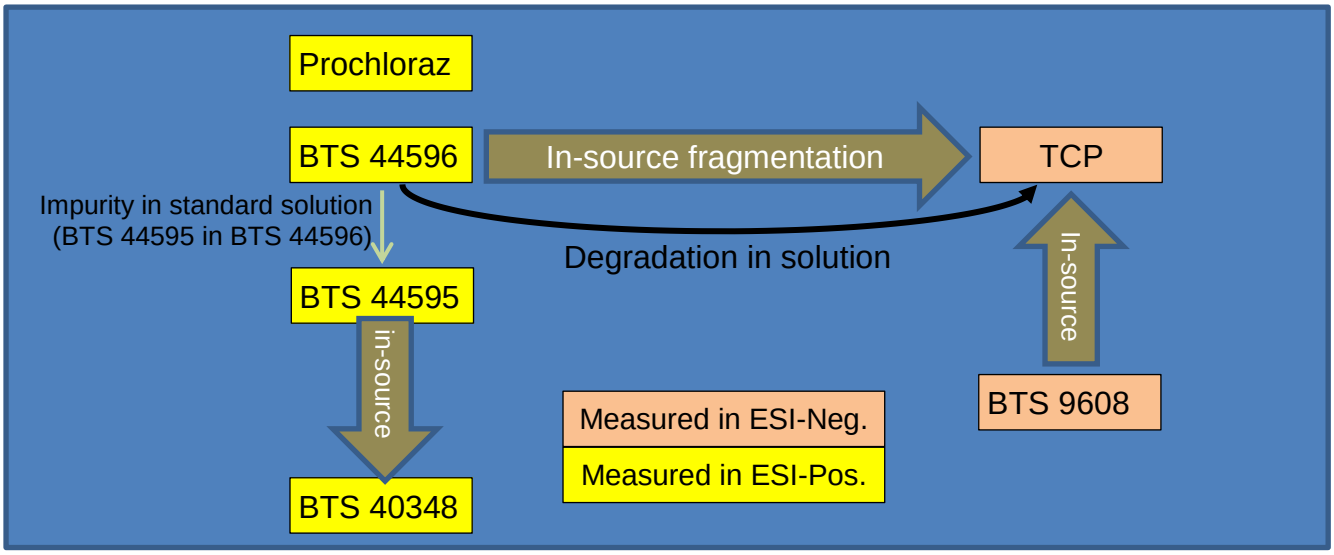
ESI positive mode		ESI negative mode	
Compound	**Sensitivity**	**Compound**	**Sensitivity**
Prochloraz	****	BTS 9608	***
BTS 44595	****	2,4,6-Trichlorophenol (TCP)	*
BTS 44596	****		
BTS 40348	***		

The analysis of the individual components is not straightforward due to the occurrence of in-source fragmentation. A good chromatographic separation of the affected components and the attention to retention times is necessary to avoid identification and quantification errors.

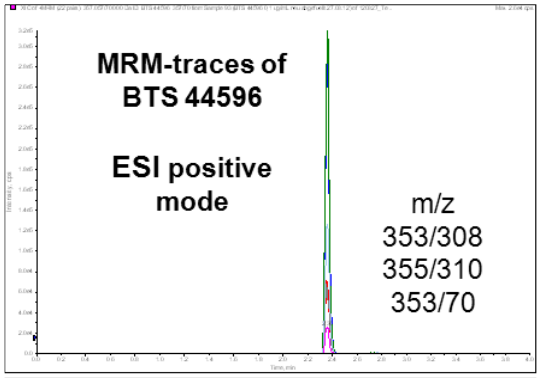
Figure 1 shows the in-source fragmentations observed using ABSciex API 5500. Prochloraz and BTS 40348 did not show any in-source fragmentation. BTS 9608 and BTS 44596 showed in-source fragmentation to 2,4,6-trichlorophenol. For BTS 44595 an in-source fragmentation to BTS 40348 was observed.

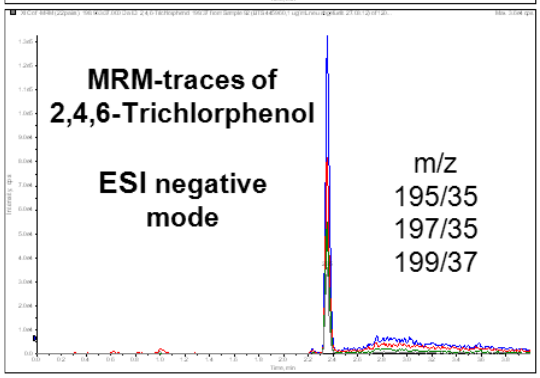
In-source fragmentations may differ depending on the conditions in different sources or the parameters set.
BTS 44596 additionally showed a small impurity of BTS 44595 in the standard solution as well as a tendency towards degradation to TCP in acetonitrile solutions (see more details under “Stability”).

Figure 1: In-source fragmentations of prochloraz metabolites using ABSciex API 5500



Although BTS 44596 is measured in the ESI-pos. mode, it is important to check its in-source fragmentation in the ESI-neg. mode to ensure that it will not co-elute with TCP or BTS 9608.



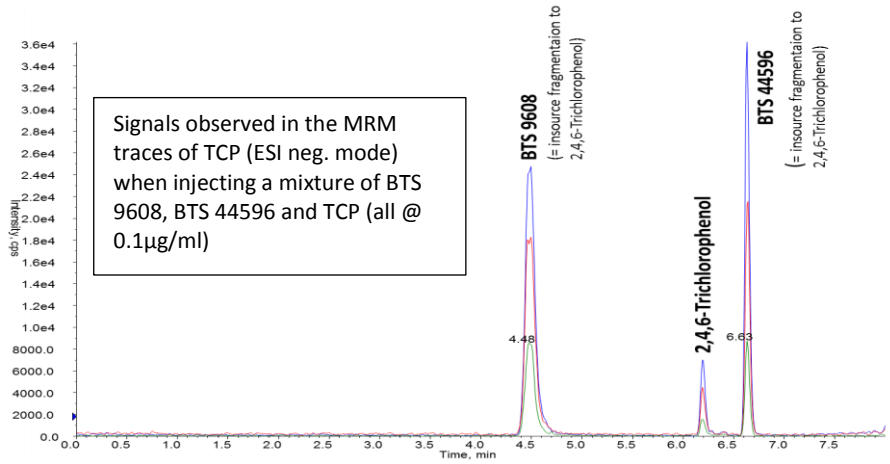


The chromatograms to the left demonstrate the in-source fragmentation of BTS 44596 to TCP. Injection of a fresh BTS 44596 solution resulted in a peak at the MRM-traces of TCP but at the retention time where BTS 44596 is expected rather than where TCP is expected. This indicates an in-source fragmentation of BTS 44596 to TCP. To ensure correct detemination these two compounds have to be separated chromatographically.
This kind of in-scource fragmentations, where one analyte of interest degrades to another but with these two analytes normally being measured under different LC-MS/MS conditions (different mode and/or LC conditions) are quite trickly and may remain unnoticed especially when these compounds are not present in the same calibration mixtures and thus not injected at the same time. Extracts of real samples can, of course contain both substances simultaneously.

Injection of the same BTS 44596 solution after one week showed an additional effect described under “stability”.

An in-source fragmentation to TCP was also observed for BTS 9608. Thus chromatographic separation should be ensured for all three compounds (see Figure 2).

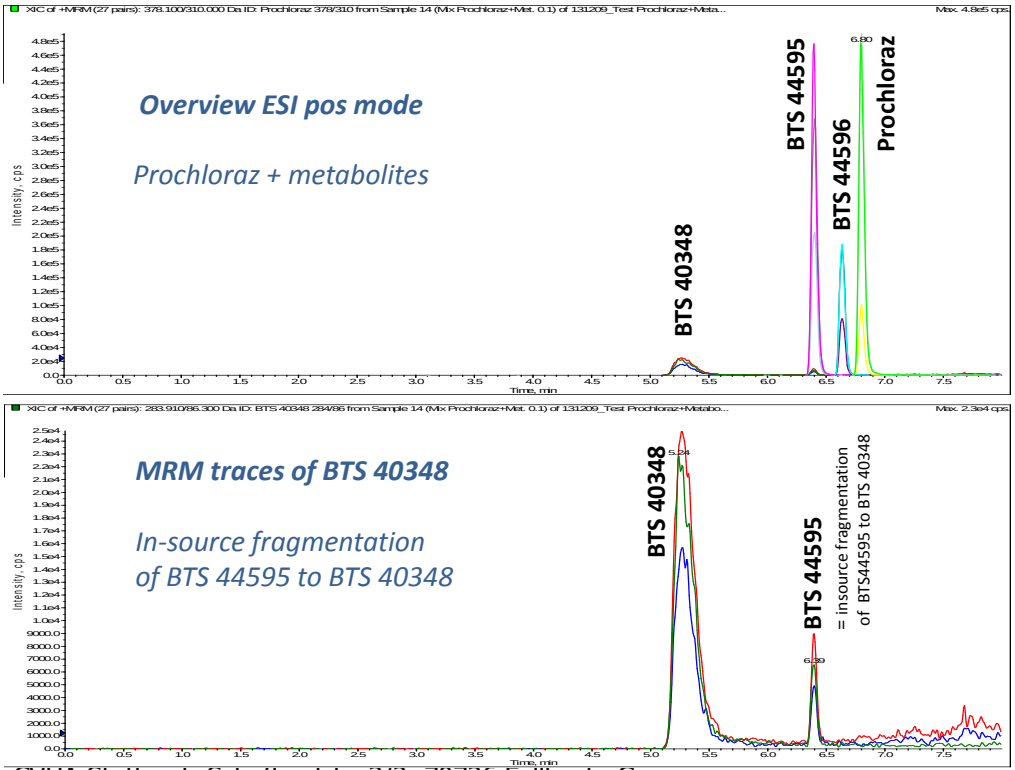
Figure 2: In-source fragmentation of BTS 9608 and BTS 44596 to TCP



Interestingly the TCP-signals resulting from in-scource fragmentation of BTS 9608 and BTS 44596 were stronger than those obtained when injecting TCP itself at the same concentration

The in-scource fragmentation of BTS 44595 to BTS 40348 is demonstrated in Figure 3. Also here chromatographic separation is crucial.

Figure 3: In-source fragmentation of BTS 9608 and BTS 44596 to TCP



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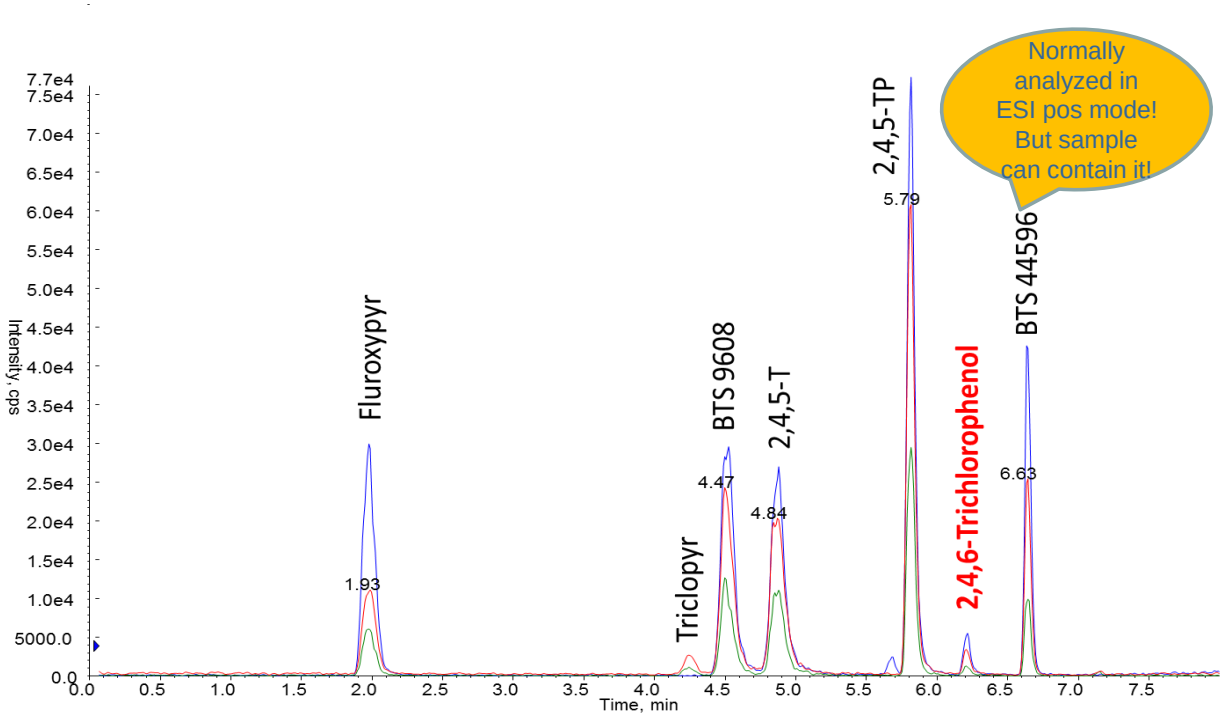
3. Further Interferences in LC-MS/MS

Other compounds, entailing a trichlorophenyl moiety may also interfere with 2,4,6-TCP. Some known examples are 2,4,5-TP and 2,4,5-T, which show an in-source fragmentation to 2,4,5-trichlorophenol, which has the same transitions as 2,4,6-trichlorophenol.

Although not containing any TCP moiety Fluroxypyr and Triclopyr also experience in-source fragmentation to products that give signals at the MRM-traces of 2,4,6-TCP. Also here a chromatographic separation of these compounds from 2,4,6-TCP is obligatory (see Figure 4).

Figure 4: MRM traces of 2,4,6-TCP when injecting a Mix of BTS 9608, BTS 44596, 2,4,6-trichlorophenol, Fluoroxypyr, Triclopyr, 2,4,5-T and 2,4,5-TP (all @ 0.1 µg/mL)

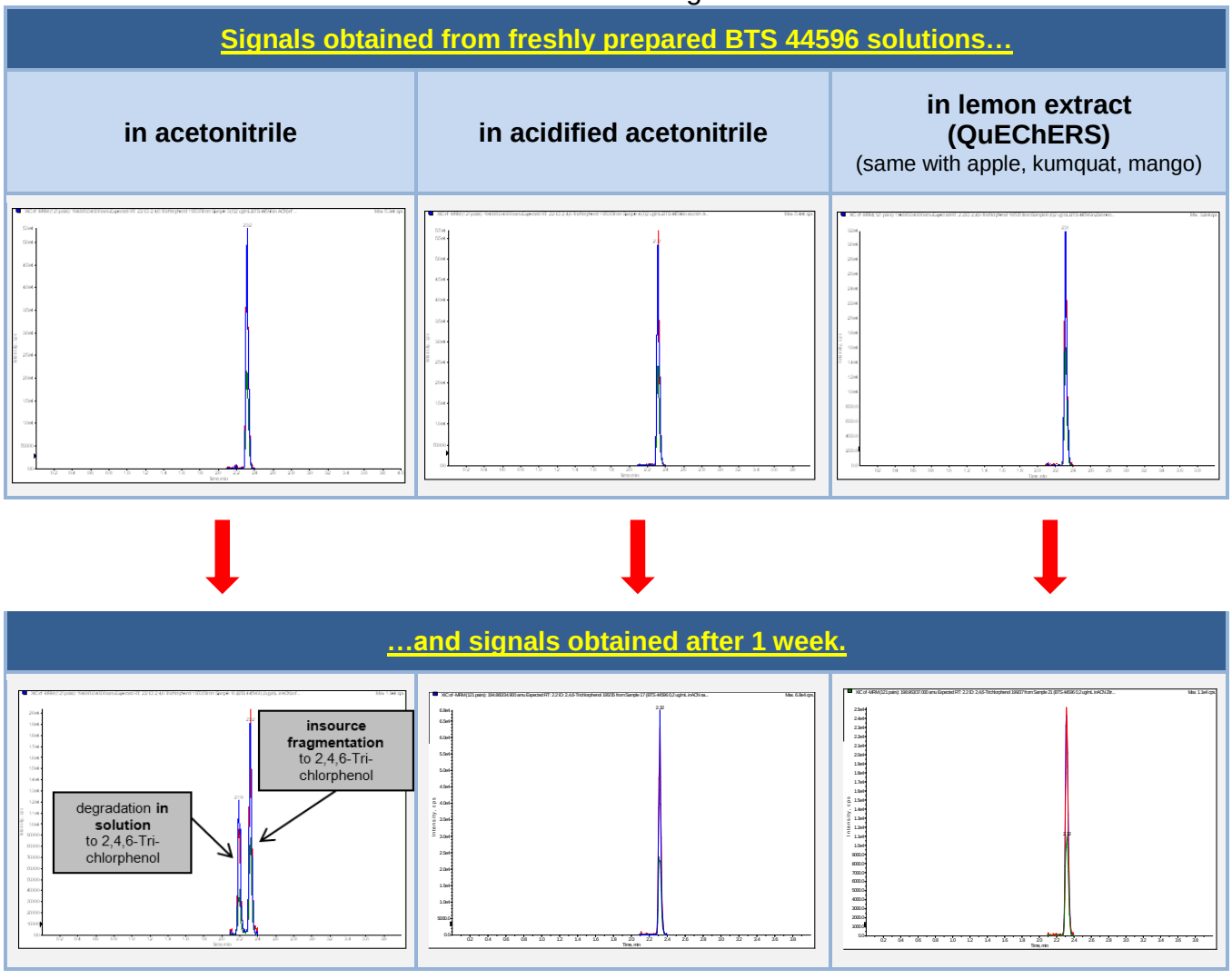


Also here the signal of 2,4,6-TCP is smaller than the signals produced by other similarly concentrated compounds via in-source fragmentation (all @ 0.1 µg/mL).

4. Stability in Solution

When injecting a 1-week old BTS 44596 solution (0.1 µg/mL in acetonitrile) an additional peak showed up at the MRM-traces of TCP. The later eluting peak was due to the in-source fragmentation of BTS 44596 to TCP as described earlier. The first eluting peak had the same retention time as TCP itself indicating degradation of BTS 44596 to TCP in solution. BTS 44596 was stable when stored in acetonitrile containing 0.4% acetic acid. Stock and working solutions of BTS 44596 in acetonitrile have thus to be acidified to prevent degradation. In QuEChERS raw extracts (without PSA cleanup) no degradation was observed. Figure 5 demonstrates these aspects.

Figure 5: Signals obtained when injecting a freshly prepared and a 1- week old BTS 44596 solution. All measurements were done in the ESI neg. mode at the MRM traces of TCP



Validation data for prochloraz and its metabolites BTS 40348, BTS 44595, BTS 44596 and BTS 9608:

						Recoveries		
Compound	**Commodity Group**	Spiking Level or Range (mg/kg)	Analytical Method (No of recov.)	Determination Technique (No of Recov.)	No of different Commod.	No of Recov.	AVG (%)	RSD (%)
Prochloraz	**High Water Content Commodities**	<= 0.01 (0.002; 0.005)	QuEChERS (citrate) (10)	LC-MS/MS (10)	1	10	89	9
		0.1	QuEChERS (citrate) (7)	LC-MS/MS (7)	1	7	105	5
	High Acid Content Commodities	<= 0.01 (0.002; 0.005; 0.01)	QuEChERS (citrate) (30)	LC-MS/MS (30)	4	34	102	9
		0.1	QuEChERS (citrate) (25)	LC-MS/MS (25)	4	40	100	6
	Dry Commodities (cereals, dry pulses)	0.01	QuEChERS (citrate) 10)	LC-MS/MS (10)	1	10	91	6
		0.1	QuEChERS (citrate) (5)	LC-MS/MS (5)	1	9	98	15
	High Fat Content Commodities (fatty/wet)	0,01	QuEChERS (citrate) (5)	LC-MS/MS (5)	1	5	92	3
		0.1	QuEChERS (citrate) (5)	LC-MS/MS (5)	1	5	95	9
	High Fat content Commodities (fatty/dry)	0.01	QuOil (15)	LC-MS/MS (15)	1	5	91	13
		0.05	QuOil (14)	LC-MS/MS 14)	3	9	85	8
		0.1	QuOil (14)	LC-MS/MS 14)	3	14	88	7
BTS 40348	**High Water Content Commodities**	<= 0.01 (0.005; 0.01)	QuEChERS (citrate) (11)	LC-ToF-MS (11)	2	11	97	5
		0.1	QuEChERS (citrate) (2)	LC-MS/MS (2)	1	2	96	2
	High Acid Content Commodities	0.1	QuEChERS (citrate) (1)	LC-ToF-MS (1)	1	1	93	
	Dry Commodities (cereals, dry pulses)	<= 0.01 (0.005; 0.01)	QuEChERS (citrate) (10)	LC-ToF-MS (10)	1	10	105	9
BTS 44595	**High Water Content Commodities**	<= 0.01 (0.005; 0.01)	QuEChERS (citrate) (15)	LC-ToF-MS (15)	2	15	94	10
		0.1	QuEChERS (citrate) (9)	LC-MS/MS (2); LC-ToF-MS (7)	1	9	98	4
	Dry Commodities (cereals, dry pulses)	<= 0.01 (0.005; 0.01)	QuEChERS (citrate) (15)	LC-ToF-MS (15)	1	15	102	14
		0.1	QuEChERS (citrate) (5)	LC-ToF-MS (5)	1	5	80	13
	High Fat Content Commodities (fatty/wet)	0.01	QuEChERS (citrate) (4)	LC-ToF-MS (4)	1	4	89	3
	High Fat content	0.01	QuOil (5)	LC-ToF-MS (5)	1	5	71	8

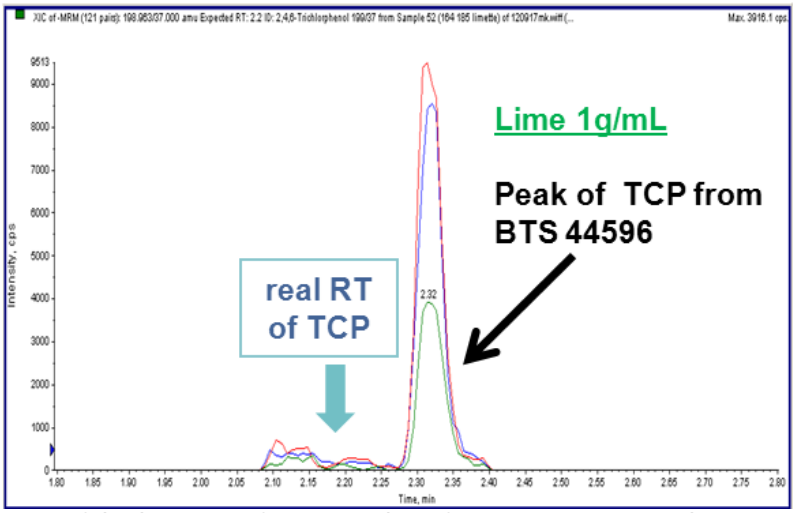
						Recoveries		
Compound	**Commodity Group**	Spiking Level or Range (mg/kg)	Analytical Method (No of recov.)	Determination Technique (No of Recov.)	No of different Commod.	No of Recov.	AVG (%)	RSD (%)
	Commodities (fatty/dry)	0.1	QuOil (5)	LC-ToF-MS (5)	1	5	78	2
BTS 44596	**High Water Content Commodities**	<= 0.01 (0.005; 0.01)	QuEChERS (citrate) (10);	LC-ToF-MS (10)	1	10	107	7
		0.1	QuEChERS (citrate) (8);	LC-MS/MS (4); LC-ToF-MS (4)	2	8	100	8
	High Acid Content Commodities	0.02	QuEChERS (citrate) (1);	LC-ToF-MS (1)	1	1	110	
	Dry Commodities (cereals, dry pulses)	<= 0.01 (0.005; 0.01)	QuEChERS (citrate) (10);	LC-ToF-MS (10)	1	10	78	25
		0.1	QuEChERS (citrate) (5);	LC-ToF-MS (5)	1	5	83	14
	High Fat Content Commodities (fatty/wet)	0.01	QuEChERS (citrate) (3);	LC-ToF-MS (3)	1	3	119	7
	High Fat content Commodities (fatty/dry)	0.01	QuOil (4);	LC-ToF-MS (4)	1	4	92	10
		0.1	QuOil (5);	LC-ToF-MS (5)	1	5	89	6
BTS 9608	**High Water Content Commodities**	0.1	QuEChERS (citrate) (2);	LC-MS/MS (2)	1	2	102	3
	High Acid Content Commodities	0.01	QuEChERS (citrate) (5);	LC-MS/MS (5)	1	5	99	5
		0.1	QuEChERS (citrate) (5);	LC-MS/MS (5)	1	5	105	4
	Dry Commodities (cereals, dry pulses)	<= 0.01 (0.005; 0.01)	QuEChERS (citrate) (10);	LC-MS/MS (10)	1	10	40	8
	High Fat Content Commodities (fatty/wet)	0.01	QuEChERS (citrate) (5);	LC-MS/MS (5)	1	5	107	7
	High Fat content Commodities (fatty/dry)	0.01	QuOil (5);	LC-MS/MS (5)	1	5	105	7
		0.1	QuOil (5);	LC-MS/MS (5)	1	5	93	2

Data source: http://www.eurl-pesticides-datapool.eu **go to "Validation Data"**

5. Analysis of real samples with prochloraz treatment history

Risk of false positive TCP results in LC-MS/MS

Real samples typically contain very little, if any, 2,4,6-trichlorophenol (TCP). As explained earlier there is however great risk of false positive results of TCP both in GC-MS (due to degradation of prochloraz and all its metabolites in the injector) and LC-MS/MS (due to degradation of BTS 44596 + BTS 9608 in the ion source).
In LC-MS/MS the risk of false positives is quite high since BTS 44596 is quite often contained in samples with prochloraz treatment history and since the TCP signals generated upon in-source fragmentation are quite intense. Good chromatographic separation and stable retention times are thus essential to avoid false positives of TCP.



Compound	Level (mg/kg)
Prochloraz	0.87
BTS 44595	0.036
BTS 44596	0.14
BTS 40348	not measured
BTS 9608	0.005
TCP	n.d. *

*Note: If the in-source-fragmentation of BTS 44595 to TCP is erroneously taken as TCP it would be calculated to 0.075 mg/kg.

Calculation of prochloraz (sum) using different approaches

Different types of samples (mango, lime, papaya, champignons, pineapple, avocado, pomelo) with positive findings of prochloraz were analyzed by GC-MS (CI-neg.) and LC-MS-MS. The results obtained were compared and added up using different approaches to total prochloraz (according to the existing residue definition), in order to demonstrate errors that may be made in practice.

For GC-calibration separate matrix-matched standard solutions for prochloraz and TCP were used in all cases. Please note that GC-results are associated with a higher uncertainty due to the poor reproducibility of the conversion into TCP.

For calculations the respective molecular weight ratios of prochloraz and the metabolites were used.

Mango

Results	LC-results (mg/kg)		GC-results (mg/kg)	
	Result of component	… calculated as prochloraz	Result of component	… calculated as prochloraz
Prochloraz	1.35	1.35	0.313	0.313
BTS 40348	0.071	0.095	-	-
BTS 44595	0.004	0.005	-	-
BTS 44596	0.030	0.032	-	-
BTS 9608	n.q. < 0.001	0	-	-
TCP	n.d.	0	0.372	0.710

n.d. = not detected; n.q.= not quantified

Calculation of prochloraz (sum) by different approaches – different approach, different result

Approach	Results considered in SUM calculation						Remark	Sum prochloraz (mg/kg)
	Prochloraz	BTS 44595	BTS 44596	BTS 40348	BTS 9608	TCP		
1	GC					GC	Underestimation due to incomplete transformation to TCP in GC	1.02
2	GC	LC	LC	LC	LC	GC	Despite overlapping determination of TCP-moiety underestimation of sum due to complete degradation of parent in GC with low transformation yields to TCP	1.16
3	LC	LC	LC	LC	LC	GC	Overestimation of sum due to overlapping determination of TCP-moiety	2.19
4	LC	LC	LC	LC	LC	LC	Best estimate for prochloraz (sum) according to existing RD using LC	**1.48**
5						GC/LC (following hydrolysis)	Prochloraz (sum) according to existing RD (involving Hydrolysis))	Not conducted
6	LC	LC	LC				Prochloraz (sum) according to RD proposed by EFSA	1.39
7	LC	LC	LC	LC			Possible alternative RD (including BTS 40348)	1.48

Lime

Results	LC-results (mg/kg)		GC-results (mg/kg)	
	Result of component	… calculated as prochloraz	Result of component	… calculated as prochloraz
Prochloraz	0.60	0.60	-	-
BTS 40348	0.10	0.133		
BTS 44595	0.005	0.006		
BTS 44596	0.020	0.021		
BTS 9608	n.q. < 0.001	0		
TCP	n.q. (< 0.01)	0	0.215	0.410

n.d. = not detected; n.q.= not quantified

Calculation of prochloraz (sum) by different approaches – different approach, different result

Approach	Results considered in SUM calculation						Remark	Sum prochloraz (mg/kg)
	PRZ	BTS 44595	BTS 44596	BTS 40348	BTS 9608	TCP		
1	GC					GC	Underestimation due to incomplete transformation to TCP in GC	0.41
2	GC	LC	LC	LC	LC	GC	Despite overlapping determination of TCP-moiety underestimation of sum due to complete degradation of parent in GC with low transformation yields to TCP	0.57
3	LC	LC	LC	LC	LC	GC	Overestimation of sum due to overlapping determination of TCP-moiety	1.17
4	LC	LC	LC	LC	LC	LC	Best estimate for prochloraz (sum) according to existing RD using LC	**0.76**
5						GC/LC (following hydrolysis)	Prochloraz (sum) according to existing RD (involving Hydrolysis))	Not done
6	LC	LC	LC				Prochloraz (sum) according to RD proposed by EFSA	0.63
7	LC	LC	LC	LC			Possible alternative RD (including BTS 40348)	0.76

Pineapple

Results	LC-results (mg/kg)		GC-results (mg/kg)	
	Result of component	… calculated as prochloraz	Result of component	… calculated as prochloraz
Prochloraz	0.089	0.089	0.080	0.080
BTS 40348	0.010	0.013		
BTS 44595	0.001	0.001		
BTS 44596	0.003	0.003		
BTS 9608	n.d.	0		
TCP	n.d.	0	0.017	0.032

n.d. = not detected; n.q.= not quantified

Calculation of prochloraz (sum) by different approaches – different approach, different result

Approach	Results considered in SUM calculation						Remark	Sum prochloraz (mg/kg)
	PRZ	BTS 44595	BTS 44596	BTS 40348	BTS 9608	TCP		
1	GC					GC	Good match due to moderate transformation to PRZ to TCP in GC and low levels of other metabolites	0.11
2	GC	LC	LC	LC	LC	GC	Overestimation of sum due to overlapping determination of TCP-moiety	0.13
3	LC	LC	LC	LC	LC	GC	Overestimation of sum due to overlapping determination of TCP-moiety	0.14
4	LC	LC	LC	LC	LC	LC	Best estimate for prochloraz (sum) according to existing RD using LC	**0.11**
5						GC/LC (following hydrolysis)	Prochloraz (sum) according to existing RD (involving Hydrolysis))	Not done
6	LC	LC	LC				Prochloraz (sum) according to RD proposed by EFSA	0.09
7	LC	LC	LC	LC			Possible alternative RD (including BTS 40348)	0.11

The following table shows a compilation of residue findings of prochloraz and its metabolites in real samples (analysed between July 2014 and July 2016):

Commodity	Country of Origin	Prochloraz mg/kg	BTS 40348 mg/kg	BTS 44596 mg/kg	BTS 44595 mg/kg	BTS 9608 mg/kg
Avocado	Chile	0.013	n.d.	n.d.	n.d.	n.d.
Avocado	Peru	0.009	n.d.	n.d.	n.d.	n.d.
Avocado	South Africa	0.43	0.11	0.054	n.q.	0.034
Avocado	South Africa	2.1	0.05	0.067	0.021	n.d.
Avocado	Israel	0.62	0.046	0.11	n.d.	0.02
Banana leafs	Thailand	0.006	0.006	n.d.	n.d.	n.d.
Garlic	Germany	0.038	n.d.	n.d.	n.d.	0.006
Garlic	Spain	0.006	n.d.	n.d.	n.d.	n.q.
Gojiberries Dried	unknown	0.039	0.009	0.019	0.012	n.d.
Gojiberries Dried	China	0.005	0.009	0.025	0.034	n.d.
Kumquat	Spain	0.006	n.q.	n.d.	n.d.	n.d.
Kumquat	Israel	0.005	n.q.	n.d.	n.d.	n.d.
Kumquat	Israel	n.q.	0.011	n.d.	n.d.	n.d.
Lemon	Spain	0.84	0.52	0.061	0.014	n.q.
Lemon	Spain	0.64	0.22	0.079	0.018	n.q.
Lemon	Spain	0.21	0.056	0.065	0.009	n.q.
Lemon	Spain	n.d.	0.041	0.091	0.029	n.d.
Lettuce Lamb's-	unknown	n.d.	0.006	n.d.	n.d.	n.d.
Lettuce Oak leaf-	Italy	0.033	n.q.	n.d.	n.d.	n.d.
Lime	Brasil	0.016	n.q.	n.q.	n.d.	n.d.
Lime	Brasil	0.007	n.d.	n.d.	n.d.	n.d.
Mango	Brasil	0.005	n.q.	n.d.	n.d.	n.d.
Mango	Brasil	2.1	0.85	0.072	n.q.	n.q.
Mango	South Africa	1.3	0.23	0.14	0.02	n.d.
Mango	Peru	2	0.21	0.05	0.006	n.d.
Mango	Germany	0.5	0.17	0.012	n.q.	n.q.
Mango	Mexico	1.7	0.15	0.076	0.016	n.q.
Mango	unknown	0.35	0.15	0.014	n.q.	n.d.
Mango	Brasil	0.26	0.081	0.053	0.008	n.d.
Mango	Peru	0.17	0.08	0.021	0.007	n.d.
Mango	Peru	0.35	0.073	0.009	n.q.	n.d.
Mango	Senegal	1.1	0.063	0.046	0.01	n.q.
Mango	Peru	0.86	0.057	0.038	0.009	n.q.
Mango	Peru	0.16	0.05	0.022	n.d.	n.d.
Mango	Peru	0.63	0.044	0.026	n.q.	n.d.
Mango	Israel	0.13	0.041	0.005	n.q.	n.d.

Commodity	Country of Origin	Prochloraz mg/kg	BTS 40348 mg/kg	BTS 44596 mg/kg	BTS 44595 mg/kg	BTS 9608 mg/kg
Mango	Cote d' Ivoire	0.14	0.03	0.006	n.d.	n.d.
Mango	Germany	0.15	0.024	0.009	n.d.	n.d.
Mango	Peru	0.2	0.021	0.008	n.d.	n.d.
Mango	Brasil	0.1	0.021	0.011	n.q.	n.d.
Mango	Peru	0.14	0.019	n.q.	n.d.	0.028
Mango	Peru	0.15	0.017	0.011	n.q.	n.d.
Mango	Israel	n.d.	0.013	n.q.	n.d.	n.d.
Mango	Brasil	0.073	0.013	0.005	n.q.	n.d.
Mango	Peru	0.09	0.012	0.005	n.d.	n.q.
Mango	Peru	0.039	0.012	0.006	n.d.	n.d.
Mango	Cote d' Ivoire	0.1	0.011	0.007	n.d.	n.d.
Mango	Peru	0.086	0.01	0.005	n.q.	n.d.
Mango Dried	unknown	n.q.	0.013	n.d.	n.d.	n.d.
Melon	Spain	n.d.	0.01	n.d.	n.q.	n.q.
Melon	Spain	n.q.	0.005	n.d.	n.d.	n.d.
Mushroom Golden Oyster-	Poland	n.d.	n.q.	n.d.	n.d.	0.013
Mushroom Oyster-	Poland	n.d.	0.01	n.d.	n.d.	n.q.
Mushroom White-	Germany	0.034	n.q.	n.q.	n.q.	n.q.
Mushroom White-	Poland	0.027	n.q.	n.d.	n.q.	n.q.
Mushroom White-	Poland	0.02	n.q.	n.d.	n.d.	n.q.
Mushroom White-	Poland	0.02	n.q.	n.d.	n.d.	0.01
Mushroom White-	Poland	0.017	n.q.	n.d.	n.d.	n.q.
Mushroom White-	Germany	0.01	n.q.	n.d.	n.q.	n.q.
Mushroom White-	Poland	0.008	n.q.	n.d.	n.q.	n.q.
Mushroom White-	Poland	0.006	n.q.	n.d.	n.d.	n.d.
Mushroom White-	Poland	0.005	n.q.	n.d.	n.d.	n.q.
Mushroom White-	Poland	0.005	n.q.	n.d.	n.d.	n.d.
Mushroom White-	Poland	0.005	n.d.	n.d.	n.d.	n.d.
Mushroom White-	Germany	n.q.	0.011	n.d.	n.d.	0.008
Mushroom White-	Poland	n.q.	0.006	n.d.	n.d.	n.d.
Mushroom White-	Poland	0.027	0.006	n.d.	n.q.	n.q.
Mushroom White-	Poland	n.q.	0.005	n.d.	n.q.	n.q.
Mushroom White-	Poland	n.q.	0.005	n.d.	n.d.	n.q.
Mushroom White-	Poland	0.085	0.005	n.d.	n.q.	n.q.
Mushroom White-	Poland	0.013	0.005	n.d.	n.d.	0.007
Mushroom White-	Poland	0.007	0.005	n.d.	n.d.	n.q.
Mushroom White-, Dried	Poland	0.12	0.047	n.d.	n.q.	0.015
Mushrooms, Mixed, Dried	unknown	0.015	0.012	n.d.	n.d.	n.d.
Papaya	Brasil	0.095	0.15	n.d.	n.d.	n.q.

Commodity	Country of Origin	Prochloraz mg/kg	BTS 40348 mg/kg	BTS 44596 mg/kg	BTS 44595 mg/kg	BTS 9608 mg/kg
Papaya	Brasil	0.067	0.044	n.d.	n.d.	0.005
Papaya	Ecuador	0.36	0.039	n.q.	n.q.	0.017
Papaya	Ecuador	0.72	0.031	0.011	n.q.	0.012
Passion Fruit	Colombia	n.d.	0.011	n.d.	n.d.	n.d.
Pineapple	Costa Rica	0.068	n.q.	n.d.	n.d.	n.d.
Pineapple	Costa Rica	0.055	n.q.	n.q.	n.d.	n.d.
Pineapple	Ghana	6.5	0.46	0.12	0.023	n.q.
Pineapple	Ghana	3.4	0.19	0.054	0.014	n.q.
Pineapple	Ghana	1.9	0.15	0.037	0.005	n.q.
Pineapple	Ghana	1.6	0.11	0.053	0.009	n.q.
Pineapple	Costa Rica	0.47	0.11	0.027	0.006	n.d.
Pineapple	Costa Rica	0.3	0.08	0.01	n.q.	n.d.
Pineapple	Ghana	1.1	0.077	0.052	0.009	n.d.
Pineapple	Costa Rica	0.38	0.075	0.036	0.005	n.d.
Pineapple	Costa Rica	0.08	0.064	0.015	n.d.	n.d.
Pineapple	Ghana	0.85	0.059	0.013	n.d.	n.q.
Pineapple	Costa Rica	0.39	0.043	0.006	n.q.	n.d.
Pineapple	Costa Rica	0.12	0.042	n.q.	n.d.	n.d.
Pineapple	Costa Rica	0.086	0.023	n.q.	n.d.	n.d.
Pineapple	Costa Rica	0.36	0.022	0.02	0.005	n.d.
Pineapple	Costa Rica	0.052	0.013	n.q.	n.d.	n.d.
Pineapple	Costa Rica	0.11	0.011	n.d.	n.d.	n.d.
Pineapple	Costa Rica	0.066	0.011	n.q.	n.d.	n.d.
Pineapple	Costa Rica	0.033	0.006	n.d.	n.d.	n.d.
Plum	Germany	n.q.	0.006	n.d.	n.d.	n.d.
Pomegranate	Turkey	3.2	0.15	0.017	0.01	n.d.
Pomegranate	Turkey	0.73	0.055	0.012	n.q.	n.d.
Pomelo	China	0.03	n.q.	n.d.	n.d.	n.d.
Pomelo	China	0.013	n.q.	n.d.	n.d.	n.d.
Pomelo	China	0.009	n.q.	n.d.	n.d.	n.d.
Pomelo	China	0.007	n.q.	n.d.	n.d.	n.d.
Pomelo	China	0.13	0.03	0.009	n.q.	n.d.
Pomelo	China	0.091	0.03	0.023	n.q.	n.d.
Pomelo	China	0.058	0.021	0.011	n.q.	n.d.
Pomelo	China	0.045	0.021	0.015	0.006	n.d.
Pomelo	China	0.008	0.021	0.006	n.q.	n.d.
Pomelo	China	0.1	0.019	n.d.	n.d.	n.d.
Pomelo	China	n.d.	0.017	0.007	n.d.	n.d.
Pomelo	China	0.22	0.017	n.d.	n.d.	n.d.

Commodity	Country of Origin	Prochloraz mg/kg	BTS 40348 mg/kg	BTS 44596 mg/kg	BTS 44595 mg/kg	BTS 9608 mg/kg
Pomelo	China	0.1	0.017	n.q.	n.d.	n.d.
Pomelo	China	0.053	0.016	n.q.	n.d.	n.d.
Pomelo	China	0.044	0.014	n.d.	n.d.	n.d.
Pomelo	China	0.041	0.008	0.011	n.d.	n.d.
Pomelo	China	0.062	0.007	n.d.	n.d.	n.d.
Quince	Italy	0.023	0.009	0.016	0.005	n.d.
Sour Cherries Canned	unknown	n.q.	0.024	n.d.	n.d.	n.q.
Sour Cherries Canned	unknown	n.q.	0.009	n.d.	n.q.	n.q.
Sour Cherries Canned	unknown	n.d.	0.006	n.d.	n.d.	n.q.
Sour Cherries Canned	unknown	n.d.	0.005	n.d.	n.d.	n.q.
Starfruit	Malaysia	n.d.	0.019	n.d.	n.d.	n.d.
Starfruit	Malaysia	n.q.	0.007	n.d.	n.d.	n.d.
Maximum residue		6.5	0.85	0.14	0.034	0.034

n.d.: not detected

n.q.: traces, <0.005 mg/kg;

The following tables give an overview about the numbers of samples within various matrix group that were found to contain residues of prochloraz and tested metabolites at levels ≥ 0.005 mg/kg. The residue levels of the metabolites are expressed as prochloraz. For each of the components the mean share to the sum (sum of all residues expressed as prochloraz) was calculated to give an impression of the importance of each component.

		Prochloraz			
Matrix Groups	**Samples Analysed**	**No. of samples w. residues ≥ 0.005 mg/kg**	**Mean Residue mg/kg ****	**Max. Residue mg/kg**	**Mean share of sum*****
Leafy Vegetables	832	2	0.020	0.033	
Fruiting Vegetables	735	0		0	
Sprouting Vegetables	254	2	0.022	0.038	
Mushrooms	114	15	0.019	0.085	63%
Mushrooms Dried	49	2	0.068	0.12	
Pome Fruits	244	1	0.023	0.023	
Stone Fruits	311	5	0.63	2.1	72%
Citrus Fruits	260	23	0.12	0.84	68%
Exotic Fruits	370	52	0.69	6.5	72%
Fruits Processed	170	2	0.022	0.039	
Miscellaneous*	1353	0			
Overall	**4692**	**104**	**0.41**	**6.5**	**64%**

		BTS 40348 expressed as prochloraz (f=1.333)			
Matrix Groups	**Samples Analysed**	**No. of samples w. residues ≥ 0.005 mg/kg**	**Mean Residue mg/kg ****	**Max. Residue mg/kg**	**Mean share of sum*****
Leafy Vegetables	832	2	0.008	0.007998	
Fruiting Vegetables	735	2	0.010	0.013	
Sprouting Vegetables	254	0			
Mushrooms	114	9	0.009	0.015	26%
Mushrooms Dried	49	2	0.039	0.063	
Pome Fruits	244	1	0.012	0.011997	
Stone Fruits	311	4	0.071	0.15	22%
Citrus Fruits	260	18	0.080	0.69	23%
Exotic Fruits	370	53	0.113	1.13	24%
Fruits Processed	170	7	0.014	0.031992	
Miscellaneous*	1353	0			
Overall	**4692**	**98**	**0.082**	**1.13**	**29%**

		BTS 44596 expressed as prochloraz (f=1.065)			
Matrix Groups	**Samples Analysed**	**No. of samples w. residues ≥ 0.005 mg/kg**	**Mean Residue mg/kg ****	**Max. Residue mg/kg**	**Mean share of sum*****
Leafy Vegetables	832	0			
Fruiting Vegetables	735	0			
Sprouting Vegetables	254	0			
Mushrooms	114	0			0%
Mushrooms Dried	49	0			
Pome Fruits	244	1	0.01704	0.017	
Stone Fruits	311	3	0.082005	0.12	4%
Citrus Fruits	260	11	0.037	0.097	7%
Exotic Fruits	370	39	0.031	0.15	3%
Fruits Processed	170	2	0.023	0.027	
Miscellaneous*	1353	0			
Overall	**4692**	**56**	**0.034**	**0.15**	**4%**

		BTS 44595 expressed as prochloraz (f=1.157)			
Matrix Groups	**Samples Analysed**	**No. of samples w. residues ≥ 0.005 mg/kg**	**Mean Residue mg/kg ****	**Max. Residue mg/kg**	**Mean share of sum*****
Leafy Vegetables	832	0			
Fruiting Vegetables	735	0			
Sprouting Vegetables	254	0			
Mushrooms	114	0			0%
Mushrooms Dried	49	0			
Pome Fruits	244	1	0.006	0.006	
Stone Fruits	311	1	0.024	0.024	0%
Citrus Fruits	260	5	0.018	0.034	1%
Exotic Fruits	370	16	0.012	0.027	0%
Fruits Processed	170	2	0.027	0.039	
Miscellaneous*	1353	0			
Overall	**4692**	**25**	**0.014**	**0.039**	**1%**

		BTS 9608 expressed as prochloraz (f=1.747)			
Matrix Groups	**Samples Analysed**	**No. of samples w. residues ≥ 0.005 mg/kg**	**Mean Residue mg/kg ****	**Max. Residue mg/kg**	**Mean share of sum*****
Leafy Vegetables	832	0			
Fruiting Vegetables	735	0			
Sprouting Vegetables	254	1	0.010	0.010	
Mushrooms	114	4	0.017	0.023	11%
Mushrooms Dried	49	1	0.026	0.026	
Pome Fruits	244	0			
Stone Fruits	311	2	0.047	0.059	2%
Citrus Fruits	260	0			0%
Exotic Fruits	370	4	0.027	0.049	1%
Fruits Processed	170	0			
Miscellaneous*	1353	0			
Overall	**4692**	**12**	**0.025**	**0.059**	**3%**

* Miscellaneous: various matrix groups not found to contain residues of prochloraz or its metabolites ≥ 0.005 mg/kg, e.g. berries (595 samples analyzed), root vegetables (190), processed vegetables (167), fruit juices (72), potatoes (89), cereals (90), cereal products (39)
** Mean Residue mg/kg: mean concentration of all results ≥ 0.005 mg/kg
*** Sum = sum of residue levels of prochloraz and its metabolites BTS 40348, BTS 44595, BTS 44596 and BTS 9608, each expressed as prochloraz

6. Conclusions:

Prochloraz and all its studied metabolites are amenable to the QuEChERS method. Prochloraz, BTS 44595, BTS 44596 and BTS 40348 can be analyzed via LC-MS/MS in the ESI-pos. mode. 2,4,6-TCP and BTS 9608 need to be analysed in the ESI neg. mode. Provided that certain prochloraz metabolites and other pesticides, which are known to undergo in-source fragmentation and to interfere in analysis of other prochloraz metabolites, are chromatographically well separated, LC-MS/MS is the simplest way to analyze prochloraz (sum). If in-source fragmentation is not considered and chromatographic separation is not optimized there is a high risk of false positive findings of 2,4,6-TCP. As BTS 9608 and 2,4,6-TCP are if at all only present at low levels in real samples. Thus, analysis in the ESI-negative mode may be skipped.

During GC analysis prochloraz and its metabolites partly convert to TCP in the hot injector. The reproducibility of this conversion varies depending on various factors. Analysis via GC is thus tricky. GC results of TCP should not be added to any results obtained by LC to avoid redundancies and overestimation of prochloraz (sum).

Analysis of real samples showed that BTS 40348 is, next to prochloraz, the main part of the residue. Its formation seems to be promoted during fruit processing. The non-inclusion of BTS 40348 in the residue definition proposed by EFSA (sum of prochloraz, BTS 44595 and BTS 44596 expressed as prochloraz) inevitably leads to a strong deviation to the currently existing residue definition of total prochloraz (*sum of prochloraz and its metabolites containing the 2,4,6-trichlorophenol moiety expressed as prochloraz*). This aspect needs reconsideration. In fact it seems worthwhile considering the establishment of a a residue definition entailing merely the parent and the metabolite BTS 40348.

BTS 44596 degrades to 2,4,6-TCP in pure acetonitrile. Acidification is necessary.

Observations at a glance:

Compound	QuEChERS amenable	Ranking of residue levels in market samples (5 = best)	Degradation rate to TCP in GC	LC-MS/MS behaviour			Degradation in solution	
				Amenable to ESI pos.	Amenable to ESI neg	In-source fragmentation	Degradation in ACN	Degradation in QuEChERS extracts
Prochloraz	✓	*****	**medium**	✓		not observed	not observed	not observed
BTS 44595	✓	*	**very high**	✓		**YES to BTS 40348**	not observed	not observed
BTS 44596	✓	**	**high**	✓		**YES to TCP**	**YES to TCP (acidify ACN)**	not observed
BTS 40348	✓	***	**high**	✓		not observed	not observed	not tested yet
BTS 9608	✓ (skip cleanup w. PSA sorbent)	*	**minor**		✓	**YES to TCP**	not observed	not observed
TCP	✓	-	-		✓	not observed	not observed	not observed

References:

[1] Conclusion on the peer review of the pesticide risk assessment of the active substance prochloraz, European Food Safety Authority (EFSA), Parma, Italy, EFSA Journal 2011; 9(7):2323

Appendix:

Materials:

Prochloraz (purity 99.0%) purchased from Dr. Ehrenstorfer (Cat #:C16290000)

BTS 44596 (*Prochloraz desimidazole-formylamino*) (purity 98.2%)
– friendly donation by BASF; also available at Dr. Ehrenstorfer

BTS 40348 (purity 99.6%) - friendly donation by BASF

BTS 44595 (*Prochloraz desimidazole-amino*) (purity 98.0%)
- friendly donation by BASF; also available at Dr. Ehrenstorfer

BTS 9608 *(2,4,6-Trichlorophenoxyacetic acid)* (purity 99.2%)
- friendly donation by BASF; also available at Dr. Ehrenstorfer

2,4,6-Trichlorophenol (purity 99.5%) purchased from Dr. Ehrenstorfer (Cat #:C17774600)

Instrumentation details:

<table>
<tr><th colspan="2">GC-MS (CI-neg.) method:</th></tr>
<tr><td>GC</td><td>GC: Agilent Typ 6890N, Injector: Gerstel CIS 4</td></tr>
<tr><td>MS</td><td>Agilent Typ 5973, run in CI negative mode</td></tr>
<tr><td>m/z</td><td>Prochloraz: 375.0, 377.0, 214.0
2,4,6-Trichlorophenol: 197.9, 199.7, 196.0</td></tr>
<tr><td>Column</td><td>Agilent 19091J-433 HP-5 5% Phenyl Methyl Siloxane</td></tr>
<tr><td>Injection volume</td><td>3 µL, solvent vent mode</td></tr>
<tr><td>Injection</td><td>
<table>
<tr><th>Ramps</th><th>°C/min</th><th>Next °C</th><th>Hold min</th></tr>
<tr><td>Initial</td><td>-</td><td>50</td><td>0.8</td></tr>
<tr><td>Ramp 1</td><td>720.00</td><td>300</td><td>5.00</td></tr>
<tr><td>Ramp 2</td><td>720.00</td><td>320</td><td>10.00</td></tr>
<tr><td>Ramp 3</td><td>0.00</td><td>-</td><td>-</td></tr>
</table>
<u>PTV-System:</u>
Injection at 50 °C – split valve open – vent flow 40 mL/min
– keep for 0.5 min
at 0.5 min – close split valve
at 0.8 min – start heating
at 2 min – reopen split valve – split flow at 41.1 mL/min
at 6 min – activate gas saver mode – split flow 20 mL/min</td></tr>
</table>

Oven program	<table><tr><th>Ramps</th><th>°C/min</th><th>Next °C</th><th>Hold min</th></tr><tr><td>Initial</td><td>-</td><td>50</td><td>2.33</td></tr><tr><td>Ramp 1</td><td>30.00</td><td>220</td><td>0.00</td></tr><tr><td>Ramp 2</td><td>5.00</td><td>260</td><td>0.00</td></tr><tr><td>Ramp 3</td><td>20.00</td><td>280</td><td>15.00</td></tr><tr><td>Ramp 4</td><td>0.00</td><td>-</td><td>-</td></tr></table>
Flow	Helium 1.7 mL/min, constant flow
Transfer line	270 °C
Detector	MSD-CI, source temperature 150 °C

LC-MS/MS method:	
LC	WATERS Acquity UPLC
MS/MS	ABSCIEX API 5500 Q-Trap, run in ESI negative/ positive mode
MRMs	**Prochloraz** 376/308, 376/266, 378/310 (ESI pos) **BTS 44595** 325/282, 325/129, 327/284 (ESI pos) **BTS 44596** 353/308, 353/70, 353/310 (ESI pos) **BTS 40348** 282/86, 282/44, 284/86 (ESI pos) **BTS 9608** 253/195, 253/35, 255/197, 257/199 (ESI neg) **2,4,6-Trichlorophenol** 195/35, 197/35, 199/37 (ESI neg)
Column	Acquity BEH C18, 2.1x100 mm, 1.7 µm
Pre-column	Acquity BEH C18, 2.1x5 mm, 1.7 µm
Mobile Phase	A: 0,01% acetic acid in purified water + 5% acetonitrile B: 0,01% acetic acid in acetonitril
Gradient	<table><tr><th>Time min</th><th>Mobile Phase A %</th><th>Mobile Phase B %</th></tr><tr><td>0</td><td>80</td><td>20</td></tr><tr><td>4</td><td>70</td><td>30</td></tr><tr><td>7</td><td>10</td><td>90</td></tr><tr><td>8.5</td><td>10</td><td>90</td></tr><tr><td>8.6</td><td>80</td><td>20</td></tr><tr><td>13.5</td><td>80</td><td>20</td></tr></table>
Flow	0.4 mL min^{-1}
Injection volume	2 µL, partial loop with needle overfill
Column temperature	40°C

Document History

Date	Action	Changes
18.04.14	Publication of V1	
08.11.14	Publication of V2	- Correction of content of 3 tables und chapter 5. Analysis of real samples with prochloraz treatment history titles Calculation of prochloraz (sum) by different approaches – different approach, different result - Inclusion of residue data on the metabolite **BTS 40348**
30.07.2016	Publication of V3	- Correction of typing errors and general text revision - Update of validation data table - Update of residue findings table - Inclusion of an overview of the residue situation in some relevant matrix groups