Talanta 81 (2010) 894–900

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Development of an analytical methodology to quantify melamine in milk using micellar liquid chromatography and validation according to EU Regulation 2002/654/EC

Maria Rambla-Alegre*, Juan Peris-Vicente, Sergio Marco-Peiró, Beatriz Beltrán-Martinavarro, Josep Esteve-Romero

Grup de Química Bioanalítica, Q.F.A., E.S.T.C.E., Universitat Jaume I, 12071 Castelló, Spain

ARTICLE INFO

Article history:
Received 30 October 2009
Received in revised form 8 January 2010
Accepted 17 January 2010
Available online 25 January 2010

Keywords:
Micellar
HPLC
Melamine
Milk
Validation

ABSTRACT

Melamine is a toxic triazine, illegally used as an additive in milk to apparently increase the amount of protein. A chromatographic procedure using a C18 column and a micellar mobile phase of sodium dodecyl sulphate (0.05 M) and propanol (7.5%), buffered at pH 3, and a detection set by absorbance at 210 nm, was reported for the resolution and quantification of melamine in liquid and powdered milk samples. In this work, samples were diluted with a SDS solution and were directly injected, thus avoiding long extraction and experimental procedures. Melamine was eluted in nearly 9.3 min without overlapping the protein band or other endogeneous compounds. The optimal mobile phase composition was taken using a chemometrical approach that considers the retention factor, efficiency and peak shape. Validation was performed following the European Commission's indications (European Decision 2002/657/EC), and the main analytical parameters studied were: linearity (0.02–100 ppm; $r^2 = 0.999$), limit of detection (5 ppb), intra- and inter-day precision (R.S.D. <7.6% and <9.7%, respectively) and robustness (R.S.D. <7.4% for retention time and <5.0% for area). Sensitivity was adequate to detect melamine under the safety limits proposed by the US FDA. Finally, recoveries for several milk samples were found in the 85–109% range.

1. Introduction

Melamine, also known as cyanuramide or triaminotriazine (1,3,5-triazine-2,4,6-triamine, $C_3H_6N_6$, MW = 126 g/mol), is an inexpensive nitrogen-containing industrial chemical (Fig. 1). It has been used in the manufacture of plastics, melamine-formaldehyde, for surface coating material, adhesives and flame retardants. As a fertiliser, it can be found as a metabolite of the insecticide cyromazine [1–9]. Therefore, melamine can be found in a variety of foods as a low-level contaminant by migration from package plastics [1,2,3,10]. Recently, intentional illegal adulteration by a high melamine level has been discovered in food. Due to its high nitrogen level (66 mass%), it produces an incorrect high reading in the protein content measurement based on the total protein content (the Kjedahl nitrogen determination method), that allows the final price of the product to increase [4,6,11].

Melamine should not be present in foods because they are toxic at high dose exposures, and may cause urolithasis and bladder cancer. A study performed by the US Food and Drug Administration (FDA) describes the risk to human health associated with eating products from animals that have been fed with melamine and its analogues [1]. Pet food ingredients contaminated with melamine and its related compounds have caused renal diseases and associated deaths in cats and dogs in the US [2,4,6,9,12].

In 2008, the intake of milk and infant formula which have been highly adulterated by melamine resulted in a major outbreak of renal disease (urolithiasis) and the associated deaths of numerous infants in China [3–5,11]. A safety limit of melamine ingestion has been officially set by the US FDA at 2.5 ppm for adults food [3,13], and at 1 ppm for infant formula [3,4]. However, the melamine concentration in several adulterated milk products reaches 3300 ppm, posing extreme danger to consumers [3,4]. The control of the amount of melamine in milk is of utmost importance in food safety due to the high intake of this product, especially by infants.

Several other methods based on GC/MS [14], immunoassay analysis (ELISA) [2,15], direct analysis in real time (DART) [16], matrix-assisted laser desorption ionisation (MALDI–MS) [17], electrophoresis [18], gas chromatography [19,20], ion-exchange chromatography–DAD [21], HPLC–DAD [1,2,6,7,10,22,23] and HPLC–MS [2,8,9,22,24–26] have been developed for the quantification of melamine in biologic muscle tissue [7], a large number of different food matrixes, as pet food [2,9,14–16], chard samples [8], animal feed [26], poultry meat [7], fish feed [18], yoghurt [18], fish [18,25],

* Corresponding author. Tel.: +34 964728099; fax: +34 964728066.
E-mail address: mrambla@qfa.uji.es (M. Rambla-Alegre).

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doi:10.1016/j.talanta.2010.01.034

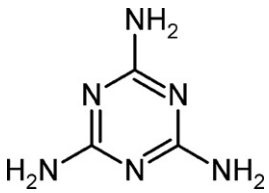

Fig. 1. Melamine structure.

rice concentrates [1], beverages [22], cereal flour [6] and eggs [7].

Milk is a complex food matrix given the presence of many compounds and the suspension caused by proteins. Various analytical methodologies for the quantification of melamine in milk have been published, which include electrophoresis [18,27], GC/MS [28], thin layer chromatography [29], HPLC with UV–visible absorption [30] or mass spectrometry detection [31,32]. However, given the complexity of the matrix, these analytical methodologies involve time-consuming extraction, preconcentration and purification steps. Moreover, because of the need of high selectivity, mobile phases are programmed as gradients, thus the analysis of a large amount of samples proves difficult. So, large volumes of pollutant organic solvents are used. Several authors have proposed the direct analysis of powdered milk by Raman spectroscopy with a very high detection limit (1%, w/w) [33]. Liquid milk has been analysed by low-temperature plasma mass spectrometry (LTP–MS-MS) [11], ultrasound assisted extractive electrospray ionisation mass spectroscopy (DAPCI–MS), but the limit of detection reached was high (0.5 ppm) due to the intense noise caused by the matrix [3], and isotope-dilution HPLC–MS, which only involves a deproteinization [34]. Moreover, these methodologies use complex, specific and expensive instrumentation.

Micellar liquid chromatography (MLC) is a liquid chromatographic methodology where aqueous solution of tensioactive over the critical micellar concentration are used as mobile phase. While the tensioactive modifies the nature of the stationary phase, micelles interact with analytes, introducing a new factor which complexes the retention mechanism. Moreover, the use of micellar mobile phases and solvents allows samples to be injected directly which expedites the analytical methodology. Competitive protein binding by monomers and micelles releases protein-bound compounds, which can then be analysed. Meanwhile, proteins and other hydrophobic substances are solubilized, and can be injected into the chromatographic system without precipitating into the chromatographic system. Moreover, they are eluted with, or shortly after, the solvent front, and they do not interfere with analytes [35,36].

Sodium dodecyl sulphate (SDS) is a widely used anionic surfactant in micellar media given its solubility in water, its low critic micellar concentration, low cost, and because it is easy to removed from the chromatographic system. Several analytical methods based on micellar media using SDS and an organic modifier have been developed to determine antioxidants in foods, such as the quantification of phenolic antioxidants in oils [37], synthetic antioxidants in milk, dietary supplements [38] meat [35], sulphonamides in milk [39] and amines in wine [40].

The aim of the work is to perform an easy, fast, accurate and reliable analytical methodology to quantify the level of melamine in milk, using mobile phases containing the anionic surfactant SDS. The analyte has to be resolved from the other compounds of the matrix with sufficient sensitivity to reach the security levels marked by the FDA. The proposed method must be validated following the European Union's indications in terms of limit of detection, sensitivity, linearity, repeatability, reproducibility, robustness and recovery. However, safety limits are not recommended by EU Regulation, so those indicated by US FDA have been taken.

2. Materials and methods

2.1. Chemicals and materials

Melamine (99% purity) was purchased from Aldrich (St. Louis, MO, USA). Sodium dodecyl sulphate (99% purity) was obtained from Merck (Darmstadt, Germany). Sodium dihydrogenophosphate monohydrate and HCl were ordered from Panreac (Barcelona, Spain). NaOH came from Scharlab (Barcelona). Methanol was bought from J.T.: Baker (Deventeer, The Netherlands) and 1-propanol came from Scharlab. Ultrapure water (Millipore S.A.S., Molsheim, France) was used to prepare the aqueous solutions and mobile phases. The characteristics of the studied milk samples are described in Tables 1 and 2.

2.2. Equipment and chromatographic conditions

A Metter-Toledo analytical balance (Greifensee, Switzerland) was used to weigh the analyte. The pH was measured with a Crison potentiometer (Barcelona) equipped with a combined Ag/AgCl/glass electrode. An ultrasonic bath was used to dissolve the standards (model Ultrasons-H, Selecta, Abrera, Spain).

Chromatographic separation was performed in an Agilent Technologies Series 1100 system (Palo Alto, CA, USA) equipped with an isocratic pump, a degasifier, an autosampler and a DAD. The stationary phase was a Kromasil C18 column with the following characteristics: pore size 100 Å, length 15 cm, internal diameter 4.6 mm, particle size 5 μm. Several mobile phases were tested by varying the SDS concentration, the amount of 1-propanol and the pH. The optimal mobile phase was an aqueous solution (0.05 M of SDS) with 7.5% of 1-propanol at pH 3, which was run in the isocratic mode with a flow of 1 mL/min at room temperature. The injection volume was 20 μL. The mobile phases and the injected solutions were filtered through 0.45 μm nylon membranes.

2.3. Chromatographic system care

Due to the use of salt solutions as a mobile phase, careful consideration is required to avoid the precipitation of SDS which would seriously damage the modules of the chromatographic system, especially needle, tubes and the column.

An essential rule of care is that the micellar mobile phase must always be running (even at low flow). Furthermore, a thorough cleaning procedure has to be maintained to keep the column in good condition. First, the system has to be cleaned by flowing 100% pure water at 1 mL/min for a minimum of 1 h in order to remove the salts. Then pure water is replaced by 100% methanol, and the column is rinsed for at least 1 h in order to totally eliminate the surfactant and strongly retained compounds.

2.4. Solution and sample preparation

SDS solutions were prepared by weighing the appropriate amount of SDS and disodium monohydrogen-phosphate. These reagents were solved in ultrapure water, the pH was adjusted to the desired value and n-propanol was added, if necessary. Finally, the solution was adjusted to the desired volume with ultrapure water, ultrasonicated and filtered.

Stock solutions with 1, 100 and 200 ppm of melamine were prepared by dissolving the appropriate amount in methanol.

Spiking the milk samples was done by adding the appropriate amount to 1 mL of liquid milk, and making it up to 10 mL with a solu-

Table 1
Characteristics and recoveries obtained for the analysed liquid milks (they were all purchased in a local supermarket).

Name	Supplier	Consumer	Spiked Concentrations (ppm)	Recovery (%) ($n=6$)
Soya milk	Liquats vegetals (Girona, Spain)	Adults	0.125	94
			0.250	92
			0.375	95
			2	98
			10	92
			20	97
Goat's milk	Lactiber Corporación Alimentaria (Resedo de Piélago, Spain)	Adults	0.125	99
			0.250	97
			0.375	96
			2	106
			10	87
			20	91
Puleva Peques 3 "Leche de Crecimiento"	Puleva Food (Granada, España)	Infants	0.050	101
			0.100	97
			0.150	94
			2	93
			10	96
			20	99
Nestle Crecimiento 1 Plus	Nestle Spain (Esplugues de Llobregat, Spain)	Infants	0.050	94
			0.100	99
			0.150	106
			2	98
			10	95
			20	88
Energy growth + Omega 3DHA	Lactiber Corporación alimentaria (Resedo de Piélago, Spain)	Infants	0.050	97
			0.100	104
			0.150	94
			2	91
			10	99
			20	104

tion of 0.05 M SDS at pH 3. The powdered milk was reconstituted by solving 4 g in 30 mL of cool water.

The validation of the analytical methodology was performed using spiked milk. By considering the 10-fold dilution in the experimental procedure, the analysed aliquot of milk spiked at the safety limits proposed by the FDA (1 ppm for infants and 2.5 ppm for adults milk), respectively, in the calibration curve.

2.5. Method validation

Validation was performed to meet the criteria specified by the European Commission Decision 2002/657/EC (2002) [41].

Linearity and sensitivity were checked by injecting the analytes at 11 different concentrations levels to cover the whole working range (0.02–100 ppm). Calibration curves of the spiked milk samples were calculated by a least squares linear regression analysis by plotting the peak area of melamine versus the analyte concentration. The limit of detection (LOD) was based on the *3 s criterion*, using a series of 10 solutions containing a low concentration of melamine. LOQ was selected as the low concentration used in the calibration curve.

Decision limits (CC_α) and detection capability (CC_β) were also calculated. CC_α was calculated by analysing 20 samples spiked with melamine at LOQ and safety limits (1 ppm for infants and 2.5 ppm for adults) of melamine. CC_α was calculated as the concentration spiked plus 1.64 the corresponding standard deviation. To obtain the CC_β values, 20 samples were spiked at the calculated CC_α levels, and analysed. The CC_β was calculated as the theoretical value of CC_α previously obtained plus 1.64 times the standard deviation [41].

Accuracy and precision were also determined by analysing three different concentration levels corresponding to 0.5, 1 and 1.5 times the proposed safety limits.

3. Results and discussion

3.1. Mobile phase selection and chromatographic conditions

3.1.1. pH selection

The pH variation of the mobile phase will affect the retention time of the weak acid or alkali compounds. The working pH range of the selected column was 1.5–9.5. However, the use of weakly basic pH allows a slow degradation of the stationary phase to reduce the working life of the column; so only acid and a neutral pH were considered.

Melamine has a pKa of 5.10 [42] with an equilibrium between two forms: one is molecular and the other is positively charged. Two pHs were tested, pH 7 where the melamine is in its molecular form, and pH 3 where the melamine is quantitatively protonated. Using optimal chromatographic conditions (Section 2.4) at pH 7, melamine almost elutes at 2.5 min (in front of the chromatogram), whereas it elutes at almost 9.1 min at pH 3, without interferences with the other peaks of the matrix. Therefore, the analyses were performed at pH 3. Retention increases at a lower pH because the electrostatic interaction between the protonated form of the melamine with the anionic SDS is higher.

3.1.2. Use of an organic modifier

Melamine is a polar compound ($\log P_{o/w} = -1.14$) [43], which means that using a C18 column and a pure micellar mobile phases would provide an adequate retention time. Three mobile phases with SDS different concentrations at pH 3 were tested. Using 0.1 M SDS, the analyte elutes at 9.27 min, a convenient time without interferences with other compounds of the matrix, but it offers poor efficiency (less than 1000) and an irregular shape.

Table 2
Characteristics and recoveries obtained for the analysed powdered milks (all the milks were for infants).

Name	Supplier	Purchased from	Preparation mode (in water)	Spiked Concentrations (ppm)	Recovery (%) ($n = 6$)
"Baby inicia, milk for suckling baby"	Hero (Alcantarilla, Spain)	Local supermarket	4 g in 30 mL	0.050	99
				0.100	97
				0.150	93
				2	104
				10	101
				20	103
"Nutriben continuación"	Alter Group (Seville, Spain)	Local chemist	4 g in 30 mL	0.050	101
				0.100	92
				0.150	97
				2	91
				10	104
				20	103
Blemil Plus 2 Forte	Laboratorios Ordesa, (Sant Boi de Llobregat, Spain)	Local chemist	4 g in 30 mL	0.050	101
				0.100	86
				0.150	91
				2	87
				10	89
				20	95
Blemil Plus 3 crecimiento	Laboratorios Ordesa, (Sant Boi de Llobregat, Spain)	Local chemist	4 g in 30 mL	0.050	99
				0.100	87
				0.150	86
				2	85
				10	104
				20	98
Blemil Plus 2 AE	Laboratorios Ordesa, (Sant Boi de Llobregat, Spain)	Local chemist	4 g in 30 mL	0.050	91
				0.100	98
				0.150	102
				2	85
				10	96
				20	105
Blemil Plus 1 Fuerte	Laboratorios Ordesa, (Sant Boi de Llobregat, Spain)	Local chemist	4.5 g in 30 mL	0.050	109
				0.100	102
				0.150	95
				2	94
				10	101
				20	97
Nutricia Almirón 1	Numil Nutrición (Valdemoro, Spain)	Local chemist	15.3 g in 100 mL	0.050	100
				0.100	103
				0.150	99
				2	95
				10	98
				20	106
Nutricia Almirón 2 Continuación	Numil Nutrición (Valdemoro, Spain)	Local chemist	16.3 g in 100 mL	0.050	94
				0.100	104
				0.150	103
				2	94
				10	96
				20	95

The addition of short-chain alcohol (1-propanol and 1-butanol) can be used to improve efficiency and to maintain adequate retention times [44]. 1-Propanol was finally selected as an organic modifier as it allows the separation of melamine and of other milk compounds in 9.3 min with greater efficiency. 1-Butanol was also tested, but the peak of the melamine overlaps with the protein band.

3.1.3. Optimisation of SDS and the amount of propanol

In order to select the best analysis conditions to detect melamine in milk, several mobile phases containing the following SDS (M)-n-propanol (%) concentrations were tested: 0.05–2.5; 0.05–12.5; 0.10–7.5; 0.15–2.5 and 0.15–12.5. Retention factor (k), efficiency (N) and asymmetry (B/A) were measured for melamine, and two unknown matrix compounds (Peaks L and O), which eluted before and after melamine in all the tested milk samples, respectively, were processed with the Michrom software [45]. The compounds retention was modelled by means of the following equation [46]:

$$k = \frac{K'_{AS}/(1 + K_{AD}\varphi)}{1 + [M](K_{AM}(1 + K_{MD}\varphi)/(1 + K_{AD}\varphi))}$$

where $[M]$ and φ are the surfactant and modifier concentrations, respectively. K_{AS} and K_{AM} correspond to the equilibrium constants between the solute in pure water and the stationary phase or micelle, respectively. K_{AD} and K_{MD} measure the relative variation in the solute concentration in pure water and micelles due to the presence of n-propanol, as compared to a pure micellar solution. The optimisation of the resolution of the three compounds was performed by measuring the overlapping fractions of each chromatographic peak, and the shape of the chromatographic peaks was also modelled to obtain the overlapping fractions and to predict chromatograms [41].

When maximum resolution-minimum analysis time criteria were applied, the mobile phase selected as being optimal was

Table 3
Inter-day and intra-day precision and accuracy of melamine.

Added Concentration (ppm)	Found[a] (mean ± SD) (ppm)	Accuracy (%)	Intra-day C.V. (%)	Found[b] (mean ± SD) (ng/mL)	Accuracy (%)	Inter-day C.V. (%)
0.05	0.049 ± 0.008	−0.9	1.8	0.051 ± 0.004	+2.0	7.6
0.1	0.091 ± 0.007	−8.6	7.6	0.096 ± 0.007	−3.5	5.8
0.15	0.139 ± 0.002	−7.3	1.5	0.135 ± 0.003	−9.8	5.0
0.125	0.117 ± 0.007	−6.0	1.4	0.116 ± 0.006	−6.8	5.4
0.25	0.254 ± 0.003	−1.7	2.1	0.24 ± 0.02	−4.1	7.8
0.375	0.397 ± 0.003	+ 6.0	1.1	0.393 ± 0.009	+4.7	2.7
2	2.08 ± 0.06	+ 4.6	2.9	2.08 ± 0.08	+3.91	6.7
20	20.6 ± 0.5	+3.1	2.4	21 ± 1	+5.9	9.7
100	99.9 ± 0.3	−0.1	0.3	99 ± 1	+1.3	6.4

[a] $n = 6$.
[b] $n = 5$.

0.05 M SDS, 7.5% 1-propanol at pH 3. In this mobile phase, melamine was adequately resolved from the other milk peaks, and the analysis could be performed in 12 min. The chromatographic parameters for melamine were: retention time, $t_R = 9.3$ min, capacity factor, $k = 13.2$, efficiency, $N = 2015$ and asymmetry factor, $B/A = 1.5$.

3.1.4. Detection wavelength optimisation

Melamine has been detected at UV–visible between 200 and 240 nm [1,2,6,10,18,21,27]. Moreover, the UV–visible spectrum of melamine in micellar media was taken by analysing spiked milk using the optimised conditions at 2 ppm. The maximum absorbance was found at 210 mn, without interferences.

3.2. Methodology validation

Validation was performed according to European Union regulation 2002/657/EC [41]. The parameters evaluated were: selectivity, linearity, LOD and limits of quantification (LOQ), precision, accuracy, decision limit (CC_α), detection capability (CC_β) and robustness.

3.2.1. Selectivity

To study the matrix effects of the possible co-eluting compounds, ten blanks of each studied milk sample were analysed. Fig. 2 shows the chromatograms obtained from analysing the milk samples both before and after contamination with 2 ppm of melamine. In the blanks, the protein band and a large number of unknown peaks appear, both before and after melamine retention, but they were sufficiently separate to avoid any overlapping. In the spiked samples, the melamine peak may be observed as sufficiently separated from other peaks, thus avoiding overlapping.

3.2.2. Linearity and sensitivity

Calibration was repeated five times (preparing the sample on each occasion) in two months, and each calibration level was analysed six times over a 2-month period. The regression curve, taken as the average of the obtained six calibration curves, was:

$$A = (5.48 \pm 0.25)[\text{melamine}] - (0.019 \pm 0.006) \qquad r^2 = 0.999$$

where A is in arbitrary units and the concentration amount is provided in ppm. LOD and LOQ were set at 5 and 20 ppb, respectively.

3.2.3. Precision and accuracy

The intra- and inter-day accuracy and precision of the proposed methodology were determined with the milk samples spiked at six concentration levels: 0.5, 1 and 1.5 times the safety limits proposed by the FDA for infants and adult food, and also at three high concentrations (2, 10 and 20 ppm). The intra-day analysis was determined by injecting aliquots of these samples six times on the same day, while the inter-day analyses correspond to the average of five measurements of the intra-day values taken over a 3-month period. The results, expressed as variation coefficients for accuracy and relative error for precision, are shown in Table 3. The data show good accuracy (intra-day −8.6 to +6.0%; inter-day −9.8 to +5.9%) and adequate precision (intra-day 0.3–7.6%; inter-day 2.7–9.7%), which are useful for routine analysis.

3.2.4. Robustness

The robustness of the method was examined by analysing a spiked milk sample ($n = 6$) by making slight changes to the following parameters: SDS concentration, percentage of propanol (%), flow rate, and pH. The variation of the sensitivity (area) and retention time was considered, and the results are shown in Table 4.

The variation of these parameters neither modified the sensitivity (R.S.D. < 5.0%) nor the retention time (R.S.D. < 7.4%) significantly. Obviously, the variation of the flow rate strongly modified the retention time. It was possible to inject around 1000 consecutive diluted and filtered samples without column damage and, consequently, without affecting the analytical performance.

3.2.5. Decision limit and detection capability

The decision limit (CC_α) indicates the limit at and above which it can be concluded with an error probability of α that a sample has a concentration over the established limits. The detection capability (CC_α) is the lowest concentration at which the method is able to detect permitted limit concentrations with a statistical certainty of $1 - \beta$ [49]. These parameters allow the assessment of the critical concentrations above which the method reliability distinguishes and quantifies a substance by taking into account the variability of the method and the statistical risk of making a wrong decision [47]. The error probabilities α and β were set at 5% [41].

In this case, three sets of CC_α and CC_β were calculated, and the LOQ and the safety limits for infants and adult milk were taken as the established limit [41]. The obtained results were:

- Spiking 20 ppb (LOQ level): $CC_\alpha = 23$ ppb and $CC_\beta = 27$ ppb.
- Spiking 0.1 ppm (infant safety limit): $CC_\alpha = 0.111$ ppm and $CC_\beta = 0.120$ ppm.
- Spiking 0.25 ppm (adult safety limit): $CC_\alpha = 0.255$ ppm and $CC_\beta = 0.280$ ppm.

The obtained values indicate that the established limits can be detected in contaminated samples.

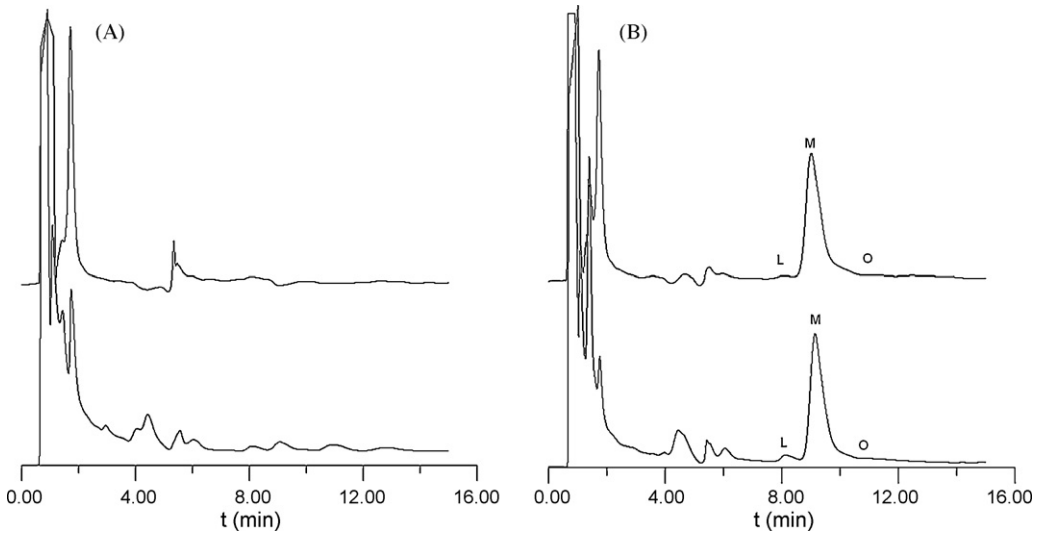

Fig. 2. Chromatograms of: (A) blank milk and (B) milk samples spiked at 2 ppm. In both cases, the top chromatogram corresponds to "Nutriben continuación", and the bottom one to Blemil Plus 2AE. Extracts were analysed following the optimised condition methodology. Peak M is melamine, and L and O are the two endogenous interferents.

3.2.6. Recovery

Recovery was calculated by analysing the spiked samples at several levels, and by comparing with the concentration provided by the suggested method. Recovery was determined for each milk sample in order to evaluate the differences between the several matrixes. The concentration levels selected for the study were 0.5, 1 and 1.5 times the safety limits proposed by the FDA, after considering the 10-fold dilution of the samples (50, 100 and 150 ppb for infants milk and 125, 250 and 375 ppb for adults milk), and three high contamination levels, 2, 10 and 20 ppm. Regarding the powdered milk samples, the results provided were obtained after they had been dissolved in water.

The results shown in Tables 1 and 2 indicate good recovery (85–109%) for all the milk samples and concentration levels. These values are in accordance with EU recommendations (EU Regulation 2002/654/EC) which establish a range of accuracy of between 80 and 110% as acceptable for concentrations over 10 ppb [41].

Table 4
Evaluation of the robustness of the MLC method.

Changes of mobile phase parameters	Level	Retention time (min)	Area (arbitrary unit)
Concentration SDS (M)			
0.045	−0.005	10.10	0.98
0.050	+0	9.27	1.03
0.055	+0.005	8.73	0.99
Mean ± SD		9.4 ± 0.7	1.00 ± 0.03
R.S.D. (%)		7.4	2.7
Amount of propanol (%)			
7.4	−0.1	9.39	0.98
7.5	0	9.27	1.03
7.6	+0.1	9.52	1.08
Mean ± SD		9.4 ± 0.1	1.03 ± 0.05
R.S.D. (%)		1.3	4.5
pH			
2.9	−0.1	9.49	1.11
3.0	0	9.27	1.03
3.1	+0.1	9.17	1.13
Mean ± SD		9.3 ± 0.4	1.09 ± 0.05
R.S.D. (%)		1.8	5.0
Flow (mL/min)			
0.9	−0.1	10.26	1.02
1.0	0.0	9.27	1.03
1.1	+0.1	8.32	0.95
Mean ± SD		9.3 ± 1.0	1.00 ± 0.04
R.S.D. (%)		10.4	4.0

4. Conclusions

Micellar liquid chromatography has been proved a suitable technique to analyse of melamine in a wide variety of milks (powdered and liquid, for adults and infants). One advantage of the procedure is the possibility of injecting diluted milk into the chromatographic system after filtration, thus avoiding long and tedious extractions. The use of chemometrical statistics allows the simultaneous optimisation of two parameters (SDS and propanol concentrations) by testing only five mobile phases. The analyte was satisfactorily resolved from the matrix in an analysis time of under 12 min. Validation was performed according to EU guidelines with satisfactory results in the linearity, selectivity, precision, accuracy, robustness and recovery studies. The limit of detection and the lineal interval range were sufficient to detect melamine in milk under the safety limits recommended by the FDA. This method meets the requirements of the "green chemistry" concept since lower quantity of organic solvents has been used. Besides, it is relatively inexpensive compared to other methods, thus making it more attractive.

Acknowledgements

This work has been supported by a project from the Spanish Ministry of Education and Science (MEC) CTQ 2007 764473/BQU and the Foundation Caixa-Castelló, Bancaixa P1-1B2006-12. María Rambla-Alegre also wishes to acknowledge the FPU grant.

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