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DEVELOPMENT OF AN ANALYTICAL METHODOLOGY TO QUANTIFY MELAMINE IN DRINKING AND WASTE-WATER BY MICELLAR LIQUID CHROMATOGRAPHY

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Because of the large potential health impact caused by deliberate contamination of the synthetic chemical melamine for human and animal consumption, the World Health Organization and the Food and Agriculture Organization of the United Nations provided a range of recommendations in order to facilitate obtaining needed data among which was the determination of the background levels of melamine in drinking and waste-water (4 December 2008). A Chromatographic procedure using a C18 column and a micellar mobile phase of sodium dodecyl sulphate (0.1M), propanol (7.5%), buffered at pH 3, and detection set by absorbance at 210 nm, was reported for the quantification of melamine in both drinking and waste water. Samples were filtered and directly injected into the chromatographic system, thus avoiding any extraction procedure. The optimal mobile phase composition was obtained by a chemometrics approach that considers the retention factor, efficiency and peak shape. Of the chromatographic peaks Melamine was eluted in nearly 6.2 min without interferences. Validation was performed following the Food and Drug Administration (FDA) guideline. The analytical parameters studied were: linearity (0.03–5 µg/mL; R^2 = 0.998), limit of detection (13 n/mL), intra- and inter-day accuracy (between 4.1 and 12.2%), intra-and inter-day precision

(less than 14.8%) and robustness (RSD < 5.1% retention time and <9.0 % for area). The proposed methodology was successfully applied for local waste and drinking water, where no melamine was found.

KEYWORDS: Melamine; Micellar liquid chromatography; Validation; Waste Water; Drinking Water

1. Introduction

Melamine, also known as cyanuramide or triaminotriazine (1,3,5-triazine-2,4,6triamine), is an inexpensive nitrogen-containing industrial chemical (Figure 1). It is produced in the manufacture of laminates, plastics, coatings, commercial filters, glues or adhesives, as well as dishware and kitchenware. The addition of melamine to different products for human and animal consumption is a fraudulent activity to obtain an incorrectly high measure of protein content based on total nitrogen (the Kjeldahl nitrogen determination method) [1], which results in a price increase of these products. After the incidents related to adulteration of feed animals, in March 2007 [2], and infant formula in China in September 2008 [3], the World Health Organization (WHO) convened a meeting of experts to review toxicological aspects of melamine, and a tolerable daily intake (TDI) of 0.2 mg/kg body weight per day was established [4].

The sources of melamine have been divided into "baseline" levels, which refer to levels in food that do not result from adulteration or misuse, and "adulteration" levels, which refer to levels in food that result from the intentional addition of melamine to food or unapproved use or misuse of melamine or substances that can degrade to form melamine. The limit between baseline and adulteration level has been set to 1 µg/mL by WHO in 2009 [4,5]. Baseline concentrations of

melamine are present in the environment and in the food-chain as a result of the widespread use of materials that contain melamine. Although data for baseline levels of melamine originating from certain sources, such as migration from tableware, were available, melamine occurrence data from other sources, such as the pesticide cyromazine or of fertilizers, were either limited or unavailable. Data on the presence of melamine in other potential sources, such as water from the industrial uses/manufacturing, were unavailable. Because of this the WHO conducted a series of recommendations for future works (e.g., background levels of melamine in drinking-water) [4].

As detailed in a previous work [6], several studies for the determination of melamine in large number of different matrices (especially milk and derived products) and different analytical methods have been published in response to the serious incidents above commented, but the quantification of melamine in water has been barely studied. A recent paper reports the detection of melamine in waste water by HPLC-DAD [7] and liquid/liquid [5] or polymer imprinted [8] extraction followed by HPLC-MS analysis. However, mass spectrometry instrumentation is quite expensive and nowadays not all the routine laboratories can afford it. In addition, the methodology involves time-consuming extraction, preconcentration and purification steps.

A methodology to quantify melamine in milk using micellar liquid chromatography (MLC) was previously developed by the authors [6]. The use of surfactant solutions above the critic micellar concentration as mobile phases shows several advantages, as the solubilization of both polar and hydrophobic compounds (avoiding their precipitation into the chromatographic system), which are eluted with, or shortly after, the solvent front, and do not interfere with analytes [9]. This allows the direct injection of the sample, which expedites the experimental procedure. Moreover, the surfactant modifies the nature of the stationary phase, and micelles can also interact with the compounds, following complex retention mechanism. Sodium dodecyl sulphate (SDS) is widely used as anionic surfactant, given its high solubility in water, its low critic micellar concentration, low cost, and because it is easy to be removed from the chromatographic system [10]. MLC using SDS as

surfactant has been successfully used to detect compounds in a wide range of complex matrices, as food [6,11,12], food extracts [13] and biological fluids [14, 15].

The aim of this work is to perform an easy, fast, accurate and reliable chromatographic procedure to quantify the level of melamine in drinking and waste water, using a micellar mobile phase. The analyte has to be resolved from the other compounds of the matrix with sufficient sensitivity to reach the recommendation levels proposed by the WHO organization (1 μg/mL) [4,5]. The proposed method was validated following the Food and Drug Administration (FDA) guideline [16], in terms of selectivity, linearity, detection and quantification limits, precision and accuracy, robustness and recoveries.

2. Materials and Methods

2.1. Chemical and materials

Melamine (99% purity) was purchased from Aldrich (St. Louis, MO, USA). The different water samples, both drinking and wastewater were collected and transferred to the laboratory by FACSA (Castellón, Spain) the company that manages water quality in the Spanish province of Castellón (see characteristics in Table 1). All the wastewater samples were collected at 2011, 11th January in the province of Castellón, and were suspected to contain melamine. Sodium dodecyl sulphate (99% purity) was obtained from Merck (Darmstadt, Germany). Sodium dihydrogen phosphate monohydrate and hydrochloric acid were ordered from Panreac (Barcelona, Spain). Ultrapure water was used throughout (Millipore S.A.S. France). Methanol was purchased from J.T. Baker (Deventeer, The Netherlands) and 1-propanol came from Scharlab (Barcelona).

2.2 Chromatographic conditions

The chromatographic system, an Agilent Technologies, series 1100 CA, USA, was equipped with an isocratic pump, a degasifier, an autosampler and a DAD (range 190–700 nm). The column used was a Kromasil C18 (150 mm × 4.6 mm i.d., pore size 100 Å and 5 µm particle size) from Scharlab. The pH of solutions was measured with a potentiometer model GLP 22 Crison (Barcelona), equipped with a combined Ag/AgCl/glass electrode. The analytical balance used was an AX105 Delta-Range (Metter-Toledo, Switzerland). The mobile phases and the injected solutions were filtered through 0.45 µm nylon membranes (Micron Separations, MA, USA). An ultrasonic bath was used to dissolve the standards (model Ultrasons-H, Selecta, Barcelona).

Several mobile phases were tested by varying the SDS concentration and the amount of the organic solvent 1-propanol. The optimum mobile phase was an aqueous solution of 0.1 M SDS–7.5 % propanol, buffered at pH 3. The flow-rate, injection volume and UV wavelength were, 1 mL/min, 20 µL and 210 nm, respectively. Chromatographic signals were acquired and processed with an Agilent ChemStation (Rev. A.10.01). Michrom software was used for chromatographic data treatment [17].

2.3 Mobile phase, standard and sample preparation

The micellar mobile phases were prepared by dissolving the appropriate amount of SDS in water and were buffered with sodium dihydrogen phosphate 0.01M at pH 3 using 0.1 M of hydrochloric acid. Then, 1-propanol, (if necessary) was added to achieve the desired concentration. Finally, water was added up to the mark-up of the volumetric flask, sonicated and filtered.

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

The water samples were spiked by adding the minimum amount of melamine solution (at µL

levels) and filling up with the corresponding sample water. Spiked samples were kept 24 h prior injection to favour the mixing between the analyte and the matrix [18].

The samples were analyzed by filtering and direct injection into the chromatographic conditions, without any other treatment.

3. Results and Discussion

3.1 Optimization of chromatographic conditions

The analysis parameters were taken from a previous work about the detection of melamine in milk by means of micellar liquid chromatography [6]. However, the separation parameters (composition of the mobile phase) were changed because the matrix compounds, which could interfere with melamine, in drinking and waste water are different from those in milk.

In a mobile phase of 0.15 M SDS at pH 3, melamine elutes at around 9.3 min, without overlapping with other compounds of the matrix, but showing poor efficiency (less than 1000 theoretical plates) [6].

The addition of a short chain alcohol can improve efficiency and maintain adequate retention time [19]. Organic solvents increase the hydrophobic character of the aqueous phase in the mobile phase, shifting the balance of the solute from the micelles and the stationary phase to the phase modifier-water. Propanol, miscible in water, remains largely outside the micelles, dissolved in the water-phase. This alcohol interacts with the interface of the micelles decreasing the mutual repulsion of the ionic heads of the surfactant monomers. Butanol is inserted into the micelles due to its low solubility in water and its particular structure that combines a polar group with a hydrophobic chain, similar to the surfactant molecule, in both cases benefits the formation of micelles and critical micellar concentration decreases. The organic solvents also influence the proprieties of the stationary phase modified by the monomer surfactant. Propanol yielded higher efficiencies but larger retention

times than butanol. However, the melamine could not be resolved from other matrix compounds with butanol. Thus propanol was preferred to optimize the separation of the melamine.

In order to select the best analysis conditions, several mobile phases containing the following SDS (M)–1-propanol (%, v/v) concentration were tested: 0.05–2.5; 0.05–12.5; 0.15–2.5; 0.15–12.5; and 0.10–7.5. Retention factors (*k*), efficiencies (*N*) and asymmetry factors (*B/A*) were measured for melamine and two unknown matrix compounds (P1 and P2), which appears in all the studied samples of water before and after the analyte, respectively. The parameters were then processed with Michrom software [17]. The retention of the compounds was modelized according to [19]:

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

where [M] and φ are concentrations of surfactant and organic solvent, 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 1-propanol, as compared to a pure micellar solution. The optimization of the resolution of the three compounds was performed following the overlapping fractions criteria [20]. The software also provides the predicted chromatograms (retention times and shape of the peaks) when the concentration of SDS and 1-propanol changes between 0.05–0.15 M and 2.5–12.5 %, respectively.

Considering the criterion of maximum resolution and minimum analysis time, the optimum mobile phase was: 0.1 M SDS–7.5% propanol at pH 3. Under these conditions, melamine was eluted in 6.2 min without interference of other matrix compounds (Figure 2B). The chromatographic parameters obtained were: $k = 6.78$: $N = 4054$ and $B/A = 1.095$.

3.2. Method validation

Method validation was done following the FDA guideline [16]. The evaluated parameters were: selectivity, linearity, detection and quantification limits, precision and accuracy, recovery and robustness. The whole method validation was performed using the sample water 4 (Table 1).

3.2.1. Selectivity

To note the matrix effects of the possible co-eluting compounds, ten blanks of each studied waste water sample were analyzed before and after being spiked with 1 µg/mL of melamine. No interference was found near the retention time of melamine. Figure 2 shows the obtained chromatograms. Melamine appears sufficiently separated from other compounds to be quantified correctly (Figure 2B).

3.2.2. Linearity

Calibration curve were obtained for nine different concentrations (six replicates), in the range of 30 ng/mL–5 µg/mL. The calibration equation (slope, intercept and regression coefficient) was constructed plotting the chromatographic peak area of melamine versus the concentration and adjusted by the least square linear regression method. To study the variability of the calibration parameters, five curves were obtained by independent measurements during 5 days (one curve each day) over a 2-month period for a different set of standards. The regression curve, taken as the average of the obtained five calibration curves, was:

Peak area = 2.27 ± 0.06 [melamine] + (-0.05 ± 0.01); $R^2 = 0.9998$

3.2.3. Detection and quantification limits

The limit of detection (LOD) was determined with *3s criterion* (three times the standard deviation of the lowest concentration solution included in the calibration curve divided by the slope). The limit of quantification (LOQ) was selected as the lowest concentration used in the calibration curve. LOD and LOQ were 13 and 30 ng/mL, respectively. The tolerable daily intake (TDI) is 0.2 mg/kg body weight per day [4].

3.2.4. Precision and accuracy

The intra-and inter-day precisions of the method were determined by analyzing the melamine at four different concentrations (50 ng/mL, 0,2, 0,5 and 1 µg/mL). The intra-day analyses were determined by injecting these four test solutions six times on the same day. The inter-day analysis was the average of six measurements of the intra-day values taken on 5 days over a 3-month period performed by different analysts and equipment at the same concentrations. The results, expressed as the percentage of the relative standard deviation (precision), and relative error (accuracy, %) for the intra- and inter-day values, are shown in Table 2. The values of accuracy (between 4.1 and 12.2%) and precision (RSD less than 14.8 %) were under the maximal accepted values by FDA guidelines (less than 15% for accuracy and precision). Thus, the developed procedure can be used in routine analyses of drinking and waste water.

3.2.5. Robustness

The robustness of the method was examined by analyzing a spiked waste water sample at 1 µg/mL of melamine (n=6) by making slight changes to the following parameters: SDS concentration, percentage of propanol (%), flow rate and pH. Results, shown in Table 3, indicate that the variations in these parameters do not significantly alter the retention time or area (less than 5.1 %) of the

studied compound.

3.3. Analysis of real samples

The proposed methodology was applied for the detection of melamine in five waste water and one drinking water samples. Waste water samples were collected in places where the presence of melamine was suspected. However, the analyte was not detected.

4. Conclusions

Results indicate that MLC can be used for the determination of melamine at background levels in waste and drinking water samples under the safety limit fixed by WHO (1 μg/mL). These data would be useful to establish the suitability of drinking water and the pollution degree of waste water. The use of a statistical chemometrics tool allows simultaneous optimization of two parameters (SDS and propanol concentrations) by testing only five mobile phases. The analyte was satisfactorily resolved from the matrix in less than 8 min. Validation was performed according to FDA guideline with satisfactory results in the selectivity, linearity, precision, accuracy, robustness and recovery studies. LOQ (30 ng/mL) is sufficient to detect melamine under the tolerable daily intake. This method meets the requirements of the "green chemistry" concept since a low amount of organic solvents has been used.

5. Acknowledgements

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Figure Legends

Figure 1. Chemical structure of melamine.

Figure 2. Chromatogram obtained from the analysis by the proposed method of the sample 4: A) blank; B) after spiking by 1 µg/mL of melamine. Marked peaks are: MEL (melamine); P1 and P2 (peaks of unidentified compounds of the matrix which are eluted before and after melamine, respectively).

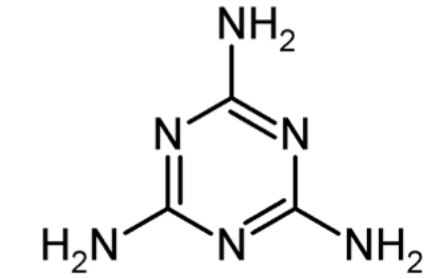

Figure 1

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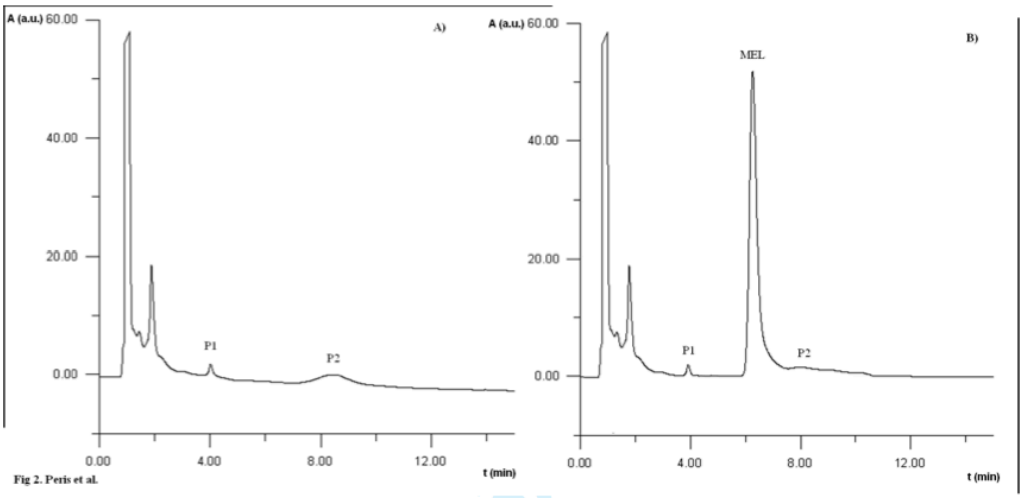

Fig 2. Peris et al.

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Table 1. Characteristics of the analyzed water samples.

Sample	Origin of the water	Origin	City of sampling
1	Waste sanitary and industrial water from an effluent from a manufacturing company of conglomerated modules clad by melamine	Police	Almassora
2	Waste industrial and home water	Iproma EDAR (water treatment company)	Almassora
3	Waste industrial and home water	Police	Onda, Betxí, Alqueries and Vila-real
4	Waste sanitary and industrial water from an effluent from a manufacturing company of conglomerated modules clad by melamine	Police	Vila-real
5	Mixture of waste water from the chemical processes in a chemical company which produces nylon and other organic compounds.	U.B.E. Corp. (Chemical company)	Castelló
6	Agua de Chovar (Bottled drinking water)	Local supermarket	Castelló

Table 3. Evaluation of the robustness of the MLC method

Changes of mobile phase parameters	Level	Retention time (min) (RSD, %)	Area (arbitrary unit) (RSD, %)
Concentration SDS (M)	0.045-0.0505	6.17±0.10 (1.7 %)	4.87 ±0.14 (2.9 %)
Propanol (%, v/v)	7.4-7.6	6.09±0.11 (1.8 %)	4.94±0.20 (4.0 %)
pH	2.9-3.1	6.13±0.08 (1.2 %)	4.3±0.4 (9.0 %)
Flow rate (mL/min)	0.95-1.05	6.2±0.3 (5.1 %)	4.7±0.2 (5.1 %)

Table 2. Inter-day and intra-day precision and accuracy of melamine

Added Concentration (µg/mL)	Found[a] (mean ± SD) (µg/mL)	Accuracy (%)	Intra-day RSD (%)	Found[b] (mean ± SD) (ng/mL)	Accuracy (%)	Inter-day RSD (%)
0.05	0.044 ± 0.004	- 12.2	14.8	0.047 ± 0.003	- 6.7	13.4
0.2	0.21 ± 0.02	+ 4.1	8.6	0.203 ± 0.003	+ 1.5	3.2
0.5	0.492 ± 0.003	- 1.6	0.4	0.493 ± 0.011	- 1.4	1.8
2	2.006 ± 0.004	+ 0.003	0.2	2.03 ± 0.04	+ 1.6	3.5

[a] n = 6, [b] n = 5