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Abstract

For pesticide residues in infant food, including infant formulae, a low default maximum residue level of 0.01 mg/kg currently applies to protect this sensitive consumer group from health risks arising from the intake of pesticides. For a small number of toxicologically critical pesticides, however, MRLs that are even lower than this default value have been established within the existing regulation to ensure food safety. Nevertheless, there are still several additional toxicologically critical pesticides with acceptable daily intake values below the health-based guidance value of 0.0026 mg/kg body weight per day that was established by EFSA in 2018, for which this default MRL may not be sufficiently protective. The aim of this study was to check the analytical feasibility of monitoring these additional critical pesticides at or below levels considered to be toxicologically safe, as this would facilitate the establishment of specific MRLs for infant formulae. The current study dealt with the analysis of 13 of those highly toxic pesticides (or their metabolites) that are not amenable to standard multi-residue methods. Additional compounds deemed relevant to milk products were also included in the project. Beyond infant formulae, milk was included, as it is a basic source of many infant formulae ingredients. The study comprised method development and validation of the concerned compounds in both types of commodities, followed by a pilot monitoring of 80 samples of infant formulae for children up to 16 weeks of age, and 54 samples of milk, to elucidate the current residue situation. Of the 13 compounds with high toxicity, none was detected in the analysed samples, except nicotine, which was detected at non-critical trace levels in more than 70 % of the samples. However, the additionally analysed compounds were frequently detected, especially chlorate, perchlorate and phosphonic acid. Overall, the results of this study revealed, that, according to the present state of knowledge, the intake of the targeted compounds through infant formulae consumption does not pose appreciable health risks to infants.

1. Introduction

In a scientific opinion by EFSA released in May 2018 [1], it was concluded that, in the case of pesticides with acceptable daily intake (ADI) values below the health-based guidance value (HBGV) of 0.0026 mg/kg body weight (bw) per day, the default MRL of 0.01 mg/kg currently applied to reconstituted infant formulae (Art. 4 of Reg. (EU) No. 2016/127 in combination with Reg. (EU) No. 609/2013) may not be sufficiently protective for children up to 16 weeks of age [2, 3]. Therefore, a number of compounds with ADI values < 0.0026 mg/kg bw per day were identified and the highest

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possible MRL that would still be considered safe for children up to 16 weeks of age was calculated. After method development and successful validation for a number of compounds at levels equal to or lower than the levels considered to be safe, a pilot monitoring study for various types and origins of infant formulae as well as milk was conducted. Table 1 shows the scope of the compounds entailed in the project together with their respective ADI value and the highest toxicologically acceptable MRL (maximum safe MRL) for infant formula. Both the consumption figures of infant formulae as well as the MRLs refer to the reconstituted (ready-to-feed) products, so the 'safe MRLs' and the maximum LOQs that would need to be reached for the various compounds were calculated on reconstituted products. For calculating the maximum LOQs for non-reconstituted powders, the preparation recipes were taken into account. The recipes of the various products were

Table 1. Scope of SRM- and MRM/SRM-compounds for the monitoring of infant formulae and milk, including the maximum safe MRL for reconstituted infant formulae considered safe for infants of up to 16 weeks of age.

	Compounds	SRM/MRM[a] Compound	Monitored in Infant food	Monitored in Milk	ADI (mg/kg bw per day)	Max. safe MRL/LOQ for reconst. products (mg/kg)	Max. LOQ for infant formula powder[b] (mg/kg)	Extraction[c]
Toxicologically critical compounds	Abamectin	MRM/SRM	✓	✓	0.0025	0.0096	0.072	A-QuEChERS
	Amitrole	SRM	✓	✓	0.001	0.0038	0.0285	QuPPe-AO
	Cotinine[d]	SRM	✓	✓	0.0008	0.0031	0.0233	QuPPe-AO
	Cyhalothrin[e]							
	γ-Cyhalothrin	MRM/SRM	✓	✓	0.0012	0.0046	0.035	A-QuEChERS
	λ-Cyhalothrin	MRM/SRM	✓	✓	0.0025	0.0095	0.071	A-QuEChERS
	Diclofop	MRM/SRM	✓	✓	0.001	0.0038	0.0285	A-QuEChERS
	Diquat	SRM	✓	✓	0.002	0.0076	0.057	QuPPe-AO
	Emamectin	MRM/SRM	✓	✓	0.005	0.0019	0.0143	A-QuEChERS
	Fentin	SRM	✓	✓	0.0004	0.0015	0.0113	A-QuEChERS
	Haloxyfop	MRM/SRM	✓	✓	0.00065	0.0025	0.01875	A-QuEChERS
	3-Hydroxy-carbofuran	SRM[f]	✓	✓	0.00015	0.0006	0.0045	A-QuEChERS
	Related compounds:							
	Carbofuran	MRM/SRM	✓	✓	0.00015			
	Benfuracarb	MRM/SRM	✓	✓	0.0035			
	Furathiocarb	MRM/SRM	✓	✓	0.0035			
	Carbosulfan	MRM/SRM	✓	✓	0.005			
	Nicotine	SRM	✓	✓	0.0008	0.0031	0.0233	QuPPe-AO
	PTU[g]	SRM	✓	✓	0.0003	0.0012	0.015	QuPPe-AO
	Topramezone	SRM	✓	✓	0.001	0.0038	0.0285	QuPPe-AO
Additionally analysed compounds	Chlorate	SRM	✓	✓	0.01	–	–	QuPPe-AO
	Cyanuric acid	SRM	✓	–	1.5[h]	–	–	QuPPe-AO
	Ethoxyquin dimer	SRM	–	✓	0.001	–	–	A-QuEChERS
	Melamine	SRM	✓	✓	0.2	–	–	QuPPe-AO
	Paraquat	SRM	✓	✓	0.004	–	–	QuPPe-AO
	Perchlorate	SRM	✓	✓	0.0003[i]	–	–	QuPPe-AO
	Phosphonic acid	SRM	✓	✓	2.25	–	–	QuPPe-AO
	Thiocyanate	SRM	✓	–	not available	–	–	QuPPe-AO
	Triazole derivative metabolites:							
	1,2,4-Triazole-acetic acid (TAA)	SRM	–	✓	1[j]	–	–	QuPPe-AO
	1,2,4-Triazole-lactic acid (TLA)	SRM	–	✓	0.3[j]	–	–	QuPPe-AO
	1,2,4-Triazol-1-yl-alanine (TA)	SRM	–	✓	0.3[j]	–	–	QuPPe-AO
	Trifluoroacetic acid (TFA)	SRM	✓	✓	0.05	–	–	QuPPe-AO

[a] SRM-compound: Compound that is not amenable to typical multi-residue methods (MRMs), i.e. Single Residue Method compound; MRM/SRM compound: Compound amenable to some MRMs, but in a modified form

[b] Based on a conversion factor of 7.5

[c] A-QuEChERS and QuPPe-AO: see '3 Materials and Methods'

[d] Cotinine was included as it is a known animal metabolite of nicotine (this compound is currently not regulated as a pesticide)

[e] λ-cyhalothrin is a 1:1 mixture of γ-cyhalothrin and its enantiomer. γ-cyhalothrin is toxicologically much more critical than its enantiomer.

[f] SRM applies when considering conjugates of 3-hydroxy-carbofuran (Note: according to Reg. (EC) 2005/396 the residue definition applying to milk (and its derivative products) is as follows: *3-OH-carbofuran (free and conjugated) expressed as carbofuran).*

[g] PTU is a degradant of propineb. It used to be regulated in infant formulae together with propineb, but since the introduction of Reg. (EU) 2021/1041 amending Reg. (EU) 2016/127 the residue definitions of Reg. (EC) 2005/396 now apply to all pesticides in (non-cereal-based) infant formulae. PTU is not regulated in Reg. (EC) 396/2005.

[h] TDI (also expressed in mg/kg bw per day) by WHO (2008)

[i] TDI (also expressed in mg/kg bw per day) by EFSA CONTAM Panel (2014) [6]

[j] Peer review EFSA [7]

Table 2. Notes on compounds that were additionally analysed during the project to supplement the scope, selected mainly on their likelihood to be encountered in milk and/or infant formulae or their ingredients

Compound	ADI (mg/kg **bw per day**)	Notes
Chlorate	0.01	Former herbicide and biocide. Currently not approved as active substance. A by-product of the disinfection of drinking water. It may contaminate food products through various pathways including irrigation (plants), drinking (animals), food processing/storage (e.g. contaminated surfaces). Toxicologically critical, as it temporarily inhibits the intake of iodine in the thyroid gland and induces oxidative stress to red blood-cells.
Cyanuric acid	1.5 [a)]	Non-regulated metabolite and hydrolysis product of various pesticides. Originates from multiple sources, e.g.: **Triazine pesticides** (incl. the herbicides terbuthylazine, atrazine, cyanazine, the fungicide anilazine and the insecticide cyromazine). From the above only terbuthylazine and cyromazine are currently in use within the EU, with the latter having lost approval but still keeping an emergency authorization. **Cyanamide-based fertilizers**: Cyanamide contained in fertilizers may convert to melamine through trimerization, which can further hydrolyze to cyanuric acid. **Urea-based fertilizers or feed**: Especially at high temperatures, urea loses ammonia converting to isocyanic acid (HNCO), which trimerizes to cyanuric acid. **Mono-, Di- and Trichloroisocyanurates**: Used as disinfectants, algaecides and bactericides. They are used in sanitation liquids and bleaching agents as well as in swimming pools (pool-tabs) to retard the loss of chlorine in chlorinated water. In water, they gradually convert to cyanuric acid. Natural formation of cyanuric acid has also been reported (e.g. in humus).
Ethoxyquin dimer	0.005	Ethoxyquin is used as an antioxidant agent in fish feed and in dried cereals. It transforms into a multitude of metabolites and reaction products, of which Ethoxyquin dimer is the most prominent in salmon. Ethoxyquin dimer is also more stable than Ethoxyquin. In infant formulae, fish oil is a frequent ingredient which aims to supplement infants with omega-3 fatty acids
Melamine	0.2	Metabolite of cyromazine (pesticide and veterinary drug). In relation to a food fraud scandal in 2008, it was revealed that melamine was used to adulterate infant formulae simulating high milk protein contents by the presence of nitrogen. May also originate from cyanamide fertilizers (trimerization of cyanamide) as well as from urea, where it is formed through trimerisation to triuret and subsequent elimination of ammonia and carbon dioxide (Note: biuret and triuret are related non-cyclic products formed from the di-and trimerisation of urea respectively). Melamine hydrolyzes to cyanuric acid via ammeline and ammelide. Regulated by Reg. 1881/2006/EC [5] as a contaminant.
Paraquat	0.004	Not approved active substance and herbicide. It was included in the scope of this study, as it is covered by the same method as diquat.
Perchlorate	0.0003 [b)]	Persistent and ubiquitous environmental contaminant. Mainly originating from fertilizers, may also be formed as a by-product in the disinfection of drinking water. Similar to chlorate, perchlorate exposure is associated with thyrotoxicity and oxidative stress. It is regulated by Reg. 1881/2006/EC [5] as a contaminant.
Phosphonic acid	2.25	Fungicide by itself, but also metabolite of fosetyl. Additional input from so-called 'leaf fertilizers' and 'plant strengtheners'. Phosphonate accumulates in perennial plants and survives over many years. Even after several years with no application, considerable levels of residues in fruits, such as blueberries, may be detected.
Thiocyanate	not available	Not approved active substance and naturally occurring in foodstuffs, especially in the plant family Brassicaceae. Various crops belonging to the Brassicaceae, such as rape and fodder cabbage are used as feeding stuff for lactating cows and may lead to elevated thiocyanate levels in milk. Furthermore, rape seed oil is a common ingredient in infant formulae. Thiocyanate also temporarily inhibits the intake of iodine in the thyroid gland.
Triazole derivative metabolites: 1,2,4-Triazole-acetic acid (TAA) 1,2,4-Triazole-lactic acid (TLA) 1,2,4-Triazol-1-yl-alanine (TA)	 1 [c)] 0.3 [c)] 0.3 [c)]	Triazole derivative metabolites (TDMs) result from the use of a large number of pesticides belonging to the group of triazole fungicides, which contain a triazole moiety in their structure. 1,2,4-Triazole is also used as a nitrification inhibitor in fertilizers and may convert to TAA, TLA and TA within the plants.
Trifluoroacetic acid (TFA)	0.05	TFA is a metabolite of numerous fluorine-containing pesticides. Moreover, it is generated during decomposition of various other anthropogenic chemicals, such as coolants and teflon. It is frequently detected in drinking and surface water and can be classified as an environmental contaminant.

[a)] TDI (also expressed in mg/kg bw per day) by WHO (2008)
[b)] TDI (also expressed in mg/kg bw per day) by EFSA CONTAM Panel (2014) [6]
[c)] Peer review EFSA (2018) [7]

largely similar, with conversion factors from powder to reconstituted product ranging between 7.52 and 7.98 (7.87 on average). Finally, it was decided to multiply the maximum MRL in the ready-to-use infant formula by the factor 7.5, resulting in the most conservative MRLs for the infant formula powder with the goal of establishing LOQs below that level.

Although the focus of the project was to examine the residue situation of toxicologically critical compounds in infant formulae, it was decided to include certain additional compounds in the scope that are known to be ubiquitous in the environment and are therefore frequently encountered in various food commodities (Table 1 and 2). Some of these compounds are of toxicological concern and others are suspected of potentially exceeding the default MRL of 0.01 mg/kg applying to infant formulae and dietary food. Where no ADI value was available, the Tolerable Daily Intake (TDI) value is noted in Table 1 and 2.

Infant formulae, also known as baby formulae, are mostly made with skimmed cows' milk or whey, which is mixed with vegetable fats, oils, emulsifiers, vitamins, minerals and stabilizing agents. The mixture is pasteurized and then dried into a powder. The products are usually sold as powders, which have to be reconstituted with water to be made into a liquid product. Some of the products are also offered on the market in reconstituted form, i.e. as ready-to-feed formulae. Infant formulae for babies below 16 weeks of age can be classified into the following types:

Type A: 'Normal'
Type B: Lactose-free (whey is replaced by e.g. isolated weigh proteins and corn syrup)
Type C: Hypoallergenic (containing extensively hydrolysed milk proteins)
Type D: Anti-reflux (containing thickening agents)
Type E: 'Comfort' (for infants with digestive problems; contains partly broken-down proteins)
Type F: lant-based, i.e. dairy-free (based on e.g. soy or rice)

2. Pilot Monitoring

Sampling: Overall, 80 samples of infant formulae, purchased in 23 countries, were collected (Table 3 and 4). These included six ready-to-use (liquid) formulae and 74 powderous infant formulae. Table 4 gives an overview of the numbers of collected samples of each type and introduces abbreviations for the types. Types B to F cover infant formulae for special needs

Table 3. Countries of sampling (21 EU and 2 EFTA countries) and types of infant formulae samples (see Table 4 for the abbreviations).

Country		Normal	L-Free	HA	AR	Comf.	Dairy-Free	Sum
EU	**Germany**	7	5		2	2	1	**17**
	Czech Republic	4	1					**5**
	Spain	3		1		1		**5**
	Belgium	4					1	**5**
	Latvia	2	2		1			**5**
	France		1	2	1			**4**
	Portugal	2	1	1				**4**
	Cyprus	3						**3**
	Denmark	1	2					**3**
	Austria	1	1					**2**
	Croatia	1	1					**2**
	Romania	1		1				**2**
	Hungary		1	1				**2**
	The Netherlands	1			1			**2**
	Sweden	1	1					**2**
	Greece			1	1			**2**
	Bulgaria	2						**2**
	Ireland			1	1			**2**
	Italy	2						**2**
	Slovenia				1			**1**
	Finland		1					**1**
EFTA	**Norway**	3	1					**4**
	Switzerland	3						**3**
Total		**41**	**18**	**8**	**8**	**3**	**2**	**80**

[a)] The IL-IS will typically correct for volume deviations. In case no IL-IS is used, volume adjustments become more important.

Table 4. Overview and numbers of collected infant formulae samples of each type. Refer to '1 Introduction' for detailed description of the infant formulae types.

Type	Abbreviation	No. of Samples			No. of Countries	
		Conventional	Organic	SUM	of Production [a)]	of Sampling
A Normal	Normal	30	11	**41**	9	17
B Lactose-free	L-Free	8	–	**8**	5	7
C Hypoallergenic	HA	18	–	**18**	5	12
D Anti-reflux	AR	6	2	**8**	6	7
E Comfort	Comf.	3	–	**3**	1	2
F Soy/rice based	Dairy-Free	1	1	**2**	2	2
Total		**66**	**14**	**80**	**10** [a)]	**23**

[a)] Where the country of production was specified on the label (10 countries of production could be identified in total)

and specific food intolerances and are thus less represented than 'normal' infant formulae. This roughly reflects the market situation. Products of type A were the most frequent (51 % of all), followed by products belonging to type C 'hypoallergenic' (23 % of all). Fourteen of the 80 infant formulae samples (18 %) were labelled as 'organic' and the rest were of conventional production.

Seventy-four of the 80 collected infant formula products were powders, which have to be mixed with water prior to consumption. The remaining six were already prepared liquid products, so-called ready-to-use formulae. Two infant formulae samples were based on goat's milk, one on soy and one on rice.

Although the samples were collected from 23 countries, they were produced in much fewer countries. The 67 products where the country of production was stated on the packaging were produced in merely 10 different countries. For the remaining 13 products, it was not possible to determine the country of production from the labelling. Most of the collected infant formulae products were produced in Germany, including many of those collected from other countries. Many of the leading brands sell their products under different brand names in different countries. In fact, some of these brands were originally produced by independent companies that were eventually taken over by one of the leading brands. An overview of milk samples and their origin is given in Table 5.

3. Materials and Methods

3.1. Reagents and Materials

Sources of analytical standards, consumables and chemicals used in this study are listed in EN 15662, the QuPPe-PO [8] or the QuPPe-AO [4] method. For validation experiments, selected milk and of infant formulae samples of various types were used. These samples were selected following preliminary experiments with the criterion, that the target compounds are absent (ideally) or present at very low levels. Please note: some of the compounds such as chlorate are ubiquitous.

3.2. Sample Preparation using QuEChERS

Two methods, the QuEChERS method (EN 15662) and the acidified-QuEChERS method (A-QuEChERS) were used. QuEChERS was conducted as described in EN 15662, using analytical portions of 2 g infant formula powder or 10 g ready-to-use infant formula or 10 g milk. The first extraction step involved 15 min shaking by a mechanical shaker. No clean-up was conducted, as only LC-MS/MS measurements were foreseen. A-QuEChERS is similar to EN 15662, but instead of pure acetonitrile, 10 mL acetonitrile containing 1 % formic acid were employed for extraction. This approach was chosen as it provided better recovery rates for certain compounds such as fentin, while other compounds were not negatively affected. Partitioning was induced by the addi-

Table 5. Overview of collected milk samples

Type	No. of Samples			No. of Countries (EU/EFTA) from which samples originated
	Conventional	Organic	Total	
Heat-Treated milk	42	2	**44**	20
Raw milk	9	1	**10**	1 (all of German origin)
Total	**51**	**3**	**54**	

tion of 4 g $MgSO_4$ + 1 g NaCl (no citrate buffer salts). Initial extraction involved 15 min shaking and a subsequent partitioning step of 2 minutes shaking by a mechanical shaker. No clean-up was conducted, as only LC-MS/MS applications were foreseen. The general analytical procedure is shown at a glance in Figure 1.

3.3. Sample Preparation using QuPPe-AO

The QuPPe-AO method was used with the analytical portions of 2 g infant formula powder and 10 g milk or ready-to-use formulae. Water was added to the tube containing the analytical portion to reach ~10 g in total, after the addition of EDTA and IS-solution; see Table 6.

An appropriate small volume (e.g. 100 µl) of the internal standard working solution (IS-WSln), containing isotopically labelled analogues of the analytes was added, followed by 10 mL acidified methanol (containing 1 % formic acid), an additional 100 µL of formic acid, and 1 mL of 10 % aqueous EDTA solution. The extraction involved 15 min shaking by a mechanical shaker. Tubes containing the raw extract were placed in a freezer (at ca. −80 °C for 30 min) and centrifuged at −5 °C for 5 min at high centrifugation speed (> 10,000 g). To remove fat and proteins, a 2 mL aliquot of the supernatant was transferred into a 10 mL centrifuge tube with screw cap, which already contained 2 mL of acetonitrile and 100 mg of C_{18}-sorbent. The tube was shaken vigorously by hand and centrifuged for 5 min at > 3,000 g. A 3 mL aliquot of the supernatant was transferred into an ultrafiltration unit (10 kDa) and centrifuged at 4,000 g for about 10 min. The filtrate was used for analysis. The general analytical procedure is shown at a glance in Figure 2.

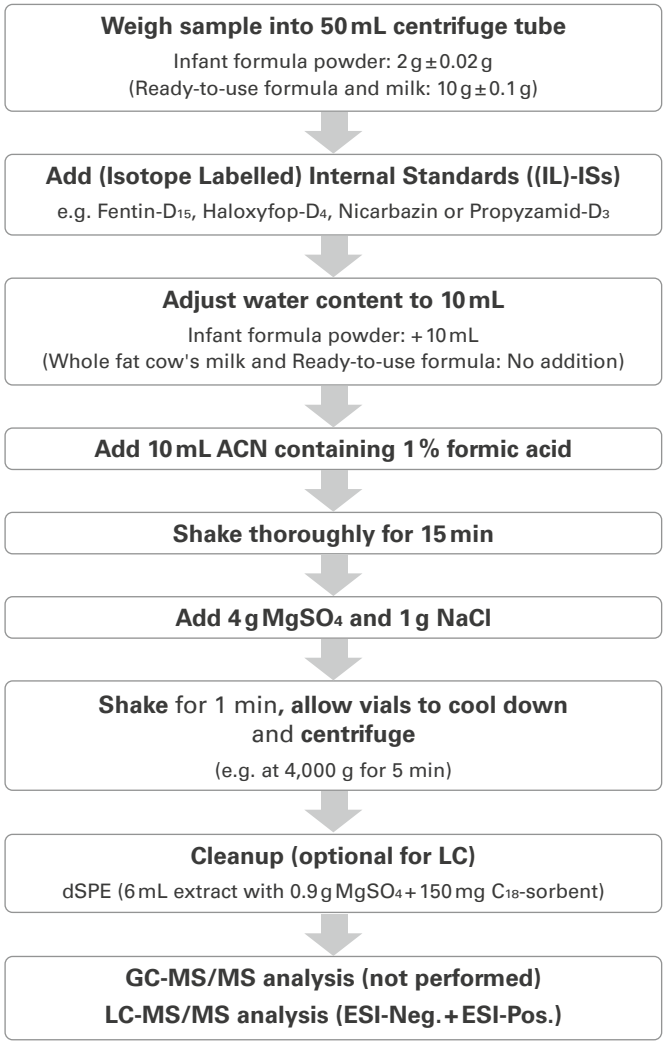

Figure 1. Workflow of A-QuEChERS

3.4. Instrumental Analysis

For the analysis of the 13 toxicologically critical SRM and MRM/SRM analytes in infant formulae, seven different LC-MS/MS methods were employed. Five of these methods involved measurement in the ESI-positive mode and for the remaining two, measurement in the ESI-negative mode. Table 7 (page 8) gives an overview of the LC-MS/MS methods used for the analysis of the targeted analytes. Detailed conditions are shown in an analytical observation report published by the EURL-SRM [9].

3.5. Method Validation

Method validation was performed on two spiking levels (low and high) for each analyte. The low level was lower than the MRL theoretically required, according to EFSA, to ensure toxicological safety for babies consuming infant formula.

Matrix-matched calibration solutions were prepared using blank extracts, at 60 % and 120 % of the spiked concentration. In the case of QuPPe analytes and fentin, the results were evaluated using the isotopically labelled analogues of the substances as internal standards. In the case of analytes covered by QuEChERS variants, chlorpyrifos D_3 (only for γ-cyhalothrin) and propyzamide D_3 (all other compounds) were used as internal standards. Detailed results of method validation and analytical performance data as well as exemplary chromatograms are shown in the EURL-SRM analytical observation report [9]. Initially, validation experiments focused on 'normal' infant formulae. In the course of sample analysis, additional validation studies were conducted to make sure that the performance criteria were also fulfilled for other types of infant formulae (i.e. for types B to F). The respective low-level was spiked and the samples were extracted in quintuplicate (n = 5). For quality control purposes, duplicate recovery experiments at the respective low-level were run with every batch of samples analysed. Samples of type A were used for this purpose. When analyzing ready-to-use infant formulae

Table 6. Adjustment of water content for infant formula

Commodity	Sample weight	Typical natural water content in g/100 g	Water to be added	Vol. of 10 % EDTA sln	Water addition may be skipped [a)]	IS-WSln added e.g.	Extra formic acid	Extraction Solvent
Infant formula powder	2 g	–	9 mL	1 mL	No	100 µL	100 µL	10 mL MeOH containing 1 % formic acid
Infant formula ready-to-use (liquid product)	10 g	85 – 87	–	1 mL	Yes	100 µL	100 µL	
Whole fat cow's milk	10 g	85	0.5 mL	1 mL	Yes	100 µL	100 µL	

[a)] The IL-IS will typically correct for volume deviations. In case no IL-IS is used, volume adjustments become more important.

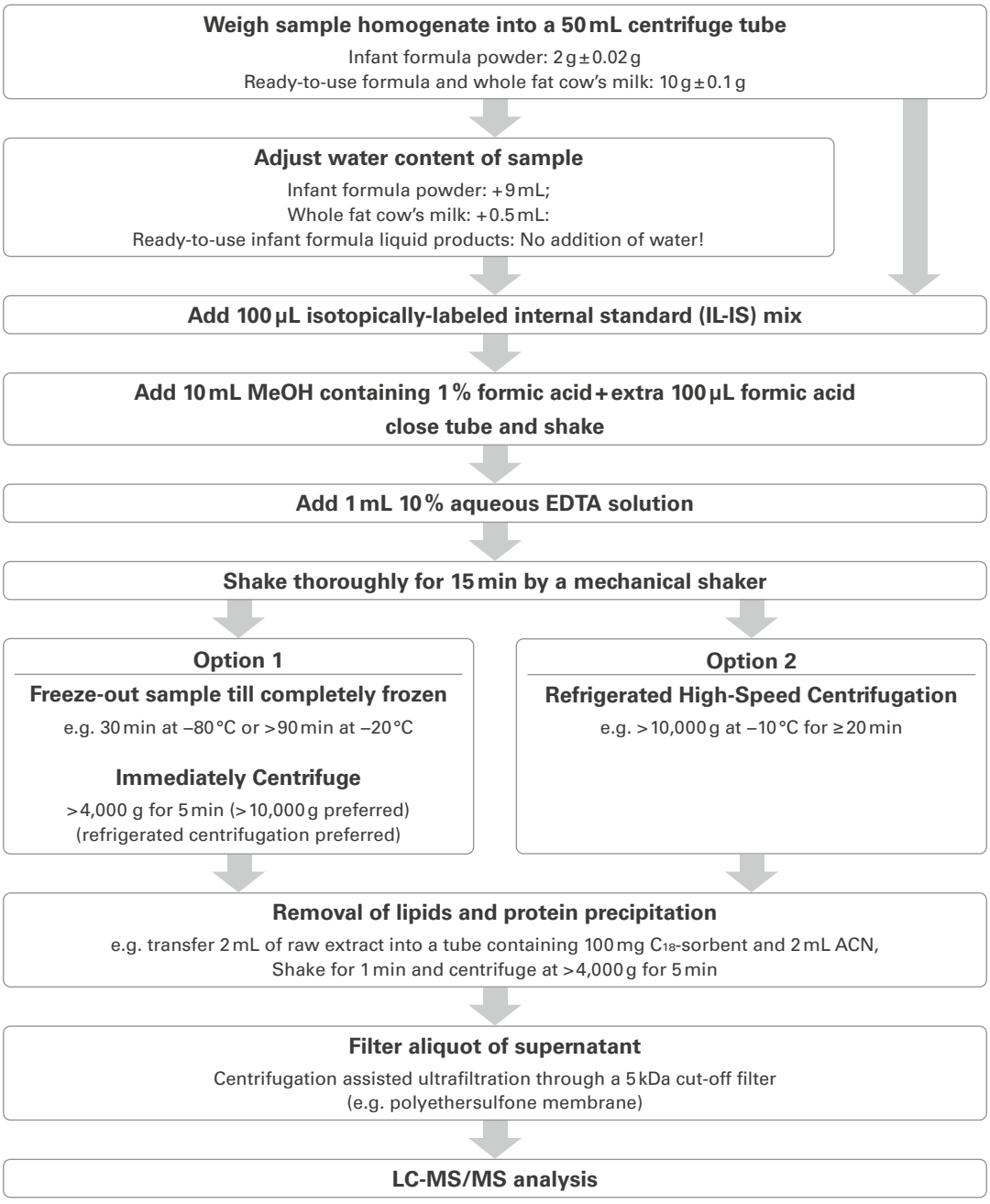

Figure 2. Workflow of QuPPe-AO for infant formula

Table 7. Overview of all analytes within the scope and the LC-MS/MS methods used. Methods 1 – 7 for the 13 toxicallogically critical compounds, and methods 8 – 11 for the additionally analysed compounds.

Instrumental Method			
Analyte	Internal Standard	Analytical Column	MS mode
Method 1			
Avermectin B1a	Propyzamide D_3	Acquity UPLC BEH C_{18}	MS/MS ESI(+)
Emamectin B1a			
Ethoxyquin dimer			
3-Hydroxy-carbofuran			
Method 2			
γ-Cyhalothrin	Chlorpyrifos D_{10}	ChiralArt Cellulose-SB	MS/MS ESI(+)
Method 3			
Fentin	With CEN QuEChERS the use of Fentin D_{15} as IL-IS helps to correct for recovery. With A-QuEChERS, where recovery rates are high, propyzamide D_3 is also a suitable IS.	Zorbax 3,5 µm; Eclipse XDB-C_{18}	MS/MS ESI(+)
Method 4			
Diclofop (free acid)	Propyzamide D_3	Acquity UPLC BEH C_{18}	MS/MS ESI(–)
Haloxyfop			
Method 5 (QuPPe-PO (M 4.2): 'Quats & Co.' on BEH Amide [8])			
Amitrole	Amitrole $^{15}N_2$ $^{13}C_2$	BEH Amide	MS/MS ESI(+)
Cotinine	Cotinine D_3		
Nicotine	Nicotine D_4		
Melamine	Melamine $^{15}N_3$		
PTU	PTU D_6		
Method 6 (QuPPe-PO (M 4.1): 'Quats & Co.' on Obelisc R [8])			
Diquat	Diquat D_8	Obelisc R	MS/MS ESI(+)
Paraquat	Paraquat D_8		
Method 7 (QuPPe-PO (M 1.6): 'Glyphosate & Co.' on Torus DEA [8])			
Topramezone	MPPA D_3/Propyzamide D_3	Waters Torus™DEA	MS/MS ESI(–)
Method 8 (QuPPe-PO (M 11): Anionic Pesticides by Ion Chromatography [8])			
Trifluoroacetic acid (TFA)	Trifluoroacetic acid $^{13}C_2$	AS19	IC-MS/MS ESI(–)
Method 9 (QuPPe-PO (M 1.4): 'PerChloPhos' [8])			
Chlorate	Chlorate $^{18}O_3$	Hypercarb	MS/MS ESI(+)
Perchlorate	Perchlorate $^{18}O_4$		
Phosphonic acid	Phosphonic acid $^{18}O_3$		
Thiocyanate	Thiocyanate ^{13}C ^{15}N		
Method 10 (QuPPe-PO (M 10): 'Triazole derivative metabolites (TDMs)' on Torus DEA [8])			
Triazole acetic acid	1,2,4-Triazole acetic acid $^{13}C_2$ ^{15}N also D_2	Hypercarb	MS/MS ESI(+)
Triazole lactic acid	1,2,4-Triazole lactic acid $^{13}C_2$ ^{15}N also D_2		
Triazole alanine	1,2,4-Triazole-1yl-alanine $^{13}C_2$ ^{15}N also D_2		
Method 11 (QuPPe-PO (M 1.3): 'Glyphosate & Co.' on Hypercarb [8])			
Cyanuric acid	Cyanuric acid $^{13}C_3$	Hypercarb	MS/MS ESI(–)

Table 8. Overview of ADI values of toxicologically critical SRM analytes, required LOQs, spiking level for powder and corresponding spiking level in reconstituted product (ready-to-use).

Analyte	ADI (mg/kg bw per day)	Required LOQ (mg/kg)	Low spiking level			High spiking level		
			on powder (mg/kg)	calculated on reconst. product [a] (mg/kg)	Pct. of required LOQ	on powder (mg/kg)	calculated on reconst. product [a] (mg/kg)	Pct. of required LOQ
Abamectin	0.0025	0.0096	0.05	0.0067	69 %	0.25	0.0333	347 %
Amitrole	0.001	0.0038	0.02	0.0027	69 %	0.1	0.0133	347 %
Cotinine	0.0008	0.0031	0.005	0.00067	22 %	0.025	0.0033	108 %
γ-Cyhalothrin [b]	0.0012	0.0048	0.032	0.0043	92 %	–	–	–
Diclofop (free acid)	0.001	0.0038	0.025	0.0033	87 %	0.125	0.0167	433 %
Diquat	0.002	0.0076	0.05	0.0067	87 %	0.25	0.0333	433 %
Emamectin	0.005	0.0019	0.01	0.0013	70 %	0.05	0.0067	350 %
Fentin	0.0004	0.0015	0.01	0.0013	87 %	0.05	0.0067	433 %
Haloxyfop	0.00065	0.0025	0.015	0.0020	80 %	0.075	0.0100	400 %
3-Hydroxy-carbofuran	0.00015	0.00058	0.004	0.00053	92 %	0.02	0.0027	462 %
Nicotine	0.0008	0.0031	0.02	0.0027	87 %	0.1	0.0133	433 %
PTU	0.0003	0.0012	0.005	0.00067	58 %	0.025	0.0033	289 %

[a] Calculated based on the conservative conversion factor of 7.5
[b] Validations were conducted by spiking λ-cyhalothrin at a level ensuring that both γ- and λ-cyhalothrin were below the safe MRL.
[c] In the absence of topramezone ILIS, MPPA D_3 was tested as it showed negligible matrix effects that were, moreover, largely compensated by matrix-matched calibrations. It thus merely served to correct for volume deviations.

(belonging to types A and C), the accompanying duplicate recovery experiment was conducted with a suitable sample of type C.

In the case of infant formulae, the lowest spiking levels for validation experiments were chosen to remain below the calculated maximum safe MRL, which depends on the ADI of each compound. Table 8 gives an overview of the low and high spiking levels and their relationship to the highest acceptable MRL.

In the case of milk, the lowest spiking level in validation experiments was 0.002 mg/kg for all QuEChERS amenable compounds and 0.01 mg/kg for QuPPe amenable compounds. Also here, matrix-matched calibration solutions were employed at 60 % and 120 % of the spiked concentration.

It was shown that A-QuEChERS is suitable for all QuEChERS-amenable compounds (avermectin B1a, emamectin B1a, 3-hydroxy-carbofuran, ethoxyquin dimer, γ-cyhalothrin, fentin, haloxyfop, diclofop (free acid) and topramezone), whereas the citrate-buffered QuEChERS was not suitable for the analysis of fentin. It was therefore decided to conduct sample analysis and additional method validation only using A-QuEChERS.

After observing that the analysis of milk samples did not show any positive findings of triazole derivative metabolites, it was decided not to continue the analysis with infant formulae. Validations in infant formulae were also skipped.

More details on individual analytical aspects are given in the analytical observation report on the present project published by the EURL-SRM [9].

3.6. Analysis of Samples used in the Pilot Monitoring

For the analysis of the infant formulae and milk samples, matrix-matched calibrations at 60 % and 120 % of the respective validated low-level were prepared using extracts of heat-treated and raw milk. Separate matrix-matched calibration standards were prepared for ready-to-use products.

For the additionally analysed compounds (some of which are ubiquitous contaminants, e.g.: chlorate, perchlorate, phosphonic acid and TFA), calibration standards were prepared at 100 %; 200 % and 400 % of the lowest validated level, as lower levels would be too affected by background levels of these ubiquitous compounds.

4. Results

Overall, 80 samples of infant formulae and 54 samples of milk were analysed. Of the 13 targeted compounds with high toxicity, none were detected in the analysed samples except for nicotine. Several additional compounds deemed relevant to milk products were also included in the scope, irrespective of their toxicological profile. Among these compounds, there were several positive findings. On the other hand, chlorate, perchlorate, phosphonic acid and thiocyanate were detected in infant formulae at levels exceeding the LOQ, with chlorate additionally being detected above the LOQ in milk.

4.1. Residue findings in infant formula samples

4.1.1. Polar compounds analysed by QuPPe-AO

Table 9 shows the results for the targeted compounds in infant formulae, broken down by the different types of products, and divided into organic and conventional. Abbreviations used for the sample types are explained in Table 4

Illustrated is the number of analysed samples, the number of positive samples, the share of results below LOQ and the median of the positive results in mg/kg. Where only two numerical results exist, the mean value is given. Also given in the table is the maximum MRL that would still be considered safe for children up to 16 weeks of age. All results refer to the reconstituted products with a conservative factor of 7.5 (instead of the mean factor of 8) being applied.

Nicotine was detected and quantified at trace levels in 48 of the 80 analysed samples of infant formulae. However, all detected values were below the LOQ of 0.0027 mg/kg reconstituted product (i.e. 0.02 mg/kg powder), and thus at levels that are still considered safe. The median concentration of all (semi-quantitatively) determined levels was at 0.0004 mg/kg (reconst. product). The highest detected level was ~ 0.0009 mg/kg (reconst. product) in a normal infant formula sample.

TFA was detected at trace levels below the LOQ of 0.0067 mg/kg (reconst. product) in 59 of the 80 analysed infant formula samples. The median concentration of all (semi-quantitatively) determined levels was 0.0008 mg/kg (reconst. product). The highest detected level was ~ 0.005 mg/kg (reconst. product) in a hypoallergenic infant formula sample.

Chlorate was detected and quantified in all infant formulae samples with levels exceeding the LOQ of 0.0027 mg/kg in more than 90 % of the cases. The median concentration of all quantified levels > LOQ was 0.0036 mg/kg (reconst. product). The highest detected level was 0.041 mg/kg (reconst. product) in a normal infant formula sample.

Perchlorate was detected and quantified in 53 of 80 samples of infant formulae. In 25 of these samples, perchlorate levels exceeded the LOQ of 0.0013 mg/kg, with a median concentration of all quantified levels > LOQ being 0.0013 mg/kg (reconst. product). The highest detected level was 0.014 mg/kg (reconst. product) in a hypoallergenic infant formula sample.

Phosphonic acid was detected and quantified in all samples of infant formulae with levels exceeding the LOQ of 0.0067 mg/kg in 12 cases. The median concentration of all quantified levels > LOQ was 0.0036 mg/kg (reconst. product). The highest detected level was 0.048 mg/kg (reconst. product) in a hypoallergenic infant formula sample.

Thiocyanate was detected and quantified in 79 of the 80 infant formulae with levels exceeding the LOQ of 0.067 mg/kg in 66 cases. The median concentration of all quantified levels > LOQ was 0.36 mg/kg (reconst. product). The highest detected level was 1.87 mg/kg (reconst. product) in a normal infant formula sample.

Although the number of ready-to-eat products was low, not allowing proper statistical evaluation, there seems to be a trend for higher levels of perchlorate (type C) and phosphonic acid (type A and C), compared to reconstituted powders, see also supplementary material.

In the case of thiocyanate there seems to be a trend for higher levels in type A und type D products, which may be related to the higher proportions of whey in these products. On the other hand, type B products (Lactose-free) seem to have lower levels, which can be explained by the non-use of whey in their production.

The 14 organic products analysed did not show any specific trend as regards the levels of the quantified compounds.

For the compounds most frequently encountered in infant formulae (chlorate, nicotine, perchlorate, phosphonic acid, TFA and thiocyanate), the 50th percentile (median) concentration of all samples and of the samples with quantified levels as well as the 95th percentile concentration of all samples was calculated. These figures are shown in Table 10 (page 12). The values shown refer to the reconstituted products. The 95th percentile was calculated using the excel formula = QUANTIL.EXCL(), representing a more conservative approach from the risk assessment point of view (worst case).. Thiocyanate, which is taken up through feed and which is also naturally formed in the body, showed ca. 100-fold higher levels compared to the other compounds (median concentration of all samples 0.36 mg/kg based on the reconstituted products). Among the other targeted compounds, chlorate was found to show the highest overall median concentration (0.0062 mg/kg).

4.1.2. Non-polar (A-QuEChERS amenable) compounds

None of the following A-QuEChERS-amenable highly toxic compounds was detected in any of the samples of infant food formula: 3-hydroxy-carbofuran, abamectin, γ-cyhalo-

Table 9. Overview of results in infant formulae samples of the 6 types with distinction between conventional (conv.) and organic (org.) products.

Toxicol. critical compounds	LOQ [a] in mg/kg of reconst. product	Max. safe MRL in mg/kg of reconst. product	Normal (n = 41)		L-free (n = 8)	HA (n = 18)	AR (n = 8)		Comf. (n = 3)	Dairy-free (n = 2)		Total (n = 80)	
			Conv. (n = 30)	Org. (n = 11)	Conv. (n = 8)	Conv. (n = 18)	Conv. (n = 6)	Org. (n = 2)	Conv. (n = 3)	Conv. (n = 1)	Org. (n = 1)	Conv. (n = 66)	Org. (n = 14)
			No. of Positive Samples (Median of determined levels or respective sub-population in mg/kg reconstituted product) [b]										
Amitrole	0.0027	0.0038	0	0	0	0	0	0	0	0	0	0	0
Cotinine	0.00067	0.0031	0	0	0	0	0	0	0	0	0	0	0
Diquat	0.0067	0.0076	0	0	0	0	0	0	0	0	0	0	0
Nicotine	0.0027	0.0031	30 [c] (0.0005)	11 [c] (0.0004)	0	3 [c] (0.0002)	3 [c] (0.0002)	1 [c] (0.0003)	0	0	0	36 (0.0004)	12 (0.0003)
PTU	0.00067	0.0012	0	0	0	0	0	0	0	0	0	0	0

Add. analysed compounds	LOQ [a] in mg/kg of reconst. product	Type of result	Normal (n = 41)		L-free (n = 8)	HA (n = 18)	AR (n = 8)		Comf. (n = 3)	Dairy-free (n = 2)		Total (n = 80)	
			Conv. (n = 30)	Org. (n = 11)	Conv. (n = 8)	Conv. (n = 18)	Conv. (n = 6)	Org. (n = 2)	Conv. (n = 3)	Conv. (n = 1)	Org. (n = 1)	Conv. (n = 66)	Org. (n = 14)
			No. of Positive Samples (Median of determined levels or respective sub-population in mg/kg reconstituted product) [b]										
Chlorate	0.0027	Positives	30 (0.0092)	11 (0.0049)	8 (0.0025)	18 (0.0042)	6 (0.0074)	2 (0.0050)	3 (0.0057)	1 (0.014)	1 (0.0046)	66 (0.0066)	14 (0.0049)
		Pos. ≥ LOQ	30 (0.0092)	11 (0.0049)	3 (0.0047)	14 (0.0045)	6 (0.0074)	2 (0.0050)	3 (0.0057)	1 (0.014)	1 (0.0046)	57 (0.0075)	14 (0.0049)
Melamine	0.0027	Positives	7 (0.0006)	0	0	0	0	0	0	0	0	7 (0.0006)	0
		Pos. ≥ LOQ	0	0	0	0	0	0	0	0	0	0	0
Paraquat	0.0067	Positives	0	0	0	0	0	0	0	0	0	0	0
		Pos. ≥ LOQ	0	0	0	0	0	0	0	0	0	0	0
Perchlorate	0.0013	Positives	22 (0.0006)	10 (0.0015)	8 (0.0017)	4 (0.0043)	6 (0.0019)	2 (0.0022)	0	0	1 (0.0058)	40 (0,0009)	13 (0.0019)
		Pos. ≥ LOQ	2 (0.0019)	7 (0.0019)	7 (0.0017)	2 (0.011)	4 (0.0030)	2 (0.0022)	0	0	1 (0.0058)	14 (0.0019)	10 (0.0022)
Phosphonic acid	0.0067	Positives	30 (0.0029)	11 (0.0026)	8 (0.0062)	18 (0.0041)	6 (0.003)	2 (0.003)	3 (0.0061)	1 (0.0085)	1 (0.016)	66 (0.0037)	14 (0.0027)
		Pos. ≥ LOQ	2 (0.015)	1 (0.013)	3 (0.0085)	3 (0.0072)	0	0	1 (0.0069)	1 (0.0085)	1 (0.016)	12 (0.0078)	2 (0.0144)
TFA (Trifluoro-acetic acid)	0.0067	Positives	28 (0.0007)	10 (0.001)	4 (0.0004)	8 (0.0009)	6 (0.0007)	2 (0.001)	0	1 (0.003)	0	47 (0.0007)	12 (0.0011)
		Pos. ≥ LOQ	28 (0.0007)	10 (0.001)	4 (0.0004)	8 (0.0009)	6 (0.0007)	2 (0.001)	0	1 (0.003)	0	47 (0.0007)	12 (0.0011)
Thio-cyanate	0.067	Positives	30 (0.55)	11 (0.45)	8 (0.088)	18 (0.067)	6 (0.60)	2 (0.38)	3 (0.25)	0	1 (0.02)	65 (0.3542)	14 (0.4062)
		Pos. ≥ LOQ	30 (0.55)	10 (0.46)	6 (0.092)	11 (0.086)	5 (0.63)	2 (0.38)	2 (0.36)	0	0	55 (0.4215)	12 (0.4291)

[a] LOQ = LSVL (Lowest Successfully Validated Level) – Validation at lower levels was not attempted
[b] Including levels <LOQ that are to be considered semi-quantitative. In case of n = 2, the mean value was calculated; in case of only one result this result is shown.
[c] all findings < LOQ

Table 10. Calculated 50th and 95th percentiles for the most frequently detected compounds in infant formula

Compounds	Positive Results	Whole Population	
	Median	Median	95th Percentile
Chlorate	0.0062	0.0062	0.0246
Nicotine	0.0004	0.0002	0.0009
Perchlorate	0.0013	0.0006	0.0047
Phosphonic acid	0.0036	0.0036	0.0152
TFA	0.0008	0.0006	0.0021
Thiocyanate	0.3647	0.3644	0.8014

thrin, diclofop, emamectin, fentin, haloxyfop, topramezone. Ethoxyquin dimer, which was additionally tested, as many infant formulae contained fish oil as an ingredient, was also not detected in any of the samples of infant food formula.

4.2. Residue Findings in Milk Samples

4.2.1. Polar compounds analysed by QuPPe-AO

Table 11 shows the results for the analysed compounds in heat-treated and raw milk. Illustrated is the number of positive samples and their medians in mg/kg. In case of only two positive samples, the mean is indicated.

Table 11. Overview of results in milk samples with distinction between conventional and organic products.

Compound	LOQ [a] [mg/kg]	Heat Treated Milk		Raw Milk	
		Conventional (n = 42)	Organic (n = 2)	Conventional (n = 9)	Organic (n = 1)
		No. of Positive Samples (M: Median of determined levels in mg/kg) [b]			
Amitrole	0.01	0	0	0	0
Chlorate	0.01	32 [14 ≥ LOQ; 18 < LOQ] (M 0.005)	2 [both < LOQ] (M 0.004)	9 [all < LOQ] (M 0.001)	1 [< LOQ] (0.001)
Cotinine	0.01	0	0	0	0
Cyanuric acid	0.005	3 [all > LOQ] (M 0.0081)	0	0	0
Diquat	0.01	0	0	0	0
Melamine	0.005	4 [all < LOQ] (M 0.0013)	0	2 [< LOQ] (M 0.0031)	0
Nicotine	0.01	36 [all < LOQ] (M 0.001)	2 [both < LOQ] (M 0.0008)	6 [all < LOQ] (M 0.001)	1 [< LOQ] (0.0009)
Paraquat	0.01	0	0	0	0
Perchlorate	0.01	23 [all < LOQ] (M 0.001)	1 [< LOQ] (0.0004)	4 [all < LOQ] (M 0.001)	1 [< LOQ] (0.003)
Phosphonic acid	0.05	39 [all < LOQ] (M 0.007)	2 [all < LOQ] (M 0.006)	0	0
PTU	0.01	0	0	0	0
Triazole acetic acid (TAA)	0.05	0	0	0	0
Triazole alanine (TA)	0.05	0	0	0	0
Triazole lactic acid (TLA)	0.05	0	0	0	0
Trifluoroacetic acid (TFA)	0.01	42 [all < LOQ] (M 0.005)	2 [both < LOQ] (M 0.004)	9 [all < LOQ] (M 0.006)	1 [< LOQ] (0.006)

[a] LOQ = LSVL (Lowest Successfully Validated Level) – Validation at lower levels was not attempted
[b] including levels < LOQ that are to be considered semi-quantitative

Nicotine was detected at trace levels in 45 of the 54 analysed samples of milk. However, all detected values were below the LOQ of 0.01 mg/kg milk, and thus at levels that are still considered safe.

TFA was detected at trace levels in all milk samples. Also, melamine was detected at trace levels in 6 of the 54 analysed milk samples.

Chlorate was positive in 44 of the 54 analysed milk samples and exceeded the LOQ of 0.01 mg/kg in 12 of these samples, with a median of 0.029 mg/kg.

Perchlorate was also positive in 29 of the 54 analysed milk samples, but only at trace levels, below the LOQ.

Phosphonic acid was positive in 41 of the 54 samples, but in all cases only at trace levels, below the LOQ.

4.2.2. Non-polar compounds analysed by A-QuEChERS

As in infant food formula, also in milk, none of the following A-QuEChERS amenable compounds was detected: 3-hydroxy-carbofuran, abamectin, γ-cyhalothrin, diclofop, emamectin, ethoxyquin dimer, fentin, haloxyfop, and topramezone.

5. Conclusion

With the aim of checking whether highly toxic compounds can be effectively monitored in infant formulae for children up to 16 weeks of age, a pilot program was conducted to monitor infant formulae from the market. Analytical methods were hereby developed and applied to various samples of infant formulae and milk. The focus was on the analysis of compounds not amenable to multi-residue methods that show a high toxicity (low ADI-values), and for which the default MRL for infant formulae of 0.01 mg/kg is considered insufficiently safe for children of the above-mentioned age.

Of the 13 compounds with high toxicity that were tested, none could be detected in the analysed samples except for nicotine, which was found at trace levels in 48 of the 80 samples of infant formulae and in 45 of the 54 samples of milk. However, all encountered levels were below the toxicologically critical threshold (and the LOQ), and are thus considered safe. On the other hand, residues of the additionally analysed compounds, mainly contaminants, were frequently detected in infant formulae and milk. Most positive findings concerned chlorate, which was found at quantifiable levels in 90 % of samples of infant formulae at levels above the LOQ. Moreover, perchlorate, phosphonic acid and thiocyanate were each tested positive in at least 50 % of the analysed samples. Overall, however, the results of the pilot study did not reveal serious toxicological risks for infants up to the age of 16 weeks arising from the consumption of infant formulae (concerning the compounds included in this study and based on the actual toxicological knowledge). In the laboratory, purified water was added to the powders for analysis. In households, tap or typically bottled water is used to reconstitute infant formula powders. Depending on the contamination of the water used by the consumers, which can vary considerably, the exposition of infants to certain ubiquitous compounds (such as chlorate, perchlorate and TFA) through the consumption of infant formulae is expected to be higher than the exposition suggested by the results of the pilot monitoring. It should be further noted, that infants are additionally exposed to considerable amounts of nicotine, when smokers live in the same household.

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Supplementary material

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