EURL-SRM – Analytical Observations Report

concerning the following…

- **Compound(s)**: Chloridazon-Desphenyl
- **Commodities**: Honey
- **Extraction Method(s)**: QuPPe
- **Instrumental analysis**: LC-MS/MS; SFC-MS/MS, LC-QToF

Risk of False Positives of Chloridazon-Desphenyl in Honey by LC-MS/MS

Version 1 (last update: 14.04.2023)

Background information / Initial Observations:

Within the frame of an EURL-SRM pilot monitoring study on pesticide residues in honey, it was noticed, that a remarkably large share of samples analyzed by QuPPe and LC-MS/MS (via method M4.2), showed signals faking the presence of chloridazon-desphenyl.

While measuring honey samples, it was noticed, that the three acquired MRM-traces of chloridazon-desphenyl showed peaks at the expected retention time with two of them being within the expected ion-ratio. The third trace, however, showed a very large signal, that was initially interpreted as originating from the matrix. This led to a large number of fake chloridazon-desphenyl "findings", mostly at "levels" exceeding the MRLs for chloridazon (sum). The unusually high frequency of apparent findings and apparent MRL-violations (despite chloridazon being phased out, at least in the EU), together with the strongly interfered MRM-transition, raised the need to run confirmatory analyses by other techniques to check the presence of this compound.

Chloridazon used to be a popular herbicide and was used e.g. in beet cultivation. However, due to massive findings of its degradation products in underground water, its use was eventually restricted. Thereafter the EU-approval was not renewed and expired by the end of 2018, with the period of grace for sales ending in mid-2020, and the end of use of stocks terminating by the end of 2020.

Chloridazon degrades into two main metabolites, chloridazon-desphenyl and chloridazon-methyl-desphenyl. For chloridazon-desphenyl, the half-life in soil is reported at 235.5 days.

Due to the persistence and leachability of chloridazon-desphenyl, findings are still reported in surface and underground water[1]. Findings in food of plant origin are also still occasionally reported, and findings in organic products were also initially of concern[2].

[1] Jan Chabera et al.; The effect of chronic exposure to chloridazon and its degradation product chloridazon-desphenyl on signal crayfish Pacifastacus leniusculus; Ecotoxicology and Environmental Safety
Volume 208, 15 January 2021, 111645; https://doi.org/10.1016/j.ecoenv.2020.111645

[2] Labor Friedle / Relana (June 2020): **file:///C:/Users/anastassiades/Downloads/Kundeninformation%20Chloridazon.pdf**

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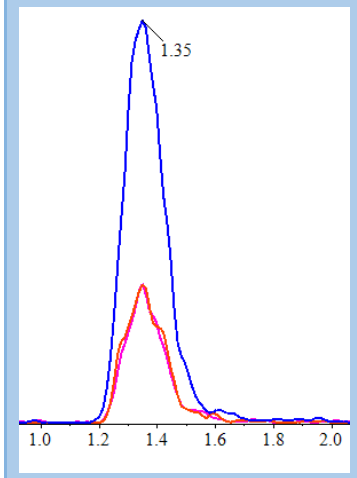
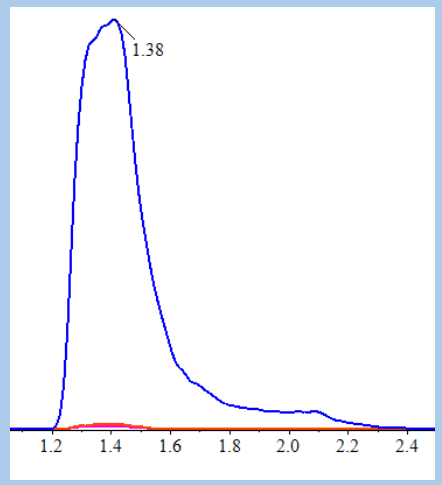
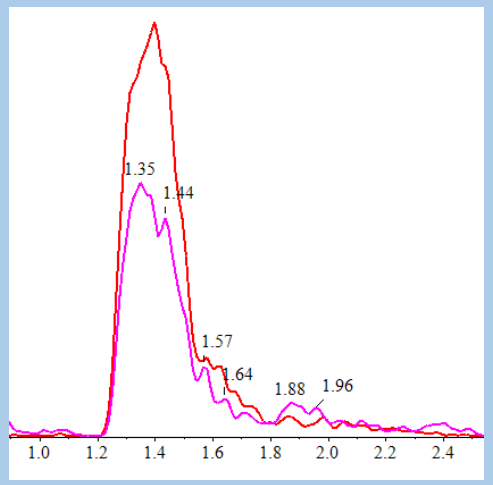
Table 1: Chromatogram(s) showing, why signals were initially miss-assigned to chloridazon-desphenyl. Signals of blossom honey No. 17 ('EU + non-EU') are compared with signals of standards based on tomato- and honey-extracts. The colors of the MRM-traces and the Ion-ratios are shown in the tables below.

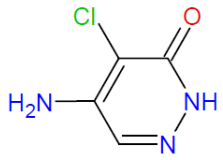
Chloridazon-desphenyl peaks in HONEY-based std. (0.025 µg/mL)	Extract of Honey No. 17 (initially suspected to contain chloridazon-desphenyl)	
	All MRMs	Without MRM 146/117
		

Color	m/z	m/z 146/117 signal set as 100	m/z 146/66 signal set as 100
blue	146/117	**100**	
red	146/66	33	**100**
pink	148/119	28	85

Color	m/z	m/z 146/117 signal set as 100	m/z 146/66 signal set as 100
blue	146/117	**100**	
red	146/66	0.9	**100**
pink	148/119	0.6	69

Compound details:

Chloridazon-desphenyl (CAS: 6339-19-1)
IUPAC: 5-amino-4-chloro-2,3-dihydropyridazin-3-one
Synomym: Pyrazone

Parameter	Value		
Molecular Mass	145.55 g/mol		
Exact mass	145.0042895 Da		
Pka	Strongest acidic pKa: 11.2 Strongest basic pKa: 3.17	Computed by Chemicalize.org	
LogD	-1.32 at pH 4 to 10		
Residue definition EU	Chloridazon (sum of chloridazon and chloridazon-desphenyl, expressed as chloridazon) Note: this RD became applicable in 2017 and has led to findings and MRL exceedances		
Approval status	**Chloridazon is not approved in the EU**		
ADI / ARfD	ADI 0.1 mg/kg bw/day		

Materials and Instruments

See QuPPe method. In addition, filters of 5 to 10 µm particle size were used to remove pollen.

Extraction

For the extraction of the honey samples, the QuPPe method was employed. It involves weighing 5.0 g of honey into a 50 mL centrifuge tube, volume adjustment by adding 7.5 mL of water, addition of 10 mL acidified methanol (containing 1% formic acid), extraction by automated shaking, centrifugation and filtration.
The raw extracts of some honey samples were found to cause clogging of the 0.2 µm filters, typically used in QuPPe. This is mainly attributed to the differences in the amount of pollen contained. One way to circumvent this difficulty is to employ high-speed, preferentially cooled, centrifugation at > 10,000 g. Another way is to use syringe/disc filters of 5-10 µm pore size, as pollen grains usually have particle sizes > 10 µm. These 5-10 µm pore size filters may be either used as pre-filters followed by a 0.2 µm filtration (the two filters can be stacked), or as a replacement of 0.2 µm filters.

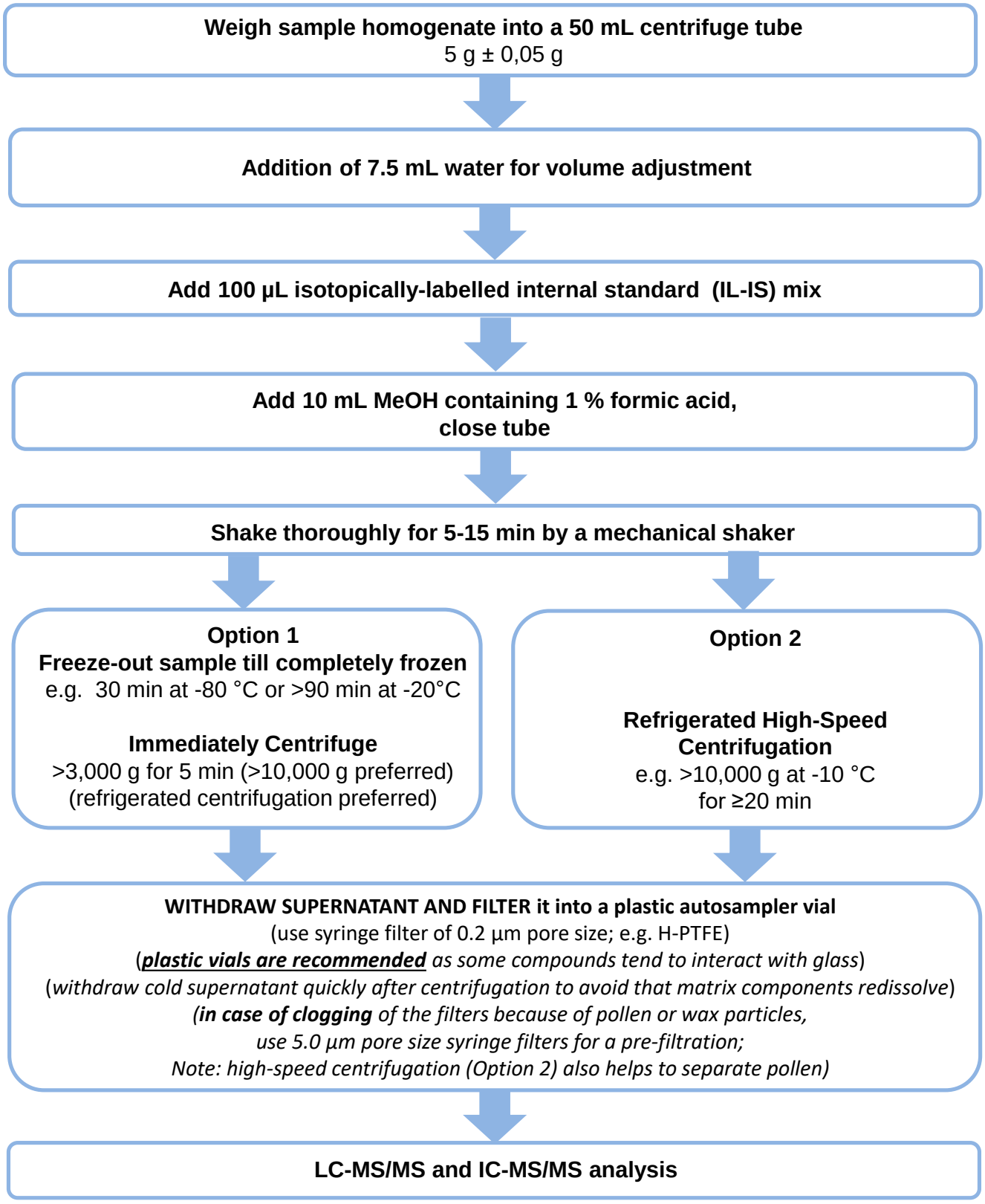


Figure 1: QuPPe method for honey samples at a glance.

Measurement conditions

Measurement was conducted by LC-MS/MS or by SFC-MS/MS or by LC-ToF, all in the ESI-positive mode. Details on the measurement conditions are given in Table 2 to Table 5.

Initial measurement:
Initial measurement, that has led to the false judgement of positive findings of chloridazon-desphenyl, was conducted by the QuPPe/LC-MS/MS method M 4.2, which employs a HILIC type Waters BEH Amide column (thereafter coded as **Method A**).

Confirmatory measurements:
Method B: SFC-MS/MS method on a Waters BEH Amide HILIC type column, but employing supercritical CO_2 in the gradient, see Table 3.

Method C: LC-MS/MS method on an Atlantis Premier BEH Z-HILIC column, see Table 4.

Method C-HR: This method resembled method C (same column and gradient), but a high resolution Time of Flight (ToF) mass analyzer was used, see Table 5.

Table 2: LC-MS/MS conditions for chloridazon-desphenyl using Method A (= QuPPe Method M4.2)

LC	Agilent 1290 Infinity II UHPLC						
MS/MS	SCIEX API 5500 Q-Trap, run in ESI positive mode						
Column	Acquity BEH Amide, 2x100 mm, 1.7 µm						
Pre-column	Acquity Van Guard BEH C_{18}, 2x5 mm, 1.7 µm						
Mobile Phase	A: 50 mmol NH_4formate in purified water (pH 3) B: acetonitrile						
Gradient	**Time** (min)		**Mobile Phase A** (%)		**Mobile Phase B** (%)		
	0		3		97		
	0.5		3		97		
	4.0		30		70		
	5.0		60		40		
	6.0		60		40		
	6.1		3		97		
	10		3		97		
Flow	0.5 mL min^{-1}						
Injection volume	2 µL						
Column temperature	40°C						
MRM-Transitions	**Intensity ranking**	**Parent (m/z)**	**Daughter (m/z)**	**DP (V)**	**CE (V)**	**CXP (V)**	
Chloridazon-desphenyl	1	146	117	120	31	6	
	2	146	66	120	51	2	
	3	148	119	120	31	6	
Chloridazon-desphenyl $^{15}N_2$ (IL-IS)	1	148	117	120	31	6	
	2	148	102	120	35	6	

Table 3: SFC-MS/MS conditions for chloridazon-desphenyl using Method B.

SFC	Waters Acquity UPC² System					
MS/MS	Waters Xevo TQ-Sμ, run in ESI positive mode					
Column	Viridis BEH, 3x100 mm, 1.7 µm					
Pre-column	-					
Mobile Phase	A: CO_2 B: 20 mmol NH_4formate in MeOH/H_2O 95/5					
Make-up Solvent	0.1% formic acid in MeOH/H_2O 90/10					
Make-up Solvent Flow	0.3 mL/min					
ABPR (Active Back Pressure Regulator)	124 bar					
Gradient	**Time** (min)	**Mobile Phase A** (%)	**Mobile Phase B** (%)			
	0	95	5			
	1.0	95	5			
	8.0	50	50			
	12.0	50	50			
	12.2	95	5			
	14.2	95	5			
Flow	1.5 mL min^{-1}					
Injection volume	0.5 µL					
Column temperature	55°C					
Compound	**Intensity ranking**	**Parent (m/z)**	**Daughter (m/z)**	**CV (V)**	**CE (V)**	
Chloridazon-desphenyl	1	146	117	8	22	
	2	146	54	8	24	
	3	148	119	12	26	
Chloridazon-desphenyl $^{15}N_2$ (IL-IS)	1	148	117	24	24	
	2	148	102	24	28	

Table 4: LC-MS/MS conditions for chloridazon-desphenyl using Method C

LC	Shimadzu Nexera LC 40						
MS/MS	SCIEX API 5500+ Q-Trap, run in ESI positive mode						
Column	Atlantis Premier BEH Z-HILIC, 2.1x100 mm 1.7µm						
Pre-column	Van Guard Atlantis Premier BEH Z-HILIC, 2.1x5 mm 1.7µm						
Mobile Phase	A: 5 mmol NH_4formate in H_2O B: 5 mmol NH_4formate in ACN/H_2O 95/5						
Gradient	**Time** (min)	**Mobile Phase A** (%)	**Mobile Phase B** (%)				
	0	2	98				
	2.5	2	98				
	5.0	80	20				
	6.0	80	20				
	6.1	2	98				
	11.0	2	98				
Flow	0.4 mL min^{-1}						
Injection volume	5 µL						
Column temperature	40°C						
MRM-Transitions	**Intensity ranking**	**Parent (m/z)**	**Daughter (m/z)**	**DP (V)**	**CE (V)**	**CXP (V)**	
Chloridazon-desphenyl	1	146	117	120	31	6	
	2	146	66	120	51	2	
	3	148	119	120	31	6	
Chloridazon-desphenyl $^{15}N_2$ (IL-IS)	1	148	117	120	31	6	
	2	148	102	120	35	6	

Table 5: LC-MS/MS conditions for chloridazon-desphenyl using Method C-HR

LC	Agilent 1290 Infinity II UHPLC		
MS/MS	Bruker maxis Compact, run in ESI positive mode		
Column	Atlantis Premier BEH Z-HILIC, 2.1x100 mm 1.7µm		
Pre-column	Van Guard Atlantis Premier BEH Z-HILIC, 2.1x5 mm 1.7µm		
Mobile Phase	A: 5 mmol NH_4formate in H_2O B: 5 mmol NH_4formate in ACN/H_2O 95/5		
Gradient	**Time** (min)	**Mobile Phase A** (%)	**Mobile Phase B** (%)
	0	2	98
	2.5	2	98
	5.0	80	20
	6.0	80	20
	6.1	2	98
	11.0	2	98
Flow	0.4 mL min^{-1}		
Injection volume	5 µL		
Column temperature	40°C		
MRM-Transitions	**Intensity ranking**	**Exact mass (m/z)**	**Notes**
Chloridazon-desphenyl	1	146.0116	$[M+H]^+$
	2	148.0087	$[M+H]^+$
	3	116.9976	In-source fragment
	4	167.9935	$[M+Na]^+$
	5	183.9674	$[M+K]^+$

Experiments, Observations and Discussion:

Doubts on results of Method A:

Using **Method A**, the target MRM was obviously interfered by a matrix component and it was considered that it couldn't be used neither for qualification nor for quantification. The intensity ratio of the other two MRMs was within the expected limits and an "overspike" merely increased peak area without any sign of any shoulder. Still it couldn't be excluded, that all detected MRMs originated from the matrix, and that two of the MRMs only coincidentally showed an ion-ratio fitting to chloridazon-desphenyl. Another observation raising doubts as regards the identification of chloridazon-desphenyl was, that many honey samples of different origin showed a similar pattern: three MRMs showing peaks matching with chloridazon-desphenyl in terms of form and RT with two of them showing fitting ion-ratios.

With the above aspects in mind, it was decided to run confirmatory experiments in order to exclude the possibility of a false positive result. For this, two equal aliquots of selected honey extracts were prepared, one was spiked with chloridazon-desphenyl $^{15}N_2$ (IL-IS) while the other one was spiked with both chloridazon-desphenyl and its IL-IS (at 0.1 mg/kg in all cases). These solutions were analyzed by the four methods (A, B, C and C-HR), to check if chloridazon-desphenyl could be separated from the matrix either chromatographically (Methods A, B and C) or mass-spectrometrically (Method C-HR) Table 6 to Table 9 show the chromatograms obtained by each method.

MS/MS experiments (concerning Methods A, B and C)

As can be seen in Table 6 to Table 8, all three LC-MS/MS methods employed (A, B, C) showed a very strong signal in the trace of the target MRM (m/z 146/117). In Table 6, the chromatograms of three honey extracts are shown (*Honey No. 7*, *Honey No.* 17 and *Honey No.* 72).

Honey No. 7 was originally considered not containing quantifiable levels of chloridazon-desphenyl, as only the target MRM (m/z 146/117), showed a signal.
Honey No. 17 and *Honey No.* 72, showed signals in each MRM-trace at the expected retention time. While the signal in the target MRM trace (m/z 146/117), was considered being interfered by matrix and thus not useful for quantifications. The signals of the other two MRMs (146/66 and 148/119) were within the expected ratio range. Moreover, retention time (RT) and peak shape were similar to those of the IL-IS. Therefore, it was initially assumed, that residues of chloridazon-desphenyl were detected in those two samples.

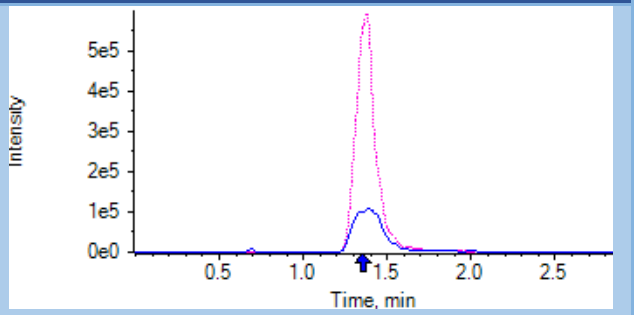
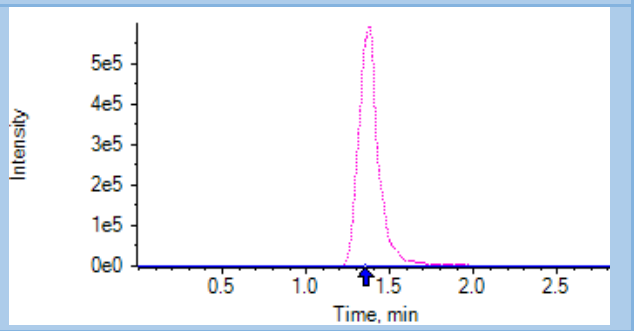
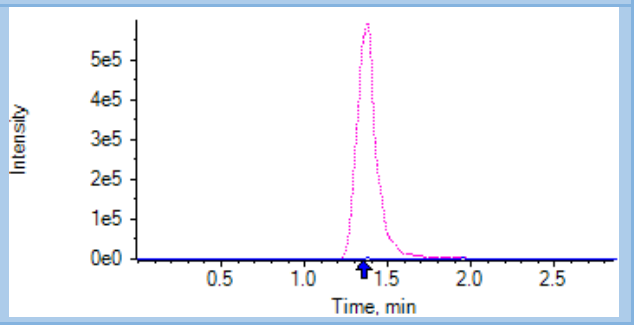
Using **Method B** (SFC-MS/MS), the interfering matrix component could be chromatographically sufficiently separated from chloridazon-desphenyl (RT 4.38 min vs. to 4.28 min respectively, see Table 7), all measured MRM-traces (146/117; 148/119 and 146/54) showed signals at 4,38 min. Unfortunately, MRM 146/66 was not acquired on this instrument and MRM 146/54, which was acquired instead, was heavily interfered and thus deemed inadequate. The analysis of *Honey No.* 17, by Method B clearly showed that the initial doubts were justified, as chloridazon-desphenyl was not detected. Only *Honey No.* 72 (see Table 7) appeared to contain chloridazon-desphenyl, however, if at all at trace levels of ~ 0.0005 mg/kg, and thus well below the LOQ.

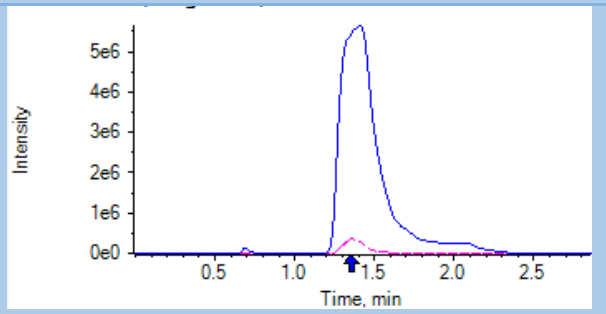
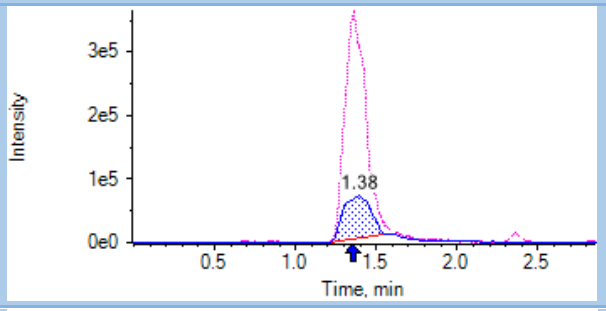
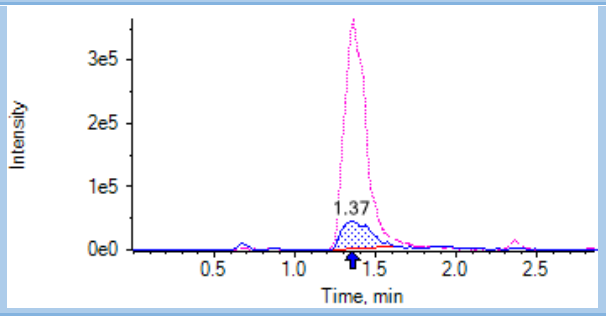
Using **Method C,** involving separation on a different column (Atlantis Premier BEH Z-HILIC) chloridazon-desphenyl could be partly separated from the matrix (RT 1.55 vs 1.41 min), thus minimizing the risk of false positives. Similarly, to Method A, the matrix component gave a very large peak at MRM 146/117, with its tail overlapping the signal of chloridazon-desphenyl. This MRM, thus could not be used for quantification or identification. In *Honey No. 7*, chloridazon-desphenyl was clearly not detected, thus confirming the results of Method A. In *Honey No.* 72, that according to Method B contained traces of chloridazon-desphenyl, signals could be seen, but identification was questionable.

LC-HR-ToF-MS experiments (Method C-HR)

To check whether the interfering matrix component could be separated mass-spectrometrically, further experiments were conducted on an LC-HR-ToF-MS instrument. The same LC-conditions, as in Method C, were used, which led to a partial separation of chloridazon-desphenyl from the critical matrix component. The chromatograms obtained are shown in Table 9.
In *Honey No. 7* strongly interfering signals were detected at the retention time of chloridazon-desphenyl (1.77 min) on mass-traces m/z 148.0087, and 116.9976. Luckily, the trace m/z 146.0116, was only negligibly interfered, which considerably minimizes the risk of false positives.
Honey No. 72 behaved quite differently. Here mass-trace m/z 146.0116 was the one showing the largest peak, but luckily this peak separated chromatographically, which also minimized the risk of a false positive result. The mass-trace m/z 148.0087 was strongly interfered and the peak did not markedly increase when injecting the extract with the overspike. Mass-trace 116.9976 gave only a weak signal even after spiking and was thus considered of limited use when dealing with low concentrations.
LC-HR-ToF-MS ion-ratios in the unspiked extracts differed much from those in the solvent-based standard, raising doubts and decreasing the possibility of a false positive result.

*Table 6: Chromatograms obtained by **Method A**; unspiked extract. The peak of chloridazon-desphenyl is shown in blue. The pink line shows the mass-trace of the IL-IS (m/z 148/117).*

Sample	Mass transition m/z	Unspiked sample extract
Honey No. 7 (originally considered not to contain quantifiable amounts of *chloridazon-desphenyl* using Method A)	146/117 (Target MRM)	
	146/66	
	148/119	

Sample	Mass transition m/z	Unspiked sample extract
Honey No. 17 (originally considered to potentially contain 0.149 mg/kg chloridazon-desphenyl using Method A)	146/117 (Target MRM)	
	146/66	
	148/119	

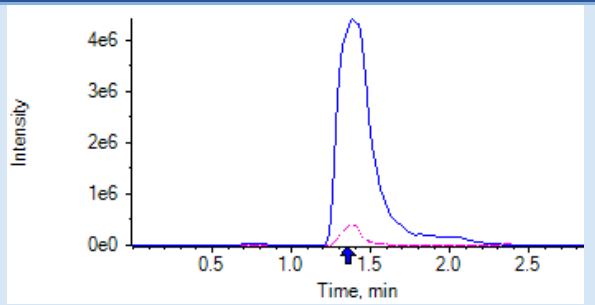
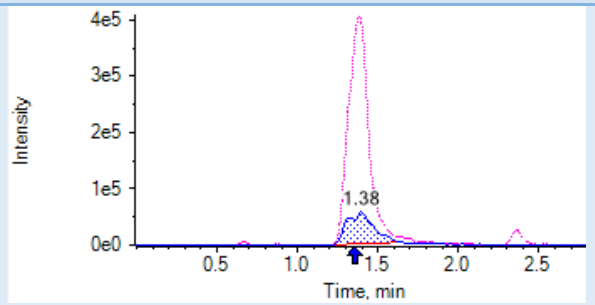
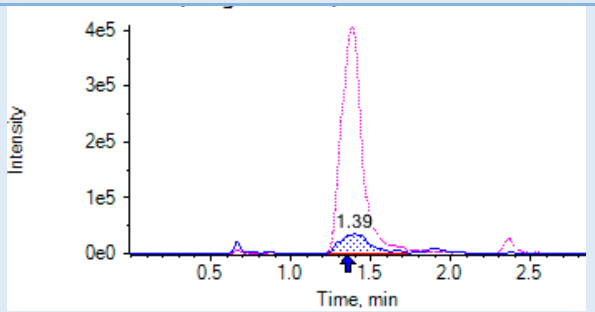
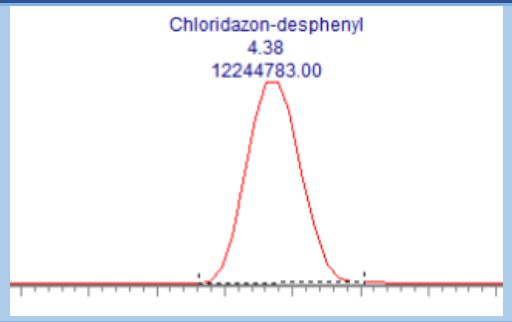
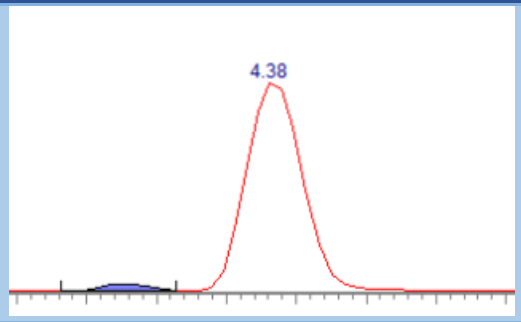
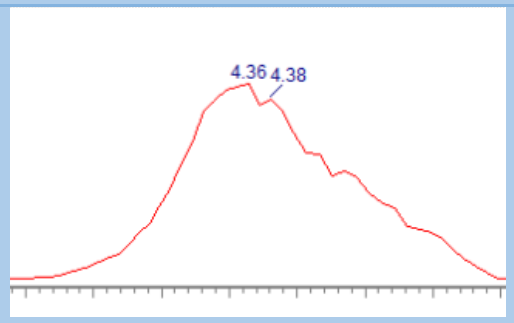
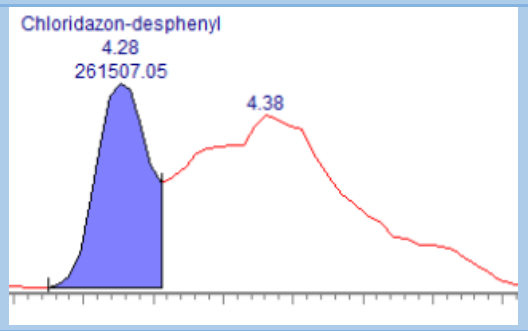
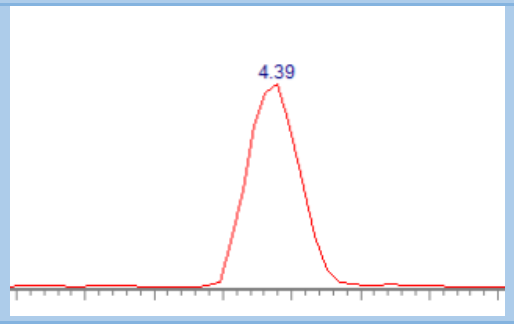
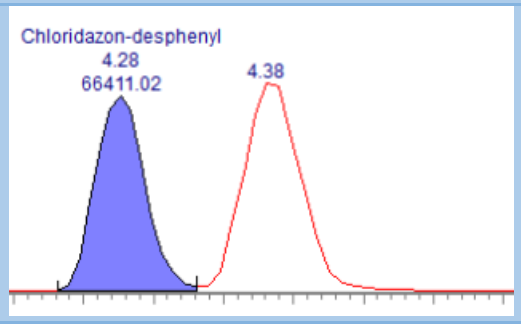
Sample	Mass transition m/z	Unspiked sample extract
Honey No. 72 (originally considered to potentially contain 0.121 mg/kg chloridazon-desphenyl using Method A)	146/117 (Target MRM)	
	146/66	
	148/119	

*Table 7: Chromatograms obtained by **Method B**; unspiked extract (left); spiked extract (right). The peak area of chloridazon-desphenyl is shown in blue. The red line represents the respective mass trace and shows matrix interferences. The IL-IS is not shown here.*

Sample No	Mass transition m/z	Unspiked sample extract	Spiked sample extract at 0.1 mg/kg
Honey No. 17 (originally considered to potentially contain 0.149 mg/kg chloridazon-desphenyl using Method A)	146/117 (Target MRM)		
	146/54		
	148/119		

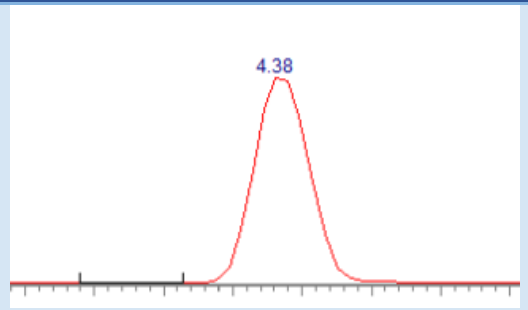
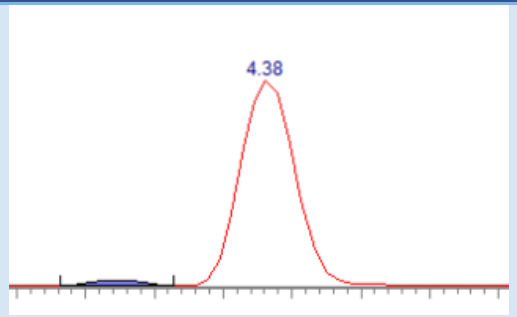
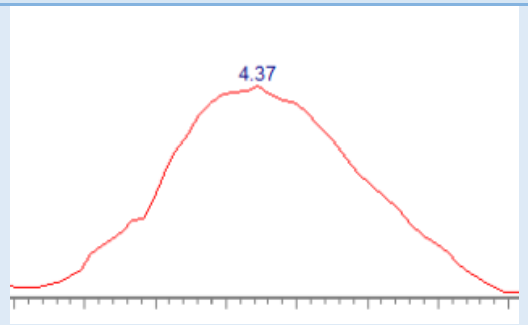
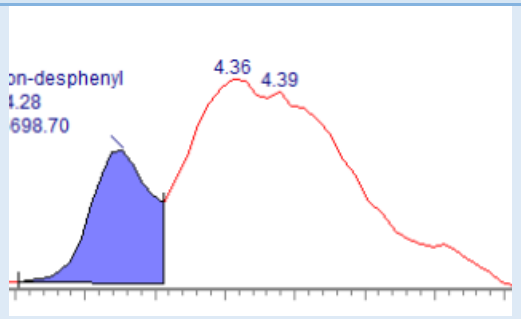
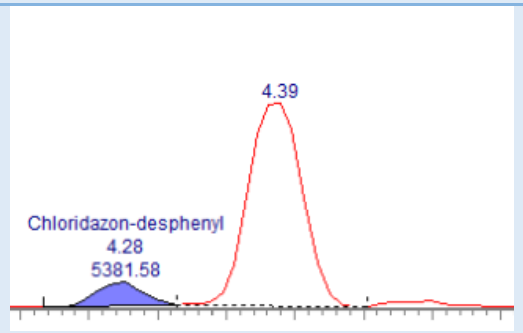
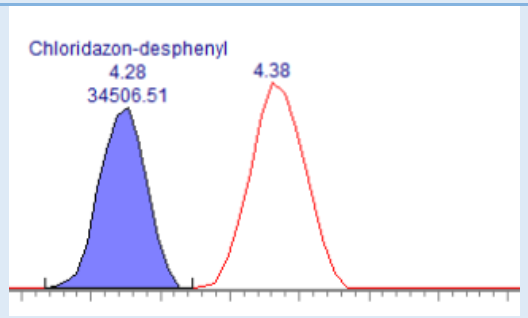
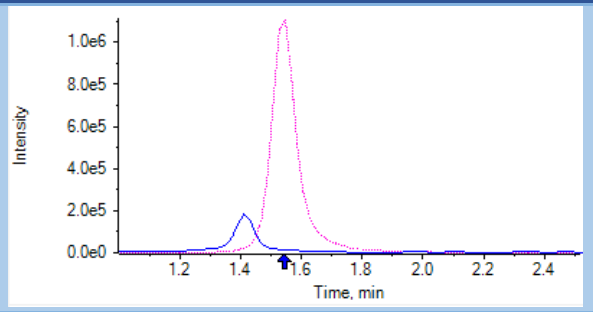
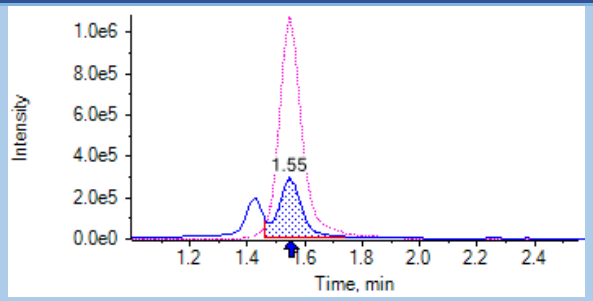
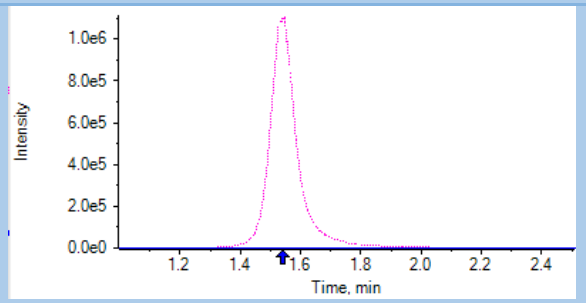
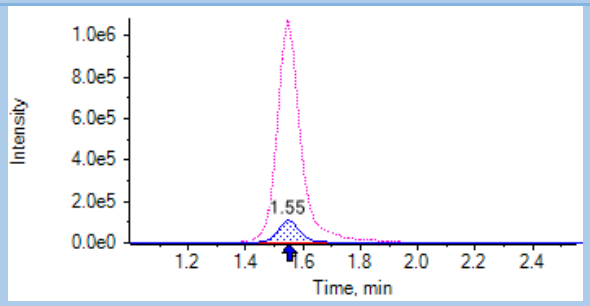
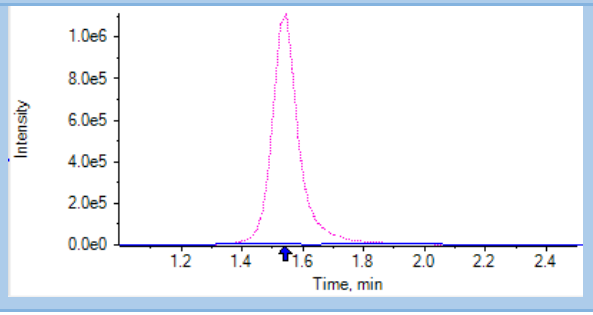
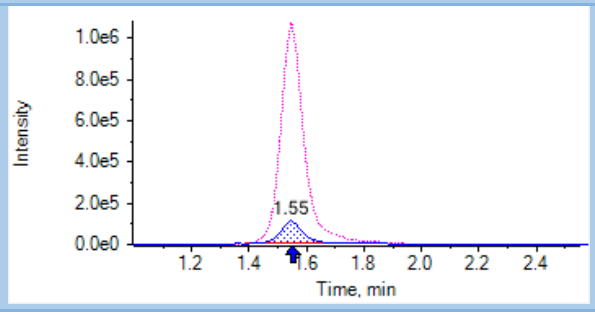
Sample No	Mass transition m/z	Unspiked sample extract	Spiked sample extract at 0.1 mg/kg
Honey No. 72 (originally considered to potentially contain 0.121 mg/kg chloridazon-desphenyl using Method A)	146/117 (Target MRM)		
	146/54		
	148/119		

*Table 8: Chromatograms obtained by **Method C**; unspiked extract (left); spiked extract (right). The peak of chloridazon-desphenyl (C-D) is shown in blue. The pink line shows the mass-trace of the IL-IS (m/z 148/117). The small blue arrow shows the expected retention time of C-D.*

Sample No	Mass transition m/z	Unspiked sample extract	Spiked sample extract at 0.1 mg/kg
Honey No. 7 (originally considered not to contain quantifiable amounts of *chloridazon-desphenyl* using Method A)	146/117 (Target MRM)		
	146/66		
	148/119		

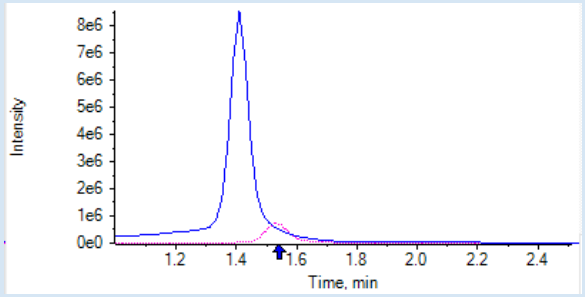
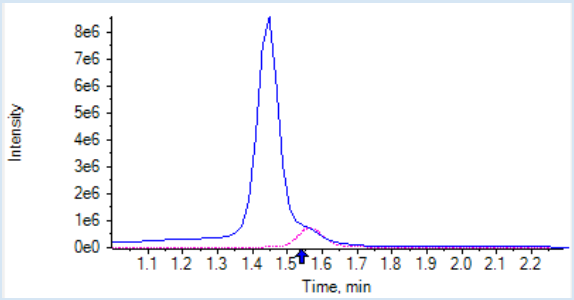
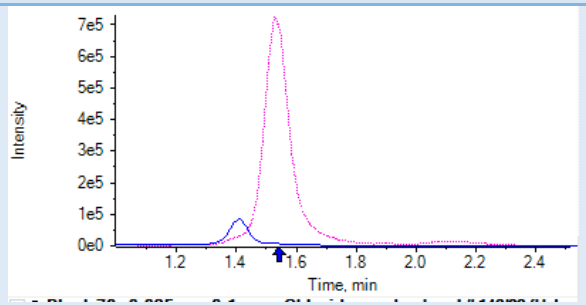
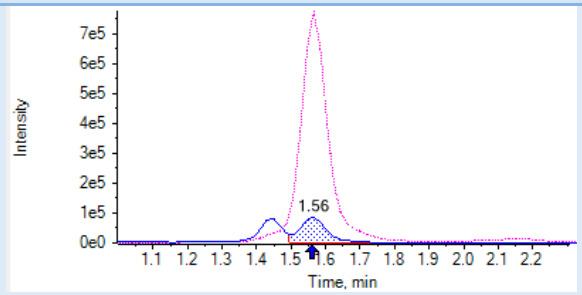
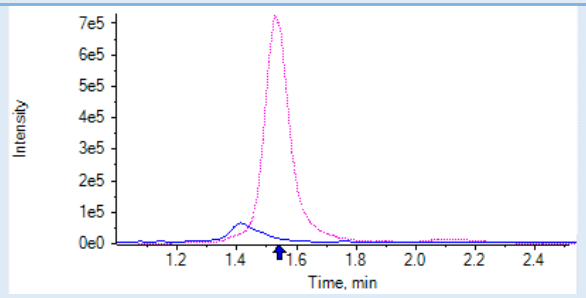
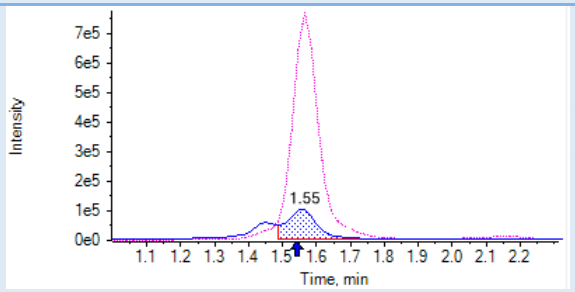
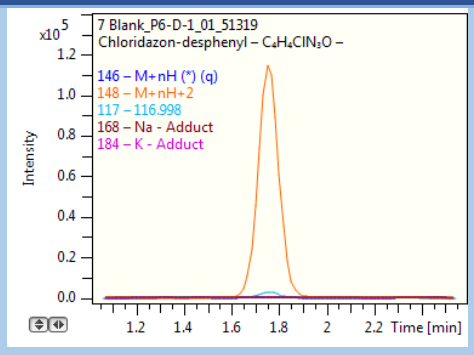
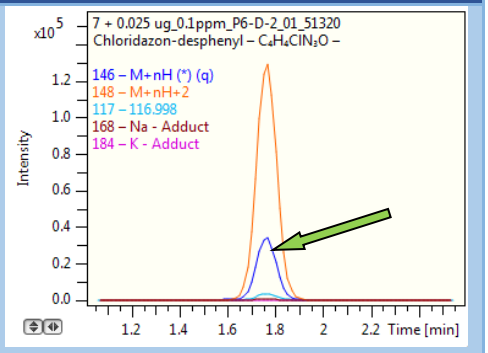
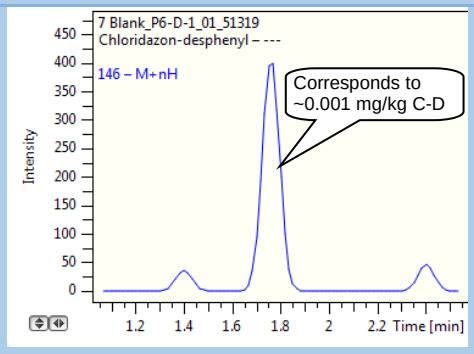
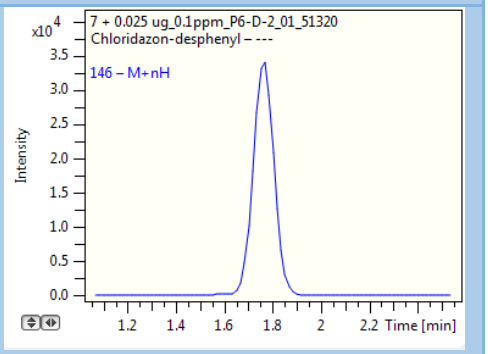
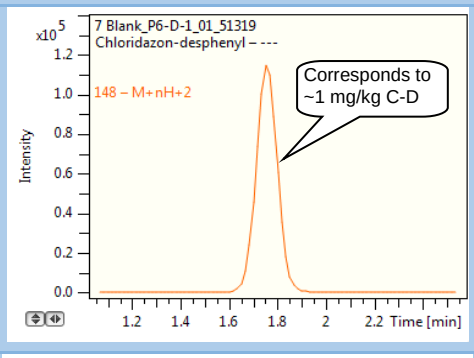
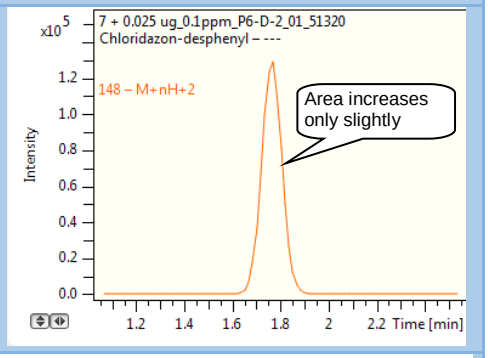
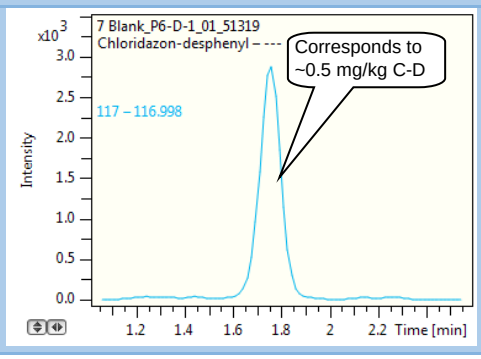
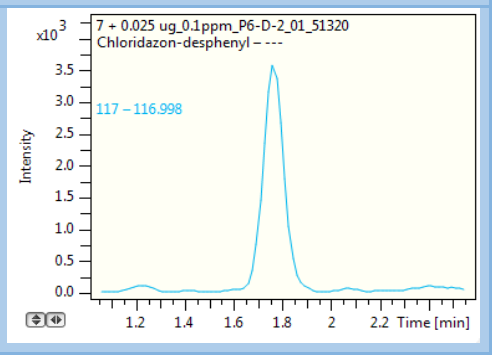
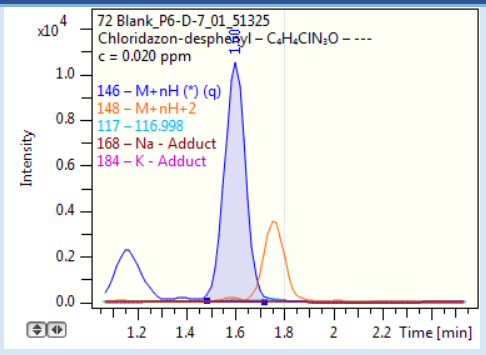
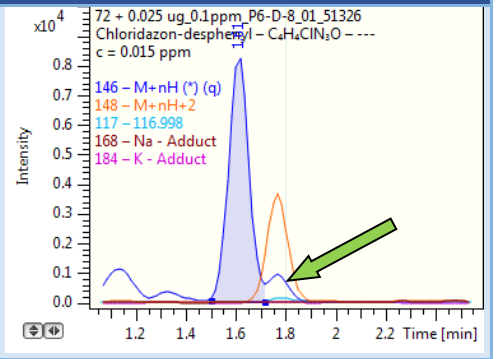
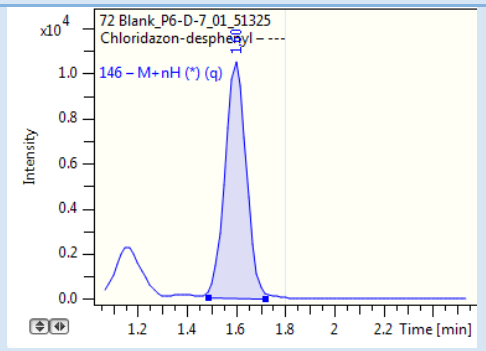
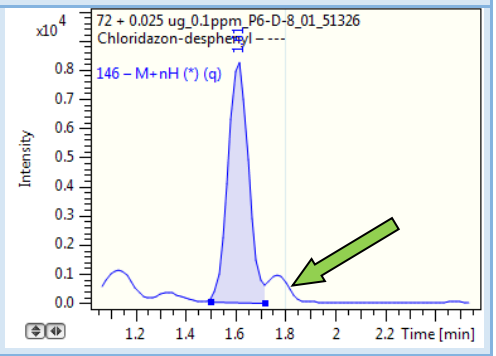
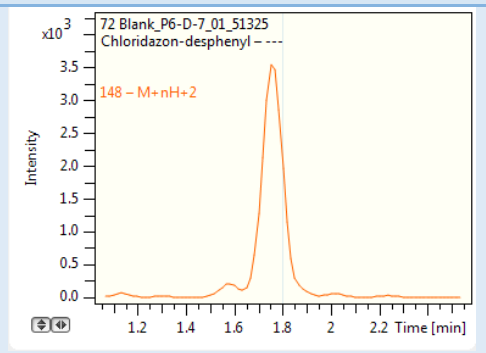
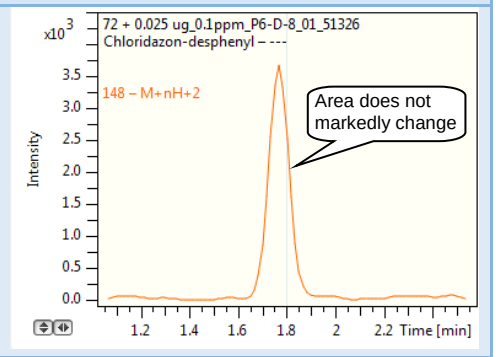
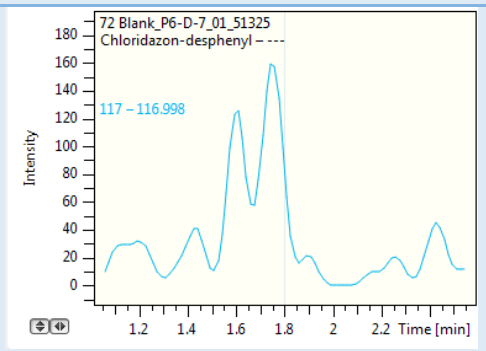
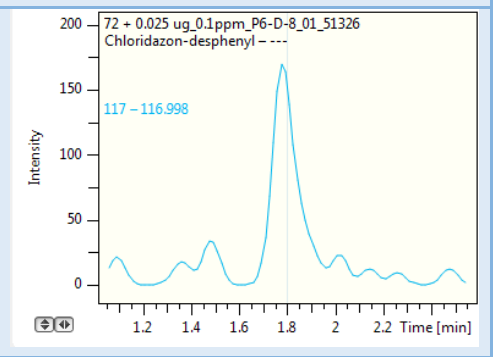
Sample No	Mass transition m/z	Unspiked sample extract	Spiked sample extract at 0.1 mg/kg
Honey No. 72 (originally considered to potentially contain 0.121 mg/kg chloridazon-desphenyl using Method A)	146/117 (Target MRM)		
	146/66		
	148/119		

Table 9: Chromatograms obtained by Method C-HR; unspiked extract (left); spiked extract (right). The peak of chloridazon-desphenyl is pointed at with a green arrow.

Sample No	Exact masses (m/z)	Unspiked sample extract	Spiked sample extract at 0.1 mg/kg
Honey No. 7 (originally considered not to contain quantifiable amounts of *chloridazon-desphenyl* using Method A)	All		
	146.0116		
	148.0087		
	116.9976		

Sample No	Exact masses (m/z)	Unspiked sample extract	Spiked sample extract at 0.1 mg/kg
Honey No. 72 (originally considered to potentially contain 0.121 mg/kg chloridazon-desphenyl using Method A)	All		
	146.0116		
	148.0087		
	116.9976		

Discussion and conclusions:

Following doubtful results for chloridazon-desphenyl in a large number of honey samples when employing the QuPPe Method M4.2 (Method A), a number of alternative approaches were checked to improve the chromatographic and/or mass-spectrometric separation.
The alternatives tested involved the use of SFC/UPC^2 (Method B), the use of a different HILIC stationary phase (Method C); and LC-ToFMS to improve mass-spectrometric selectivity through high resolution accurate mass measurements (Method C-HR). None of the above methods gave unequivocal results, which highlights that attention is needed when dealing with the analysis of chloridazon-desphenyl. It is thus recommended to evaluate all available information in order to solidify identification-judgement and to avoid false positive (or false negative) results.
Using QuPPe Method M4.2 (Method A), the interfering matrix component(s) exactly co-eluted with chloridazon-desphenyl on all measured MRMs, showing that in some cases a fitting retention time even if accompanied by a matching signal ratio of two MRM-transitions may still lead to a false judgement. This shows the importance of having a third backup mass-transition as well as knowledge about the particularities of the matrices analyzed. Overspiking with the analyte suspected to be present in the sample, did not help to increase confidence in the identification in this case. One of the MRMs of chloridazon-desphenyl was highly interfered and could not be used for identification. At the same time, however, this very intensive interference also gave a valuable hint to start questioning the identification certainty. According to the SANTE document "If the initial analysis does not provide unambiguous identification … a confirmatory analysis is required" (see also Chapter D7 of SANTE quality control procedures document [3]).
Method B (UPC^2) as well as the alternative HILIC column in method C, improved chromatographic separation thus giving value to the retention time as supporting evidence for the non-presence of chloridazon-desphenyl. Still, especially with method C, insufficient separation in combination with large signals of matrix components, compromised the ability for quantification and identification at low levels.
Despite the high-resolution power of the ToF mass analyzer, the identification of chloridazon-desphenyl in honey extracts remained uncertain, due to massive interferences on all mass traces. Interferences were different from matrix to matrix. Masses 148.0087 and 116.9976 were both interfered at the retention time of chloridazon-desphenyl, whereas mass 146.0116 showed a massive peak, but at a slightly different retention time, calling for the need for a better chromatographic separation.

To sum it up, in order to achieve reliable results, it is very important to check all available supporting evidence of identification in detail and to also take into account evidence that may be weakening identification certainty. In case of a doubtful result, the sample should be re-analyzed using a different technique for confirmation.

History

Action	When	Document Version
Initial Experiments	August 2022	
Further Experiments	Nov 2022 – Jan 2023	
Observation document placed on-line	April 2023	V1

[3] According to chapter D7 of the SANTE 11312/2021 - ANALYTICAL QUALITY CONTROL AND METHOD VALIDATION PROCEDURES FOR PESTICIDE RESIDUES ANALYSIS IN FOOD AND FEED (https://www.eurl-pesticides.eu/userfiles/file/EurlALL/SANTE_11312_2021.pdf): "Where an ion chromatogram shows evidence of significant chromatographic interference, it must not be relied upon for identification";,