EURL-SRM - Analytical Method Report

Concerning the following…

- **Compound(s)**: Lambda-Cyhalothrin (RS and SR constituent isomers)
- **Commodities**: Fruit and vegetables, cereals
- **Extraction Method(s)**: QuEChERS modified
- **Instrumental analysis**: LC-MS/MS

Analysis of Lambda- and Gamma-Cyhalothrin involving QuEChERS Extraction and Enantioselective LC-Separation of RS and SR-Isomers

Version 1 (last update: 12.04.2019)

Short Description:

A QuEChERS-based procedure involving enantioselective LC-MS/MS analysis of the two isomers of lambda-cyhalothrin is presented. Separation is achieved on a cellulose-based stationary phase covered by an immobilized chiral selector.

Background information:

Cyhalothrin, is an insecticide belonging to the group of synthetic pyrethroids. It is currently not approved for use in agriculture within the EU but it is still approved for veterinary purposes against ectoparasites such as ticks and mites. Cyhalothrin consists of 4 stereoisomers (RS, SR, RR, SS) in a 1:1:1:1 ratio. **Lambda-cyhalothrin** is a 1:1 mixture of 2 of the 4 cyhalothrin components (RS and SR). Its approval for agricultural use was renewed by Reg. 146/2016/EU. **Gamma-cyhalothrin** is constituted only by the SR-isomer, which is the insecticidally most active and also the most toxic of the 4 cyhalothrin isomers. Gamma-cyhalothrin is approved as active substance under Reg. 1334/2014/EU but no MRLs have been approved yet. Studies on various crops showed no preferential degradation/conversion between the 2 enantiomers of lambda-cyhalothrin [5].

In a focused review of 2017, EFSA identified several cases where the MRLs of lambda-cyhalothrin valid at that time would be toxicologically critical, if calculated assuming that the residue is entirely composed of gamma-cyhalothrin. EFSA concluded that lacking specific analytical methods, uses of gamma-cyhalothrin leading to residues not exceeding the MRLs for lambda-cyhalothrin could still result in consumer health risks.

Following a request by DG-SANTE EFSA prepared a scientific opinion in 2018, in which it was concluded that for infant food for children up to 16 weeks of age, the default MRL of 0.01 mg/kg applying for infant

formulae[1], may not be sufficiently protective for pesticides with ADI values that are lower than the health-based guidance value (HBGV) of 0.0026 mg/kg bw per day. Applying this calculation the default MRL of 0.01 mg/kg wouldn't be sufficiently protective for gamma- and lambda-cyhalothrin having ADI values of 0.0012 and 0.0025 mg/kg bw per day, respectively. For being sufficiently safe for children <16 weeks of age the MRLs for gamma- and lambda-cyhalothrin should thus not exceed 0.0046 and 0.0096 mg/kg respectively.

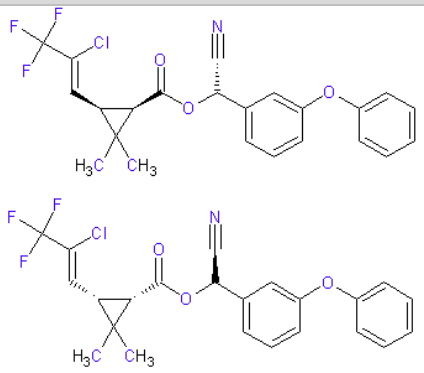
Using traditional separation techniques the constituent isomers of lambda-cyhalothrin (SR and RS) cannot be chromatographically separated. Data generated by such methods are thus of limited use for risk assessment and do not allow recognizing which of the two pesticides (lambda- or gamma-cyhalothrin) was applied in the field. For a proper control of gamma- and lambda-cyhalothrin there is a need for a method that is sensitive and at the same time able to distinguish between the two isomers of lambda-cyhalothrin. Separate quantification of the toxicologically most critical gamma-cyhalothrin (SR-isomer) is paramount for a proper risk assessment. In document SANCO/12745/2013 (rev. 10), which provides general guidance for the design of national monitoring programs, EURL-support is requested on this compound ("*As part of the outcome of the discussion held during the SC PAFF of 21-22 September 2017 it was requested that the EURLs would continue their effort to develop a routine method which can discriminate between the two substances*.").

In recent years some developments have been noticed both in LC and GC-based chiral separation technology. As thermal isomerizations may occur during GC-injection (a common phenomenon when dealing with pyrethroids containing a cyano-group in alpha position to the triangular ring), it was decided to choose LC-separations as a primary option and embark into GC-separations only if LC-MS/MS is not satisfactory in terms of enantiomeric separation or sensitivity.

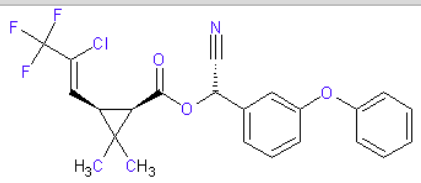
Compound details:

Name: Lambda-Cyhalothrin (CAS: 91465-08-6)

IUPAC: rac-(R)-cyano(3-phenoxyphenyl)methyl (1S,3S)-3-[(1Z)-2-chloro-3,3,3-trifluoroprop-1-en-1-yl]-2,2-dimethylcyclopropane-1-carboxylate

Parameter	Value	
Molecular Mass	449.9 g/mol	
Formula	$C_{23}H_{19}ClF_3NO_3$	
Exact mass	449.10055 Da	
Pka	not ionized [2]	
LogD	7 (20°C) [3]	
Residue definition EU	Lambda-cyhalothrin (includes gamma-cyhalothrin) (sum of R,S and S,R isomers) (F)	
Lambda-cyhalothrin is approved in…	AT, BE, BG, CY, CZ, DE, DK, EE, EL, ES, FI, FR, HR, HU, IE, IT, LT, LU, LV, MT, NL, PL, PT, RO, SI, SK, UK	
ADI / ARfD	0.0025 mg/kg bw per day / 0.005 mg/kg bw (Reg. (EU) 2016/146)	

[1] *Regulation 2006/141/EC referring to infant formulae and follow-on formulae repealed by Regulation 609/2013/EU*

Name: Gamma-Cyhalothrin (CAS: 76703-62-3) IUPAC: (S)-α-cyano-3-phenoxybenzyl (1R)-cis-3-[(Z)-2-chloro-3,3,3-trifluoropropenyl]-2,2-dimethylcyclopropanecarboxylate		
Parameter	**Value**	
Molecular Mass	449.9 g/mol	
Formula	$C_{23}H_{19}ClF_3NO_3$	
Exact mass	449.10055 Da	
Pka	not ionized	
LogD	7 (20°C)	
Residue definition EU	Currently included in the residue definition of lambda cyhalothrin	
Gamma-cyhalothrin is approved in…	BE, BG, CZ, DE, DK, FR, HR, HU, IE, RO, SK	
ADI / ARfD	0.0012 mg/kg bw per day / 0.0025 mg/kg bw (Reg. (EU) 2016/146)	

Chemicals and Consumables and Apparatus:

Please refer to the QuEChERS standard procedure (EN15662).

Analytical Standards:

Substance	CAS	Purity	Source (exemplary)
Lambda-cyhalothrin	91465-08-6	99.7%	HPC Standards GmbH
Gamma-cyhalothrin	76703-62-3	98.5%	LGC (Dr. Ehrenstorfer)
Chlorpyrifos D10	285138-81-0	97.0%	LGC (Dr. Ehrenstorfer)

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

The stock solutions of gamma- and lambda-cyhalothrin and of chlorpyrifos-D10 used in this study were prepared at 1 mg/ml by dissolving 15 mg of the compound in 1 mL acetonitrile and filling it up to 15 mL with acetonitrile. Working solutions were prepared by appropriately diluting stock solutions with acetonitrile. All other materials and chemicals used were as listed in EN 15662

Extraction and Cleanup

Extraction is conducted following the **citrate buffered QuEChERS (EN 15662)**. For this 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 Chlorpyrifos D10). Shake 15 min using a mechanical shaker. Add a mixture of 4 g $MgSO_4$, 1 g NaCl, 1 g trisodium citrate dihydrate and 0.5 g disodium hydrogen citrate sesquihydrate, shake 1 min and centrifuge. **Cleanup** via dispersive SPE using PSA-sorbent is conducted as described in EN15662, but is optional for fruits and vegetables.

LC-MS/MS Analysis

Following optimization measurement was conducted by LC-MS/MS in the ESI-pos. mode. The signals in the ESI-neg. mode were clearly less sensitive than those in the positive mode (by a factor of ca. 3) so it was deemed that measurements in the ESI-pos. mode would be more suited for achieving the desired sensitivity. As parent ion the ammonium adduct was used (m/z 467 and 469 for the 37Cl isotope). A simple isocratic elution profile was used with the mobile phase consisting of roughly 80% methanol, 20%

water and 5 mmol ammonium formate as a buffer. Table 2 and Table 3 show the measurement conditions chosen following optimization.

Table 2: Instrumentation details

LC	WATERS Acquity UPLC IClass		
MS/MS	SCIEX 5500 QTrap, run in ESI positive mode		
Column	ChiralArt Cellulose-SB, 100x4.6 mm, 3 µm		
Pre-column	None		
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	20	80
	15	20	80
Flow	0.6 mL/min*		
Injection volume	5 µL**		
Column temperature	35°C		

* Flow was reduced from 0.8 to 0.6 mL/min (for Sciex 4000QTrap) to avoid column overpressure.
** The flow-through injector operated by the default settings (pre-and post-aspirate air gaps) was used. When working with the LC-instrument connected to the Sciex 4000QTrap "partial loop with needle overfill" injection mode was used

Table 3: MRMs and MS/MS settings for gamma- and lambda-cyhalothrin (Sciex 5500 QTrap, ESI-pos.,):

Compound	Intensity ranking	Q 1	Q 3	DP	CE	CXP
Gamma-Cyhalothrin	1	467	225	71	23	12
OR	2	467	450	71	15	6
Lambda-Cyhalothrin	3*	469	452	76	13	6
Chlorpyrifos D10	-	360	199	71	23	12

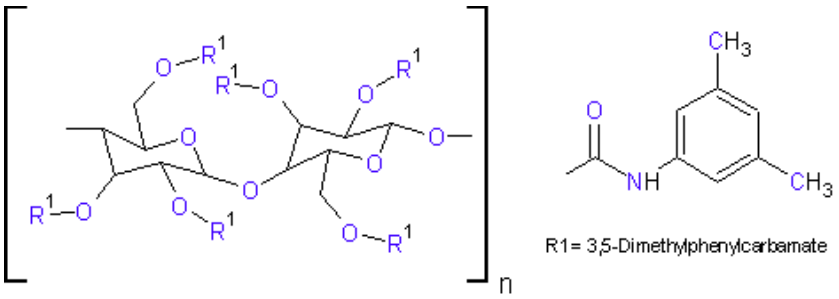
Note: Gamma-cyhalothrin and lambda-cyhalothrin share the same MRMs
* Limited sensitivity but suitable for confirmation at higher levels (e.g. >0,1mg/kg)

Experiments conducted and observations

Selection of column and gradient

A variety of LC-columns with chiral stationary phases is available on the market. After not being able to achieve satisfactory separation using columns with loosely coated (non-immobilized) chiral stationary phases, we have employed a column with immobilized chiral selectors (CHIRAL ART Cellulose-SB by YMC). Columns having the chiral selectors bonded to the support material are typically more robust and allow the use of a wider range of chromatographic solvents. The chiral sector of the column is shown in Figure 1.

Figure 1: Chiral selector of Chiral Art cellulose-SB column [1].



Applying the conditions proposed by YMC the CHIRAL ART Cellulose-SB column was able to largely separate the two enantiomers of lambda-cyhalothrin, with the gamma-cyhalothrin component eluting first. To further improve separation the parameters the influence of the following parameters on separation was tested:

- Column temperature: up to 40°C
- Mobile phase solvent: methanol/water or acetonitrile/water
- Mobile phase elution profile: gradient versus isocratic elution
- Mobile phase modifier: formic acid, acetic acid or ammonium formiate
- Mobile phase flow rate: 0.5 to 0.8 ml/min (operational pressure should be <250 bar)
- Ionization mode: ESI positive or negative
- Ion-source settings: curtain gas, temp., ionization voltage (Sciex 4000 and 5500 QTrap)
- Injection volume

Observations on mass spectrometric behavior of isomers

Two MS/MS instruments Sciex 5500 QTrap and the Sciex 4000 QTrap were employed and compared as regards the sensitivity with which they can detect lambda- and gamma-cyhalothrin. For this, separate acetonitrile standards containing 0.1 µg/mL lambda- or gamma-cyhalothrin were injected into both instruments using the same column and gradient (see Table 2). The peak areas obtained are shown in Table 1 and the peaks in Figure 1. The signals of the 5500 instrument showed a strong fronting, which may be related to the different injector type used. Using the Sciex 5500 QTrap model the absolute signal areas obtained for the target mass-transition (467>225) were ca. 20-fold larger than those of 4000 QTrap model. For the qualifier mass-transition (467>450) the signal was ca. 10-fold larger.

The ratio between the peak areas of the two mass-transitions of gamma-cyhalothrin was similar to that of lambda-cyhalothrin (see Table 4), suggesting that both isomers experience a similar MS fragmentation pattern (although there is some uncertainty due to the single injection). As the two isomers of lambda-cyhalothrin are reportedly enantiomers (mirror images), this similar mass spectrometric behavior reflects the theory.

Interestingly, in both instruments and all mass transitions the area of the gamma-cyhalothrin peak (first eluting) was slightly smaller (ca. 6% on average) than that the area of the equally concentrated lambda-cyhalothrin (when integrating both peaks together). As the number injections performed was limited, it is not possible to say whether this deviation was due to a measurement error or due to deviating standard concentrations (e.g. due to weighing error or wrong information as regards the purity of the neat standards).

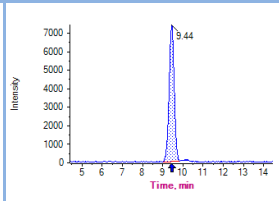
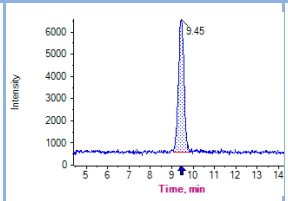
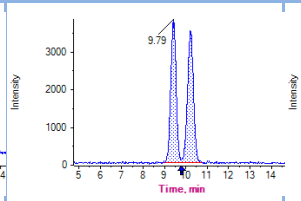
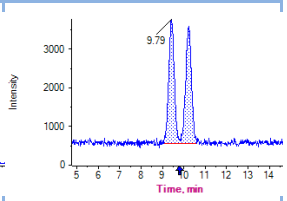
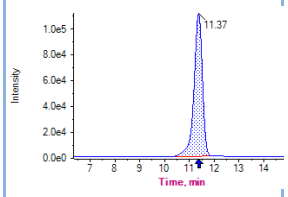
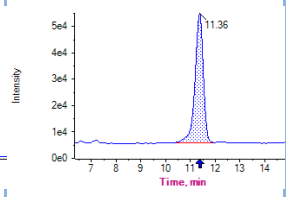
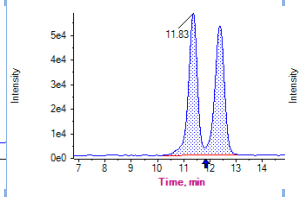
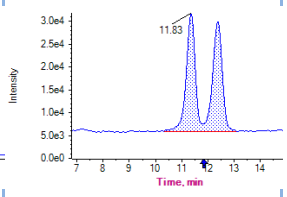
Within the lambda-cyhalothrin chromatograms it was furthermore observed, that in all chromatograms the first-eluting gamma-cyhalothrin signal was always a bit higher than that of its isomer. In the case of the measurements with the 5500 QTrap instruments, this distortion in favor of the first eluting gamma-isomer can be partly attributed to the peak fronting, but a similar distortion was also observed with the 4000 QTrap instrument, where the peaks were more symmetric and well separated. Assuming similar

chromatographic behavior of the two isomers this observation would indicate that the gamma-cyhalothrin isomer is slightly predominating within the lambda-cyhalothrin mixture. But also here more experiments would be needed to clarify the issue.

Table 4: Signals of gamma- and lambda-cyhalothrin by different instruments. Both standards at 0.1 µg/mL (n=1)

Instrument Type	MRM		Gamma-cyhalothrin (0.1 µg/mL)		Lambda-cyhalothrin (0.1 µg/mL)		Factor of Gamma / Lambda signals
			Peak areas (Single peak)	Factor MRM1/MRM2	Peak areas (sum of two peaks)	Factor MRM1/MRM2	
Sciex 4000 QTrap	1	467>225	1.31e5	1,21	1.36e5	1,17	0.96
	2	467>450	1.08e5		1.16e5		0.93
Sciex 5500 QTrap	1	467>225	2.62e6	2,28	2.76e6	2,17	0.95
	2	467>450	1.15e6		1.27e6		0.91
						Average	**0.94**

Figure 1: Sensitivity comparison of Sciex 4000 QTrap and 5500 QTrap instruments, exemplary using acetonitrile *solutions* of gamma-cyhalothrin and lambda-cyhalothrin at 0.1µg/mL each

Instrument	gamma-cyhalothrin m/z 467>225 (target)	gamma-cyhalothrin m/z 467>450	lambda-cyhalothrin m/z 467>225 (target)	lambda-cyhalothrin m/z 467>450
Sciex 4000 QTrap				
Sciex 5500 QTrap				

In a follow-up experiment and using freshly prepared stock solutions, separate standard solutions of each compound were prepared containing gamma-cyhalothrin at 0.01 and 0.1 µg/mL, lambda-cyhalothrin at 0.02 and 0.2 µg/mL and cyhalothrin at 0.04 and 0.4 µg/mL, These solutions were injected in triplicate, measuring the total peak area as well as the peak area of the well separated gamma-isomer in all cases. In the case of cyhalothrin only the gamma-isomer separated whereas the other three isomers largely co-eluted. Still, the elution profile allows distinguishing between lambda-cyhalothrin and cyhalothrin.

The measured peak areas are shown in Table 5 and some exemplary chromatograms in Figure 2. It should be noted, however, that the peak areas of gamma-cyhalothrin are associated with some uncertainty due to the non-full separation of the two isomers. The peak areas shown in Table 5 are those obtained after dividing the peaks through a vertical line at the lowest point of the peak valley, thus excluding some of the tailing of gamma-cyhalothrin and including some of the fronting of the later eluting compounds. Overall the share of gamma-cyhalothrin on the total peak area was close to the

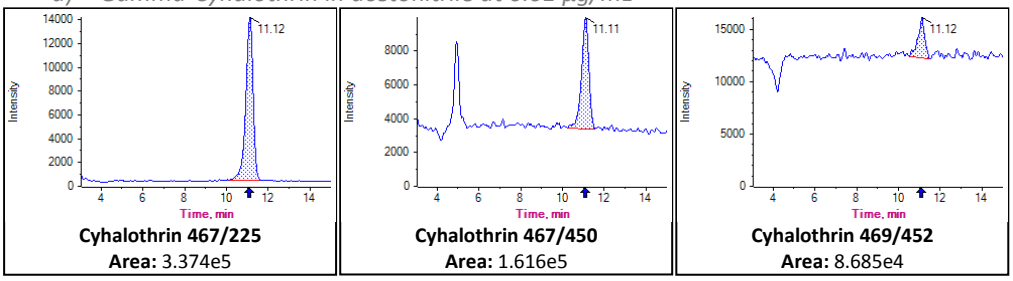
theoretical 50% in the case of lambda-cyhalothrin and close to the theoretical 25% in the case of cyhalothrin. Comparing the total peak areas of gamma- to those of lambda-cyhalothrin and cyhalothrin the gamma-cyhalothrin standards seems to have a lower concentration than expected. The deviation seems to be in the range between 12-16 % and may be due to a deviation in the purity of the standard.

Table 5: Signals obtained from the injection of gamma-cyhalothrin, lambda-cyhalothrin and cyhalothrin standards at different concentrations, and comparison of the total peak areas and the peak areas of gamma-cyhalothrin

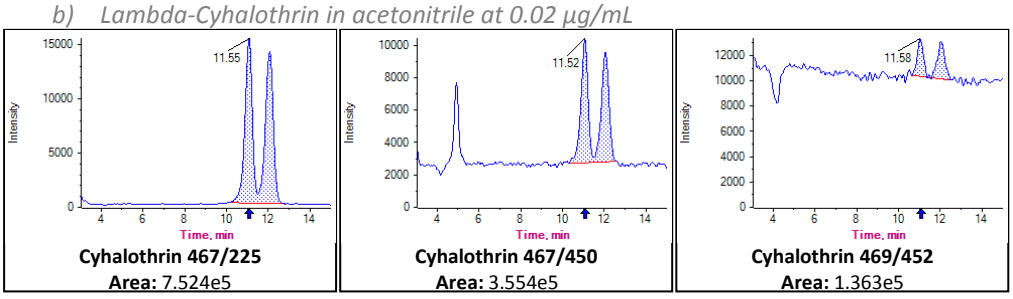
Compound	Avg. peak area gamma isomer	Avg. total peak area	Share of gamma isomer on total peak area in %	Normalized peak area of gamma-isomer	Normalized total peak area
Low level (n=3 each)					
Gamma-cyhalothrin	334,000	334,000	**100**	**100**	**100**
Lambda-cyhalothrin	389,000	770,000	50.5	114	115
Cyhalothrin	411,000	1558,000	26.4	123	117
High Level (n=3 each)					
Gamma-cyhalothrin	3,630,000	3,630,000	**100**	**100**	**100**
Lambda-cyhalothrin	4,160,000	8,090,000	51.4	108	112
Cyhalothrin	4,290,000	15,780,000	27.2	118	109

Figure 2: Exemplary chromatograms of gamma-cyhalothrin, lambda-cyhalothrin and cyhalothrin

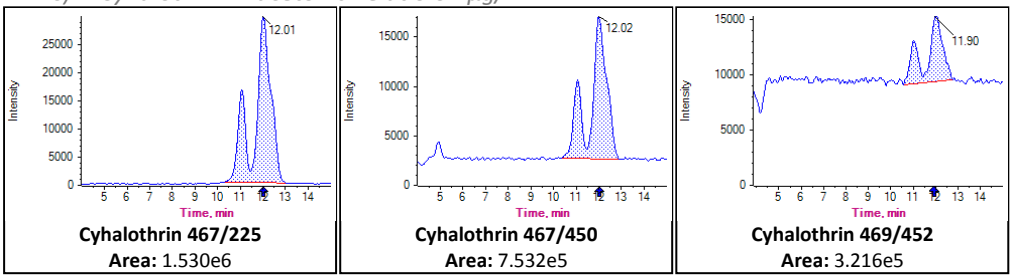
a) Gamma-Cyhalothrin in acetonitrile at 0.01 µg/mL



b) Lambda-Cyhalothrin in acetonitrile at 0.02 µg/mL



c) Cyhalothrin in acetonitrile at 0.04 µg/mL



Validation

Validation experiments were based on the standardized QuEChERS procedure (EN-15662) [4].

In a pre-experiment the impact of sample preparation and dSPE cleanup (with PSA as sorbent) on the recovery rates as well as the isomeric composition of lambda-cyhalothrin were studied. Using blank cucumber as matrix gamma-cyhalothrin was spiked at 0,005 mg/kg and separately lambda-cyhalothrin at 0,01 mg/kg. Recoveries were nearly quantitative and **neither sample preparation nor dSPE cleanup had a notable influence on the recovery rates** or **the isomeric distribution.** The results of this experiment are shown in Table 6.

Table 6: Validation of gamma-cyhalothrin respectively lambda-cyhalothrin on cucumber with PSA cleanup (ESI-pos. mode using Sciex API 5500 QTrap),

Compound	MRM used	Spiking Level* (mg/kg)	dSPE Cleanup (with PSA/C_{18})	Mean Recovery %
Gamma-Cyhalothrin	467>225	0.005	**Yes**	105
			No	108
Lambda-Cyhalothrin		0.010	**Yes**	103
			No	103

Given the high relevance of gamma- and lambda-cyhalothrin for infant formulae, the first full QuEChERS validation experiment was performed on **infant milk formula**. 2 g analytical portions were spiked either with 0.05 µg gamma-cyhalothrin, or with 0.1 µg lambda-cyhalothrin which corresponds to 0.025 and 0.05 mg/kg respectively. Calculated on the basis of the reconstituted milk (recipe: 13.4 g infant formula + 90 mL water = 103.4 g reconstituted milk) the spiking levels were 0.0032 mg/kg for gamma- and 0.0064 mg/kg lambda-cyhalothrin. Following addition of 10 mL of water extraction and partitioning followed the CEN-QuEChERS protocol. Cleanup was conducted by dispersive SPE using both C_{18} and PSA sorbents. Calibration points were prepared both in acetonitrile (solvent calibration) and on blank matrix extracts (matrix-matched) at the 60% and 120% level of the spiked concentration. Chlorpyriphos-D_{10} was used as internal standard. Table 7 shows a compilation of the results,

Table 7: Validation of gamma- and lambda-cyhalothrin on **infant formula** (ESI-pos. mode; Sciex API 5500 QTrap):

Compound	Spiking Level* (mg/kg)	No. of replicates	Mass Transition	Mean Recovery %	RSD %
Gamma-Cyhalothrin	0.0033	5	MRM1: 467>225 (T)	99	1.7
			MRM2: 467>450	103	7.7
Lambda-Cyhalothrin	0.0064	5	MRM1: 467>225 (T)	95	6.7
			MRM2: 467>450	93	7.6

* the odd numbers result from the reference to the reconstituted product. 2 g milk reconstituted according to recippee on the package results in 15,43 g of milk.

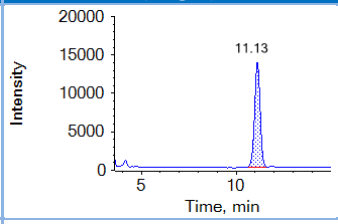
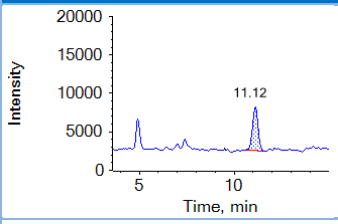
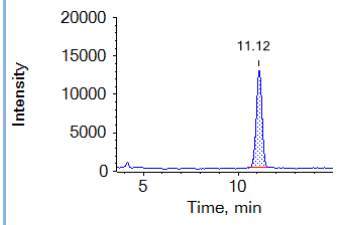
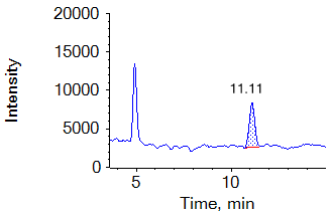
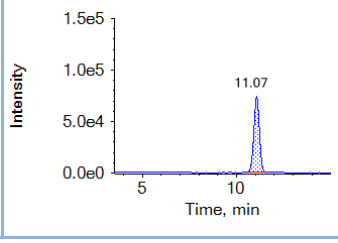
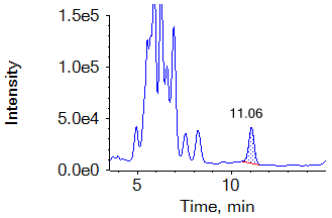
Fortunately **only insignificant matrix effects** were noticed in the LC-MS/MS analysis of infant formula extracts. At the 60% calibration level the response ratio between the solvent and matrix-matched calibration was 1.05 and 1.03 for MRM1 and MRM2 respectively, and at the 120% level it was 1.03 and 1.01.

Further QuEChERS validation experiments were conducted on blank homogenates of cucumber, orange juice and wheat flour. A summary of the validation results is given in Table 8. Chlorpyriphos-D10 was used as internal standard. Mean recoveries and RSDs were only based on the target MRM (m/z 467>225). Measurements were conducted with the more sensitive API5500 instrument. Exemplary chromatograms are shown in Figure 3.

Table 8: Recovery data for gamma-cyhalothrin from various commodities using the QuEChERS method

Matrix Group	Matrix	Sample Weight + water addition	Spiking Level (mg/kg)	QuEChERS w/o cleanup		
				n	Mean Rec.%	RSD %
High water	**Cucumber**	10 g	0.01	5	97	1.7
			0.1	5	93	1.1
High water and+ low pH	**Orange Juice**	10 g	0.01	5	95	0.7
			0.1	5	94	1.0
Dry	**Flour**	5 g + 10 mL water	0.01	5	103	1.3
			0.1	5	107	1.1

Figure 3: Exemplary chromatograms of gamma-cyhalothrin in extracts from various commodities:

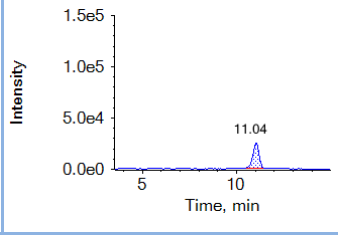
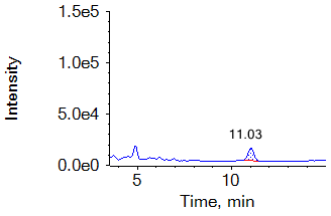
Matrix	Spiking Level (mg/kg)	gamma-cyhalothrin m/z 467>225 (target)	gamma-cyhalothrin m/z 467>450
Cucumber	0.01		
Orange Juice	0.01		
Wheat Flour	0.1		

In addition validation was conducted on sunflower seed oil using the QuOil method, which involves extraction of 2 g sample with 10 mL acetonitrile and partitioning without the addition of water and salts. dSPE cleanup is conducted on an aliquot of the extract using C_{18} sorbent. The addition of the internal standard is done following aliquotation. The validation results of this experiment are shown in Table 9 and the chromatograms in Figure 4.

Table 9: Recovery data for gamma cyhalothrin from sunflower seeds oil using the QuPPe method

Matrix Group	Matrix	Sample Weight	Spiking Level (mg/kg)	QuEChERS w/o cleanup		
				n	Mean Rec.%	RSD %
Dry and high lipid content	Sunflower seed oil	2 g	0.1	5	107	1.1
		2 g	0.01	5	95	2.1

Figure 4: Exemplary chromatograms of gamma-cyhalothrin in extracts from various commodities:

Matrix	Spiking Level (mg/kg)	gamma-cyhalothrin m/z 467>225 (target)	gamma-cyhalothrin m/z 467>450
Sunflower seed oil	0.1		

Discussion and conclusions

A procedure was developed allowing a sensitive and enantioselective analysis of gamma- and lambda-cyhalothrin. Following CEN-QuEChERS extraction the compounds were analyzed by chiral LC-MS/MS, which resulted in a chromatographic separation of the more toxic gamma-cyhalothrin (SR-isomer) from its RS-isomer. Satisfactory recovery rates and repeatabilities were achieved for gamma-cyhalothrin in cucumber, orange juice and wheat flour at 0.01 mg/kg each. In addition the method was successfully validated in infant formula at 0.0036 mg/kg (expressed on the basis of recipe-conform reconstituted product). In addition, using the QuOil method satisfactory recoveries were achieved on sunflower seed oil.

Literature

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[2] Chemicalize.org
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[5] EFSA Journal 2015;13(12):4324: Revision of the review of the existing maximum residue levels for the active substance lambda-cyhalothrin
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History

Action	When	Document Version
Initial Experiments with chiral columns	Apr 2017 and Apr-May 2018	
Experiments with YMC column	Oct - Dec 2018	
Validation Experiments	Dec 2018 - Feb 2019	
Observation document	Mar-Apr 2019	V1