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Molecularly Imprinted Dispersive-Based Extraction Method for Melamine in Milk (MIDE-HPLC-DAD)

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ABSTRACT

An easy and selective molecularly imprinted dispersive extraction method (MIDE) coupled with HPLC-DAD was developed for melamine´s analysis in commercial milk samples. The molecularly imprinted polymer (MIP) was synthetized by suspension polymerisation with melamine as template, while methacrylic acid, ethylene glycol dimethacrylate and chloroform were used as monomer, cross-linker and porogenic solvent, respectively. The analytical methodology was validated presenting a limit of detection of 0.6 mg/kg. The recovery percentage in spiked milk samples ranged from 92.9% to 102.0% with an intermediate precision of 5.0%. The analysis of real samples with MIDE revealed melamine´s presence in 20% of the cases with a maximum concentration of 9.3 ± 0.3 mg/kg, which exceeds the maximum recommended level of 2.5 mg/kg.

Indexing terms/Keywords

Melamine; molecularly imprinted dispersive extraction; molecularly imprinted polymer.

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1. INTRODUCTION

Melamine is an organic compound with high nitrogen content that is used in plastic industry and in the production of flame retardants. It has also been fraudulently used as food additive to artificially increase the measured protein content. Such incidents occurred in America in 2007 and in China 2008 after the contamination of pet food and milk products with melamine [1]. This fraudulent addition was responsible for the death of animals and children, because while in the organism, melamine can react with cyanuric acid producing insoluble compounds that tend to accumulate in kidneys causing renal failure and ultimately death [2].

In April of 2010, European Union set a tolerable daily intake (TDI) of 0.2 mg per kg of body mass. According to the Codex Alimentarius Commission the legal limit of melamine in food products cannot exceed 2.5 mg/kg for milk products and 1 mg/kg for infant formulas [3]. The concern about human exposure led to the development of methods capable of detecting and quantifying the presence of melamine in food samples [4].

Analytical approaches such as capillary electrophoresis [5], high performance liquid chromatography (HPLC) [6], gas and liquid chromatography with mass spectrometry have been developed for the determination of melamine in food samples [2, 7]. Due to the complexity of matrices as food samples, selective extraction and/or sample clean-up procedures are often necessary before chromatographic analysis. Selected examples of analytical procedures are resumed in Table 1. As expressed in this table, different approaches provide low LODs and also good recoveries. However, the key problem in food analysis is the high variability of the sample composition. This may lead to unexpected co-elution and erroneous results. An analytical method designed for one particular sample is not always applicable to other samples. Classic solid phase extraction has been seen as a typical pre-treatment procedure to improve the applicability of analytical methodologies by removing potential interferents. However, classical SPE sorbents often present lack of selectivity[8] which puts in evidence the need of materials with higher selectivity towards the analyte that could be able to increase the range of applicability and also robustness of the analytical method.

Molecular imprinting emerged as a technique to overcome the above mentioned drawbacks of classical SPE techniques. Economic aspects and simplicity of preparation made these materials very attractive, but the most promising property is the high selectivity towards the analyte. MIPs are synthetic polymers produced in the presence of a template, typically the analyte or in other cases an analogue. After polymerisation, template is removed from the polymeric structure producing cavities with specific molecular recognition for the original template[9].

Recently some authors have described the development of imprinted polymers for the extraction of melamine from food samples (Table 1). Melamine [10, 11], cyromazine [12, 13] and 2-(4,6-diamino-1,3,5- triazin-2-ylamino) ethanethiol disulphide (DTY) [14] have been selected as preferential templates to prepare MIPs typically used as selective sorbents in solid phase extraction (MISPE). In 2012, Yan et al [15], prepared an imprinted polymer for a new method called matrix solid-phase dispersion. According to the authors this technique allows to reduce the number of steps required to perform the clean-up and concentrate the analyte eliminating most of the limitations associated to typical SPE procedures. Recently, He et al [16] reported the synthesis of a magnetic molecularly imprinted polymer which was blended and stirred with diluted milk for the extraction of melamine.

Most of these imprinted materials were obtained by bulk polymerisation which is considered the easiest way to prepare MIPs. However this technique also produces heterogeneous particles as a consequence of the crushing and grounding steps applied to the bulk polymer. Another disadvantage is that only 30 to 40% of the initial polymer is suitable for application [9]. Other aspect in the preparation of MIPs selective to melamine is the use of analogous templates like cyromazine or DTY which in addition to being more expensive than melamine, were, in the case of the latter synthesized prior to the imprinting process. Based on the previous aspects a melamine imprinted polymer was developed according to the suspension protocol. The obtained MIP was successfully applied in a dispersive extraction procedure for the analysis of melamine in milk samples.

From the considered reports involving MIPs and melamine it was observed the lack of information on validation parameters like detection limits and precision of the described methods. Considering the articles where values of LOD, precision and accuracy were provided, it was verified that none of the methods was submitted to the estimation of global uncertainty.

In this work a full validation was carried out by the determination of detection limits, precision and accuracy and also the evaluation of the global uncertainty for the proposed method.

2. MATERIAL AND METHODS

2.1. Reagents and Working Solutions

Melamine, methacrylic acid (MAA), ethylene glycol dimethacrylate (EDMA), azobisisobutyronitrile (AIBN), sodium 1-heptanesulfonate and polyvinyl alcohol (PVA) (87-89%) hydrolysed with average molecular weight 130 000) were purchased from Sigma-Aldrich (St. Louis, USA). Acetic acid, chloroform and citric acid were acquired from Merck (Darmstadt, Germany). Acetonitrile and methanol of HPLC grade were from VWR (Lisbon, Portugal). All solutions were prepared with distilled water, previously filtered through 0.45 µm nylon filter membranes Supelco from Sigma-Aldrich (Sintra, Portugal).Milk samples with different nutritional content were purchased in a local market (5 based milk drinks from china and 5 medium fat milks of national provenance).

2.2. Equipment and Operating Conditions

The chromatographic analysis of melamine was performed by a system Merck Hitachi Elite LaChrom (Darmstadt, Germany) equipped with a pump L-2130, an autosampler L-2200, a diode-array detector, and acquisition system with data treatment.The analysis were performed at room temperature, using an injection volume of 100 µL. A C18 reverse-phase Purospher®STAR end capped column (250 mm x 4 mm; 5 µm) combined with a pre-Purospher®STAR column (4 mm x 4 mm; 5 µm) from Merck (Darmstadt, Germany) was used. The mobile phase was acetonitrile (10 %) and 10 mM sodium 1-heptanesulfonate with 10 mM citric acid (90 %) at flow rate of 1 mL/min. All the data was acquired in a wavelength range between 220 and 440 nm although the absorbance of melamine was measured at 240 nm.

2.3. Preparation of Imprinted and Non-Imprinted Polymer

The suspension method was applied for the polymer synthesis. First 1 mmol of melamine (solid) and 15 mmol of MAA were dissolved in 5.2 mL of porogenic agent (chloroform). Then 30 mmol of cross-linker (EDGMA), 40 mg of initiator (AIBN) and 400 mg of surfactant (PVA) were added to the pre-polymer. All reagents were dispersed in 50 mL of distilled water. The reaction took place in a 250 mL volumetric flask equipped with dual manifold, one with a rubber cap and another with a thermometer. The flask was connected to a condenser to avoid evaporation of the reagents.

Before starting the polymerization, solutions were saturated with a gentle flow of nitrogen for 10 minutes in order to remove dissolved oxygen. Bath temperature was set at 60 ^{o}C and the reaction was conducted for a period of 24 h with a constant stirring of 400 rpm.

After polymerisation, the obtained particles were filtered and the template was extracted in a Soxhlet apparatus with a mixture of methanol: acetic acid (9:1, V/V) for two periods of 24 h. The remaining acetic acid was washed from the polymer overnight with methanol. Non-imprinted polymer (NIP) was prepared in the same way as MIPs, without the addition of the template.

2.4. MIDE and Sample Preparation

Milk samples (2.5 mL) were added to a 15 mL centrifuge tube with subsequent addition of 7.5 mL of acetonitrile. Sample and solvent were mixed to promote proteins precipitation and phases were separated by centrifugation at 4000 rpm for 10 min. Supernatants were transferred to another tube containing 200 mg of MIP (or NIP) particles. Sample and particles were agitated for 10 min and then centrifuged. The supernatant was discarded and 6 mL of methanol:acetic acid (9:1, v/v) was added to elute the analyte from the particles. Solvent and MIP were mixed for 10 min with subsequent centrifugation. 500 µL of the extract were collected and evaporated under a gentle flow of nitrogen. After being reconstituted in 250 µL of distilled water the extract was analysed by HPLC-DAD.

2.5. Method Validation

The proposed MIDE-HPLC-DAD method was validated with respect to linearity by using 7 standard spiked solutions prepared from a stock melamine aqueous solution (500 mg/L) in a concentration range of (0.5 ; 20) mg/L which is equivalent to 0.6 and 23.3 mg/kg. Limits of detection (LOD) and quantification (LOQ) were estimated at signal-to-noise ratio of 3:1 and 10:1, respectively.

Precision and accuracy parameters were evaluated in triplicate at 2.3, 11.6 and 23.3 mg/kg, and applied for the determination of the method global uncertainty.

Quantification of spiked samples was performed using HPLC-DAD.

3. RESULTS AND DISCUSSION

The complexity exhibited by milk matrix is likely to affect the signal of the analytical response. Although selective sorbents like molecularly imprinted polymers could supress some of the interference problems, a matrix effect study is advisable. In order to evaluate the level of interference, three standards of spiked milk were prepared with final melamine concentration of 2.0, 10 and 20 mg/L equivalent to 2.3, 11.6 and 23.3 mg/kg. These samples were treated with acetonitrile, before extraction with MIDE. Aliquots of 500 µL were taken then evaporated and reconstituted in distilled water (250 µL) before HPLC-DAD analysis. Same procedure was applied to melamine aqueous solutions in the same concentrations.

Figure **1** puts in evidence a matrix effect with signal decrease for standards in milk attributed to matrix interferences. The decrease of the analytical response suggests the implementation of a matrix-matched calibration to make-up the matrix effect and lead to more reliable results.

In order to study the performance of the method for other samples, a different type of milk with high calcium content was extracted and compared with the previous results. The results showed no variation in the analytical response after using a different milk sample. Hence, it is proved that the presented external calibration is suitable to different types of milk samples.

Experiments were also carried out to evaluate the existence of template bleeding during the analysis of commercial samples due to the remaining of unextracted melamine in the polymer network. With that purpose non-spiked water and milk, previously analysed to confirm the absence of melamine content, were extracted by MIDE and analysed by HPLC-DAD according the procedure already described. From the analysis of the extracts it was registered a melamine content

of 0.3 mg/kg in both water and milk. In accordance to literature even with extensive washing procedures a small amount of template can remain in the polymer [17]. However, in this study the bleeding levels obtained were lower than the quantification limit of the presented method (2.1 mg/kg). Also, the fact of bleeding levels being equivalent in water and milk allowed circumventing the existence of false positives in future analysis. The bleeding "concentration" was therefore subtracted in all samples.

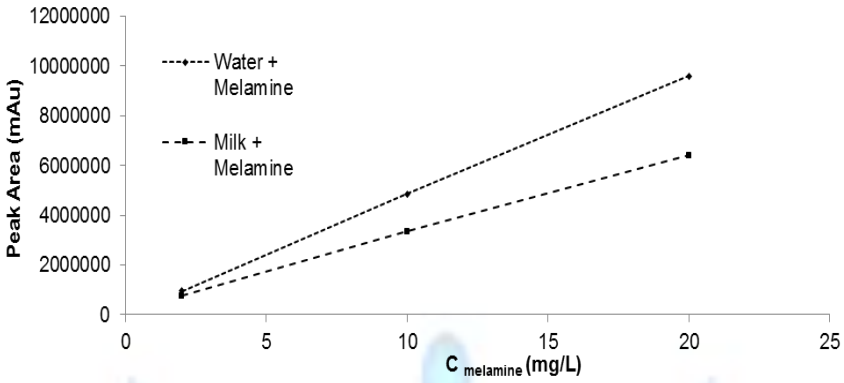

Figure 1. Analytical response for water and milk spiked with melamine.

3.1. Method Validation

A good linearity between the analytical response and the spiking level was observed with a correlation coefficient of 99.8%. Based on the signal to noise ratio it was obtained a limit of detection of 0.6 mg/kg and a limit of quantification of 2.1 mg/kg.

Method precision was evaluated by repeatability and intermediate precision. Repeatability assay was performed with three standard spiked solutions (2.3, 11.6 and 23.3 mg/kg) that were extracted and injected independently 3 times in the same day (intra-day assay). Intermediate precision was determined by the injection of the previous extracts on different days (inter-day assay). The results (Table 2) indicate repeatability values below 4.1% and an intermediate precision of 5% which suggest that the method is precise.

Accuracy of the method was determined by recovery experiments of three standards spiked with melamine at 2.3, 11.6 and 23.3 mg/kg each one analysed in triplicate. Accuracy with respective relative standard deviation values expressed in percentage are shown in Table 2.

Table 2. Validation parameters for melamine analysis by MIDE-HPLC-DAD.

Calibration Range (mg/kg)	0.6-23.3		
R^2	0.9984		
Limit of detection (mg/kg)	0.6		
	2.3 mg/kg	11.6 mg/kg	23.3 mg/kg
Repeatability (%CV)	4.1	3.7	1.3
Intermediate Precision (%CV)	10.4	2.3	2.4
Accuracy (% recovery ± RSD)	92.9 ± 11.7	102.0 ± 10.9	99.5 ± 3.3

Regarding the methods reported in literature for molecularly imprinted polymers applied in the extraction of melamine it was possible to verify that accuracy values ranged from 83 to 102.8% which contain the registered values for the proposed method.

Another important analysis is to compare the obtained recoveries with studies applying similar spiking levels in milk samples. Here it was possible to find an average recovery of 99.8% at 10 mg/L for a cyromazine imprinted polymer prepared by the suspension protocol[12] and 96.2% for a spike of 27 mg/L using a cyromazine imprinted monolith[15]. With a more affordable template the proposed MIDE method was able to produce accurate results.

Only 3 of the 8 considered reports with MIPs and melamine evaluated the precision of the described methods. In these reports authors registered values below 5.3% for repeatability assays and ≤7.2% for intermediate precision. From Table 2 it is confirmed that the average values for repeatability and intermediate precision were according to the ones described in literature.

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3.2. Assessment of global uncertainty

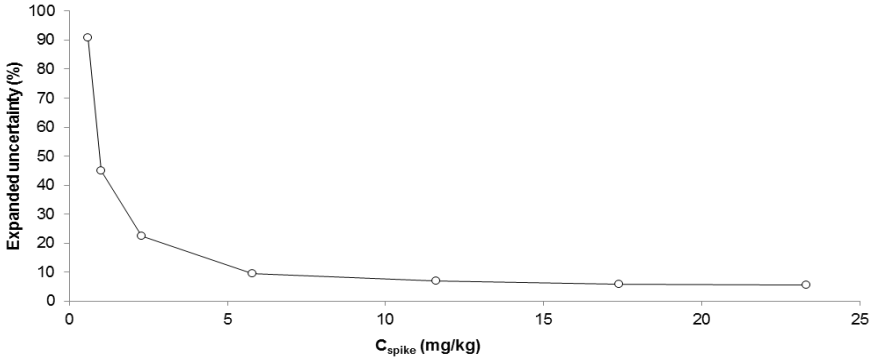
The uncertainty of the proposed method was evaluated by the bottom-up approach described in the EURACHEM/CITAC guide[18]. According to this approach, global uncertainty of an analytical methodology is dependent on individual sources of uncertainty. In this work, four sources of uncertainty were considered, namely U1, uncertainty associated to the standards preparation; U2, uncertainty associated with the calibration graph; U3 uncertainty associated with the precision and U4 uncertainty associated with accuracy. Table 3 shows the calculation procedure adopted for each source as well as the determination of global uncertainty.

Table 3. Uncertainty sources and respective calculation formula for the assessment of global uncertainty. Adapted from [19].

Source of Uncertainty			
Standard preparation (U_1)	$U_1 = u_{st}$	$u_{st} = \sqrt{\sum \left(\frac{\Delta m_i}{m_i}\right)^2}$	Δm_i- error of the measurement of a given parameter (weighing or pipetting); m_i- value measured in each action
Calibration curve (U_2)	$U_2 = s_{xo}/x_o$	$S_{xo}=\frac{S_{y/x}}{a}\sqrt{\frac{1}{m}+\frac{1}{n}+\frac{(y_o-y_{av})^2}{a^2\sum(x_i-x_{av})^2}}$ $S_{y/x}=\sqrt{\frac{\sum(y_i-y_{icalc})}{n-2}}$	s_{xo}- standard deviation of the concentration, obtained from calibration curve; b – slope of the calibration curve; m – number of replicates; n – number of standards used for calibration; y_o – experimental value of y calculated by the calibration curve; y_{av} – average of y_i values; x_i – concentration of standards; x_{av} - average of x_i values; y_i – experimental values; y_{icalc} – values calculated by the calibration curve;
Precision (U_3)	$U_3 = \frac{u_p}{y_{av}}$	$u_p = \frac{s}{\sqrt{n}}$	s – standard deviation of precision assays; n – number of assays
Accuracy (U_4)	$U_4 = u_c$	$u_c = \frac{s(\eta)}{\sqrt{n}}$	$s(\eta)$ – relative standard deviation of the average percentage recovery; n – number of assays
Global uncertainty (U)		$U = \sqrt{U_1^2 + U_2^2 + U_3^2 + U_4^2}$	

As seen in Table 4, U2 is the individual source with higher impact on the final value of global uncertainty reaching 45% for the standard of lower concentration. After this source, uncertainty related to accuracy (U4) and standards preparation (U1) are the ones with most influence on the final value of global uncertainty. Accuracy and precision source values were expressed as an average for 3 spiked standards (0.6, 11.3 and 23.3 mg/kg).

In order to ensure a confidence level of 95 %, expanded uncertainty was determined by applying a conversion factor of 2



to the global uncertainty[18]. From

Figure **2**, when concentrations approach the detection limit of the method (0.6 mg/kg) the values of expanded uncertainty rise exponentially reaching a percentage of 90%. This puts in evidence the need of carefully analyse the results around detection limit owing to high uncertainty associated to this method. Nevertheless for concentrations above the legal limits established by the European Union for melamine in food (2.5 mg/kg)[3] it can be seen that the expanded uncertainty varies from 11.2 to 2.9% for levels of melamine between 2.3 and 23.3 mg/kg.

Table 4. Values of uncertainty sources and global uncertainty for each standard.

C_{spike} (mg/kg)	% U_1	% U_2	% U_3	% U_4	% U_{global}
0.6	1.2	45.3	0.9	2.4	45.4
1.0	1.2	22.3			22.5
2.3	0.4	10.9			11.2
5.8	0.4	4.1			4.9
11.6	1.2	2.1			3.5
17.4	0.4	1.5			3.0
23.3	0.4	1.3			2.9

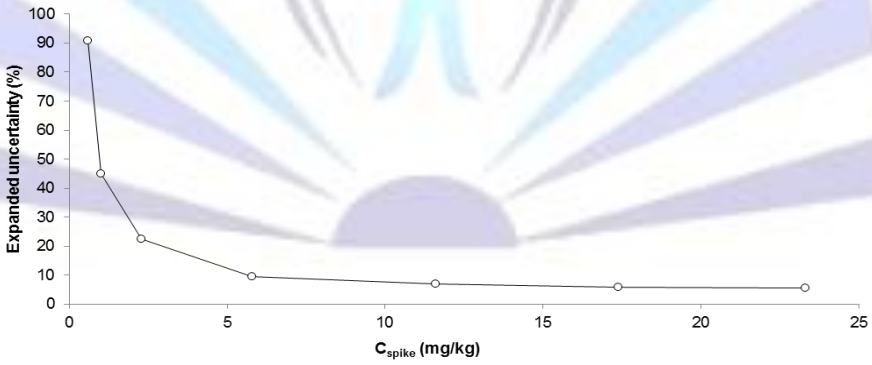


Figure 2. Expanded uncertainty associated to results.

Regarding the previous methods with MIPs and melamine, none of the reported methods was submitted to the estimation of the global uncertainty. From our point of view taking into account safety limits set by the authorities, the assessment of uncertainty associated to results should be performed for each method. Keeping this in mind we are able to say that this work succeed in presenting a fully validated method for the analysis of melamine in milk products.

3.3. MIDE of Milk Samples

The proposed MIDE-HPLC-DAD method was tested in the analysis of commercial milk samples. National and Chinese brands were acquired from local markets and extracted according to the procedure described in the Experimental section. The extracts were analyzed by HPLC-DAD to determine the levels of melamine present in samples.

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Table 5 summarizes the nutritional content of each investigated sample and melamine levels available in each product.

Analyzing the results it can be concluded that tested milk samples origin from Portugal show no evidence of melamine´s presence. However, two of the Chinese products that were analyzed exhibited detectable levels of melamine. The maximum concentration found was 9.3 ± 0.3 mg/kg of melamine which exceeds the legal limits set by European Union for these kinds of products (2.5 mg/kg).

A study about the presence of melamine in food products has already been published by our research team who examined a group of 20 food samples (soy milk powder, milk powder, soybean powder, cookies and biscuits). Samples were extracted by a solvent mixture comprising diethylamide, water and acetonitrile and then derivatized before GC-MS analysis.

This method provided a limit of detection of 0.15 mg/kg for melamine. From the application of the method, melamine was detected in 11 samples (8 of Chinese origin, and 3 of national origin) with a maximum concentration of 3.4 mg/kg.

Table 5. Melamine´s levels detected in the different milk samples.

Sample	Provenance	Sample characterization per 100mL	$C_{melamine}$ (mg/kg)
Milk	National	Proteins: 3.3 g Lipids: 0.2 g Carbohydrates: 4.9 g	ND
		Proteins: 3.3 g Lipids: 1.6 g Carbohydrates: 4.8 g	ND
		Proteins: 3.2 g Lipids: 3.6 g Carbohydrates: 4.8 g	ND
		Proteins: 3.3 g Lipids: 1.6 g Carbohydrates: 4.9 g	ND
		Proteins: 3.6 g Lipids: 2.1 g Carbohydrates: 0.7 g	ND
Milk based products	Chinese	Proteins: 1.0 g Lipids: 1.2 g Carbohydrates: 5.0 g	ND
		Proteins: 0.9 g Lipids: 1.4 g Carbohydrates: 7.9 g	9.3 ± 0.3
		Proteins: 2.3 g Lipids: 2.5 g Carbohydrates: 10.0 g	3.0 ± 0.3
		Proteins: 2.4 g Lipids: 5.5 g Carbohydrates: 11 g	ND
		No reference	ND

4. Conclusions

A melamine imprinted polymer prepared according the suspension technique was used as selective sorbent in a dispersive extraction procedure for the analysis of melamine in milk samples. The purposed MIDE-HPLC-DAD method provides a linear response for concentrations between 0.6 and 23.3 mg/kg with a limit of detection of 0.6 mg/kg. Intermediate precision was below 10.4% and recovery from spiked samples was 98.1 ± 4.8. Applying the bottom-up/Eurachem approach to the presented method an expanded uncertainty between 2.9% and 11.2% was obtained for concentrations between 2.3 and 23.3 mg/kg.

The application of the described MIDE-HPLC-DAD method to real milk samples showed the presence of melamine in some of the tested products with a maximum concentration of 9.7 ± 0.3 mg/kg.

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Table 1. Selected methods for melamine´s analysis in food products.

Analytical Method			Details				Ref.
Extraction	**Clean-up**	**Chromatographic technique**	**Matrix**	**LOD**	**Precision (RSD)**	**Accuracy**	
TCA (1 %); acetate solution (2.2 %); sonication; centrifugation	PCX-SPE	HPLC-DAD	Milk	0.030 mg/L	Repeatability: 1.5 % Intermediate Precision: 5.6 %	85.5-99.3 %	[20]
Perchloric acid (2.5 %); agitation; ACN; agitation; sonication; centrifugation	-	HILIC-UV	Milk powder	0.005 mg/L	-	-	[1]
ACN; sonicated; centrifugation	-	HPLC-UV	Dairy products	0.003 mg/L	-	73.0-96.0 %	[6]
Formic acid (2%); sonication; centrifugation; ACN; centrifugation	-	LC-ESI-MS/MS	Milk products	0.05 mg/L	-	-	[21]
TCA (1%), centrifugation; diluted with H_2O	-	LC-ESI-MS/MS	Milk	0.01 mg/L for liquid	< 8.0 %	70.0-110.0 %	[2]
H_2O; HCl; centrifugation; DCM; centrifugation	SPE: Oasis MCX	UPLC-MS/MS (i.s. $^{13}C_3$-melamine)	Milk products	0.004 mg/kg			[22]
ACN (5% DMSO); sonication; centrifugation; 0.45 □m filter, extracted with n-hexane	-	GC-CI-MS/MS after derivatization with Sylon BFT	Powdered milk	0.0002 mg/kg	< 6.8 %	72.0-93.0 %	[7]
DEA:H_2O:ACN (10:40:50, v/v/v)	-	GC-MS after derivatization by the silylating reagent	Infant formula	0.01 mg/kg	-	-	[23]
DEA:H_2O:ACN (10:40:50, v/v/v); centrifugation	-	GC-MS after derivatization with BSTFA (1% TMCS)	Dairy products	0.15 mg/kg	Repeatability: 3.8 % Inter-day: 6.0 %	70.0-146.0 %	[24]
H_20: $^{15}N_3$-cyanuric acid solution: $^{13}C_3$-melamine solution, TCA: ACN	SPE: CARB/SCX	GC-MS after derivatization with BSTFA (1% TMCS)	Dairy products	0.01 mg/kg	-	80.8-101.5 %	[25]
DEA:H_2O:ACN (10:40:50, v/v/v); sonication; centrifugation	-	GC-MS after derivatization with BSTFA (1% TMCS)	Milk products	0.002 mg/kg	-	61.4-117.2 %	[26]
TCA (5%), centrifugation	SPE: Oasis MCX	GC-MS after derivatization with BSTFA (1% TMCS) UPLC-MS/MS	Egg	0.010 mg/kg 0.005 mg/kg	< 20.0 % < 9.0 %	85.2-93.3 % 80.0-120.0 %	[27]
MeOH; sonication; centrifugation	MISPE	GC-MS after derivatization with BSTFA (1% TMCS)	Milk	0.01 mg/L	Repeatability < 5.34 % Intermediate Precision < 4.45 %	95.0-98.8 %	[12]
ACN; centrifugation	MISPE	HPLC-DAD	Dairy products	0.06 mg/L	-	95.2 %	[10]

TCA (1%), Acetate solution (2%); sonication; centrifugation; pH adjusted to 7.0 with NH_4OH (50%); centrifugation	MISPE	HPLC-UV GC-MS	Animal feed and Milk	-	-	83.4-103 %	[13]
TCA (3%); sonication; centrifugation; pH adjusted to 7.0 with NH_4OH (50%); centrifugation	MISPE	HPLC-DAD	Egg or Milk	-	-	85.6-98.8 %	[14]
Dissolved in H_20; boiled to remove fat; HAc/CH_3COONa (0.1mol/L), centrifugation	MISPE	HPLC-UV	Milk	-	-	89.8-100.6 %	[11]
Sample blended with MIP particles	MI-MSPD	HPLC-UV	Milk	0.05 mg/kg	Repeatability: 3.8 % Intermediate Precision: 4.2 %	86.0-96.2 %	[15]
-	MMIP	LC-MS	Milk	2.6 μg/L	Repeatability: 4.3 % Intermediate Precision: 7.2 %	88.0-95.8%	[16]