Advances on melamine determination by micellar liquid chromatography: A review

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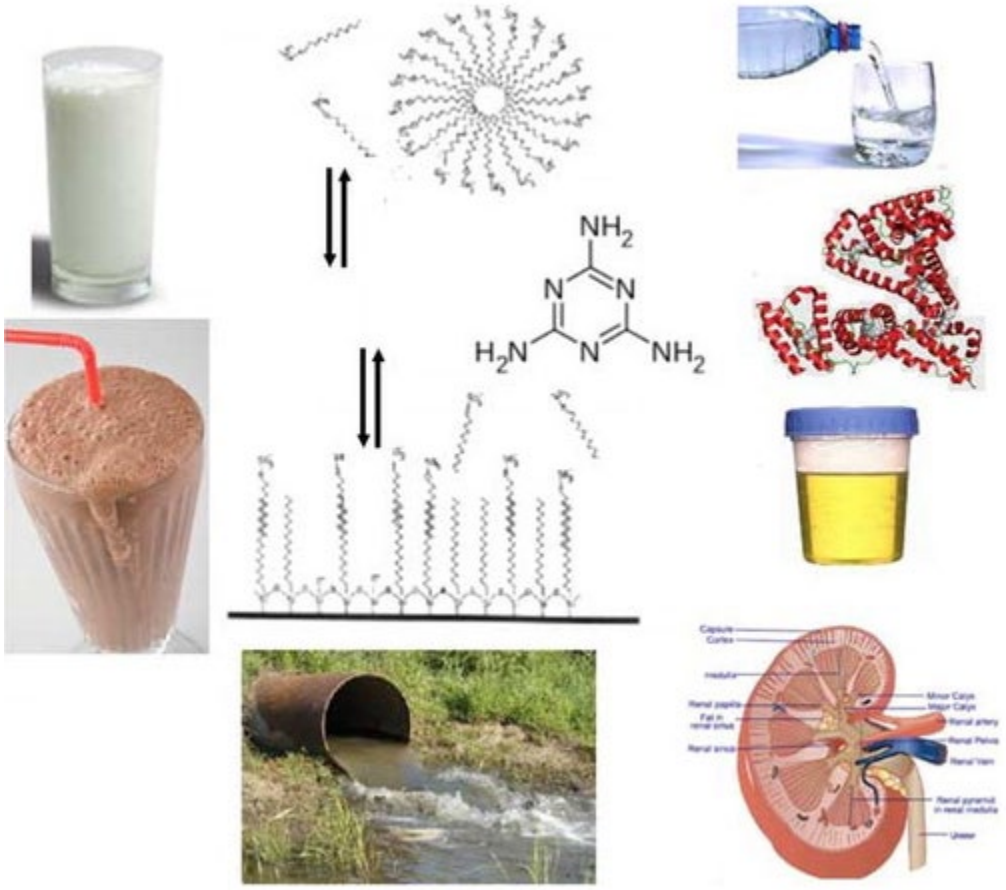
Abstract

Melamine is a toxic triazine which has been recently proven as a threat to human health. It can be ingested by several methods, being the most important the unethical adulteration of protein-rich foodstuff for economic reasons. This review presents several analytical methods, taken from the literature studies, devoted to the determination of melamine in milk, dietetic supplements, drinking and wastewater, swine kidney, plasma, and urine, using micellar liquid chromatography (MLC). We consider that the control of these samples is crucial to prevent and manage melamine intoxication. This technique has been demonstrated as an excellent tool to determine organic compounds in these matrices. We detail the optimization strategy and the obtained results in the different steps of method development, such as sample pretreatment, chromatographic separation, and validation process. The similarities and differences of the procedures have been described and discussed, as well as their advantages. The main ones were the possibility of direct injection and the efficient chromatographic elution, in spite of the complexity of the samples. Besides, it was found that the MLC procedures were fast, easy-to-handle, inexpensive, eco-friendly, safe, and useful for routine analysis. Therefore, they represent an excellent alternative to evaluate the melamine concentration in that kind of sample reliably.

Keywords: Environment; Food; Health; Melamine; Micellar



Graphical Abstract

1. Introduction

Melamine: A global threat

Melamine, also known as cyanuramide or triaminotriazine (1,3,5-triazine-2,4,6-triamine, C3H6N6, MW = 126 g/mol), is a small hydrophilic (log Po/w = -1.4) nitrogen-rich (66.6% w/w) biodegradable molecule belonging to the family of the heterocyclic organic compounds. It is a trimer of cyanamide and has three amino groups in its structure. It is a colorless to white powdered or crystalline solid and sublimates at 350° C. It is water-soluble and weakly basic with a pKa (melamine-H^+) = 5.0. It is highly stable in the main situations and decomposes under strong acidic and basic conditions as by intense heating. Melamine analogues (ammeline, ammelide, and cyanuric acid) are formed by successive substitution of amino to hydroxy groups by hydrolysis. Therefore, they can be found in raw melamine as impurities of the melamine synthesis process. Melamine can self-assembled and with analogues in high molecular weight structures by intermolecular networks of hydrogen bonds and π–π aromatic ring stacking [1,2].

Melamine is a non-expensive industrial chemical. It is mainly used as a starting material in the manufacture of synthetic resins, mostly as melamine-formaldehyde polymer, which is used in the production of a large variety of molded plastic objects, coating materials, filters, laminates, permanent press fabrics, wrinkle-free textiles, tarnish inhibitors, paper and textile finishers, tableware, paints, adhesives, and as additive in flame retardant formulations.[2–9] In agricultural backgrounds, melamine can be found in fertilizer mixtures, and as a decomposition product of cyromazine, an insecticide approved for use in many countries on animals and fields.[2,10] The population is exposed to melamine from a wide number of sources including food and environmental ones. Melamine can be found at a low level in food by migration from package [4,11,12] or from the breakdown of cyromazine.[10] It can also be found in wastewater, [13,14] from industry using this chemical as prime material or from agricultural water from an area where cyromazine has been used. If this water is inadequately purified, melamine can finally pass into the water supply system.[15]

Recently, deliberate illegal melamine adulteration was detected in different protein-rich products destined for humans, like milk and milk derivatives [5,6,16–19] and animals, as pet food[4,5,7,9,19–21] and feed for farm animals.[22] Because of its high amount of nitrogen, melamine causes an incorrectly high reading in the quantification of proteins based on the nitrogen amount (the Kjeldahl nitrogen determination method), which increases the apparent nutritional value and then the final price of the product.[16,23]

The pharmacokinetics and toxicology of melamine in mammalians are nowadays well known.[2] Melamine is rapidly absorbed orally after ingestion.[24] Melamine flows through the gastrointestinal system toward the bloodstream, from which it could be absorbed by other organs. However, melamine is unable to pass through cellular membranes.[25] Melamine is not metabolized and does not accumulate in tissues or organs. Therefore, it is quantitatively removed from the blood by the kidney and eliminated mainly unchanged in the urine in a few days. [13,26,27] Melamine has low acute toxicity by oral ingestion. It irritates the digestive tract due to the formation of melamine and melamine/analogue crystals, resulting in nausea, vomiting, diarrhea, hypertension, and edema.[2] However, the toxicity of melamine is enhanced in the urinary system, as it can react with uric acid or cyanuric acid to form calculi which can precipitate in the kidney, urethra, or bladder. The accumulation of these crystals can cause oliguria, nephrolithiasis, urolithiasis, or bladder cancer.[2,6,28] In 2007, a large number of cat and dog intoxications in the United States were related to the intake of protein-rich pet food manufactured from gluten and other cereal-based ingredients strongly contaminated with melamine.[4,5,7,9,19–21] One year later, in China, the ingestion of milk and milk-based infant formula highly contaminated with melamine (up to 3300 mg/L) resulted in a major outbreak of renal diseases in newborns and young children, and even several deaths were confirmed.[5,6,16,17–19]

It was the first outbreak of kidney failures related to melamine intoxication in humans. In both cases, melamine–uric acid stones were found in the kidney, urethra, and bladder of the patients. This event causes the launching of international sanitary alert about this toxic compound.[18,25]

Due to its toxicity, US Food and Drug Administration (FDA) has set the following maximum residue limits (MRLs) for the presence of melamine in foodstuff: 2.5 mg/kg in adult's food[6,21,25] and 1 mg/kg in infant formula.[5,6] Moreover, the World Health Organization (WHO) has established a tolerable daily intake (TDI) of 0.2 mg/kg body.[25] Therefore, the determination of melamine in milk products, dietetic supplements, and flesh must be performed for safety health reasons. Milk and supplements are taken because of their high nutritional value and as protein and mineral sources. Children formula must be carefully controlled because they are important for their growth and represent the main ingredient of infant alimentation.[5,16] Flesh samples should also be analyzed. Although not directly adulterated, melamine residues can occur, if the farm animal has been nourished with contaminated animal feed.[22] WHO also recommends the control of the level of melamine in wastewater to evaluate the danger for the environment and in drinking water for health safety reasons.[13,25] The occurrence of melamine in blood, urine, and kidney in humans and farm animals is an evidence of a strong timely or a continuous intoxication, and then to assess if they have been taken adulterated food. It can also be useful in clinical studies to determine the pharmacokinetics and toxicology of melamine.[13,27,29,30] Then, because of the different types of sources of melamine and its effect on human health, the development of economic, easy, and rapid analytical methods to reliably quantify melamine in these samples is of the utmost importance.

2. Analytical approaches for melamine in biological, food and environmental matrices

The impact and awareness of melamine threat in our society can be inferred by the high number of analytical procedures developed for its quantification in a relatively short period (2008–2015).[2,31,32] It has been determined in a wide range of matrices, like food for humans: yogurt, fish,[33] cereal gluten,[34] rice,[3,34] and soy[34] concentrates, beverages,[11] dairy products,[33,35,36] dietetic supplements,[24,34] eggs,[8,26], poultry meat,[8] royal jelly,[37] milk and milk-derived products, [5,16,19,30,33,38–48] bakery flour and goods,[42] and water in contact with kitchen and tableware made of melamine resin;[12] animal feeds: farm animal feeds,[38,49] pet food,[4,9,34,50] cereal flours,[7] fish feed;[33] biological samples: muscle tissue,[8,13] urine and plasma of pigs,[51] goat blood,[45] hen

plasma,[26] human urine,[30,52] chicken body fluids,[17] plasma,[24,29,53] urine and feces[53] of rats, and kidney[9,13,20,24,53] and other organs, such as liver, spleen, bladder, and brain[24,26,53] of several animals; and environmental samples: crop,[13] waste water,[10,13–15] and soil[10,13] samples. Melamine studies have been undertaken using a large variety of techniques, such as electrochemistry,[30] nuclear magnetic resonance (NMR),[47] immunoassay analysis (ELISA),[4,17] Raman spectroscopy,[40] direct mass spectrometry (MS),[5,16] and several kinds of sensors.[2,31,32] Because of the complexity of the matrices, higher signal-to-noise signals have been obtained using separative techniques, like electrophoresis,[33,39] thin layer chromatography,[41] gas chromatography-mass spectrometry,[10,17,19,30,34,38] and liquid chromatography. This last one can be considered, undoubtedly, the technique of choice for the quantification of melamine.[2,31,32] Indeed, many methods have been developed using different branches of HPLC, like ion exchange,[50] hydrophilic interaction,[3,8,13,20,24,29,36,44,45,46,49] turbulent flow,[35] ultraperformance liquid chromatography (UPLC)[42,43,53] and packed reverse phase, either by using ion-pairing,[11,26,37,38,42] or hydro-organic,[4,7,9,10,12,14,15,48,51,52] coupled to UV–visible absorbance diode array (DAD),[3,4,7,8,10–12,14,15,26,37,38,46,50,51] fluorescence[12,48] or MS[4,9,13,15,20,24,29,35,36,42–45,49,52,53] detection. This last instrumentation has the problem of easy contamination and high cost, considering both acquisition and maintenance, then only a few laboratories can afford it.

The determination of melamine in food, biological, and environmental samples by HPLC requires a preliminary sample processing to separate the analytes from the matrix to avoid the introduction of proteins, lipids, and other insoluble substances, which may precipitate in the chromatographic system and damage the stationary phase.[31,32] These sample pretreatments use tedious, time-consuming and multistep extraction, preconcentration and purification steps, such as leaching,[3] matrix precipitation,[8,24,29,35,42–44,46,48,53] and liquid [10,12,13,20,26,36–38,42,43,48–50,52,53] or solid [8,10,11,15,24,26,36–38,42,45,50,51] phase extraction, thus introducing additional sources of variance. In several cases, even various consecutive treatments are required. This improves the probability of loss of analyte, either by error operator, apparatus malfunction, or physicochemical change, thus reducing the recovery and increasing the uncertainty of the results. These effects can be partially corrected by the addition of an internal standard [3,13,20,35,42–44,49,52]. Besides,

they require specific instrumentation, and/or large volumes of toxic chemicals, which is harmful to the laboratory staff and the environment.[31,32] Online solid phase extraction–HPLC has been proposed to improve the automation degree, but it requires expensive and complex experimental assembly.[46] Recently, the use of micellar liquid chromatography (MLC) has been proposed as an excellent alternative for the quantification of melamine in a wide variety of matrices, such as milk,[54] dietetic supplements,[55] swine kidney,[56] waste and drinking water,[57] as well as human plasma and urine,[58] avoiding the above-described drawbacks.

3. Basic description of micellar liquid chromatography

Surfactants are amphiphilic compounds containing a hydrophobic chain (tail) bonded to a polar group (head), which can be cationic, anionic, neutral, or zwitterionic. In aqueous solutions, the surfactant monomers join to form a spherical shaped configuration: the normal-phase micelle, at concentrations over the critical micellar concentration (CMC). The nonpolar tails stay in the core to be isolated from water, whereas the polar groups are oriented to the water and configure an outer layer and are water solvated. The palisade layer is located in the intermediate region. For each surfactant, the micelles contain nearly the same number of monomers (aggregation number), and then an increase of the monomers in solution provokes an augmentation of the number of micelles. The hydrophobic interaction between the carbon chains sustains the structure of the micelle, whereas the polar groups are water solvated and interact with the aqueous environment, allowing the dispersion of the micelles throughout the solution. Therefore, micellar solutions are colloidal and microscopically heterogeneous, being composed of two distinct media: the amphiphilic micellar aggregates (micellar pseudo phase) and the surrounding bulk water or aqueous organic solvent that contains surfactant monomers in a concentration approximately equal to the CMC.[59,60] Micelles have several sites of solubilization: hydrophobic (core), hydrophilic (outer layer), miscellaneous (palisade layer), and, eventually, electrostatic (polar head for charged surfactants). Because of these multiplicities of environments, micellar solutions are able to interact with compounds in a large variety of charge, hydrophobicity and molecular mass, and consequently provoke their solubilization.[61,62]

Micellar liquid chromatography is a well-established branch of reverse phase (RP)–HPLC, which use micellar solutions as mobile phases, instead of hydro-organic ones.[63] The most used columns are those containing an octadecyl (C18)-bonded silica phase.[64] The micelles and the free surfactant monomers strongly affect the properties of both stationary and mobile phases. The monomers are adsorbed on the stationary phase, with the polar group oriented to the water, creating a structure similar to an open micelle. A polar and eventually charged outer layer appears on the stationary phase, while the inner layer turns more hydrophobic. The volume of the pores is reduced and the surface area increases resulting in an augmentation in the system pressure. Besides, the silanol groups are partially blocked facilitating the study of basic compounds. The stationary phase environment is independent of the micelle concentration, as the number of adsorbed monomers remains constant beyond the saturation point. In the mobile phase, the presence of the pseudo micellar phase introduces a new environment available for the solutes and then a secondary equilibrium. Besides, the solutes can be involved in a larger variety of interactions. These effects provoke a strong influence on the chromatographic behavior of the solutes, and complexes the retention mechanism, if compared to hydro-organic HPLC. The retention mechanism has been accurately described by the three-partitioning theory. It proposes that the analyte has available three differentiated environments and then considers three partition equilibria for the solutes: modified stationary phase ↔ bulk mobile phase; modified stationary phase ↔ micellar pseudo phase; and bulk mobile phase ↔ micellar pseudo phase. The distribution of the analyte is influenced by the hydrophobicity, charge and steric factor of the solutes, stationary phase; and micelles. As these parameters depend on the acid/basic side reactions, a buffer must be added to adjust the pH to the adequate value and maintain in constant during the whole chromatographic run.[62,65–68]

Pure micellar mobile phases hold a limited elution power because they are essentially polar. In addition, they provide relatively lower efficiencies due to the slow mass transfer between the micelle and the stationary phase. To avoid these drawbacks, a common strategy in MLC is the decrease of the polarity of the mobile phase by the addition of a water-soluble organic solvent, such as acetonitrile, methanol, ethanol, 1-butanol, or 1-pentanol (in increasing order of hydrophobicity). The solvent monomers adsorbed on the stationary phase with the hydroxy/cyano groups oriented toward the mobile phase and then increase its polarity. These

combined effects thermodynamically and kinetically impulse the transfer of the analyte from the stationary phase toward the mobile phase. Consequently, the retention, broadness and asymmetry of the peaks decrease. However, the organic solvent also may alter the characteristics, such as the structure, CMC, and aggregation number, of the micelle.[68] Beyond a specific proportion, micelles even disaggregate, as surfactant monomers remain preferably solubilized, to produce a high sub-micellar solution. This limits the maximum proportion of organic solvent useful in hybrid mobile phases. The influence of the organic solvent is enhanced as its hydrophobicity and its proportion increase and is noticeable even at low values. As long as the micelles persist in the mobile phase, the three-phase theory is also used to model the retention mechanism by considering that the organic solvent changes the value of the equilibrium constants.[62,66,69]

The retention mechanism in MLC depends on several experimental factors, such as type of column, pH, nature, and quantity of surfactant and alcohol, which must be set to provide the maximal resolution between the analytes and other compounds of the matrix at the minimum time with a good peak shape. Therefore, it is desirable to investigate the effect of these chromatographic conditions over the retention time, efficiency, asymmetry, and resolution of the peaks. The reproducibility and stability of the retention mechanism in MLC with isocratic mode have permitted the development of a mechanistic and empirical mathematical model, based on the three-partitioning theory, which permits the prediction of the chromatographic behavior from the composition of the surfactant and the organic solvent. This can expedite the resolution of complex mixtures.[67–69]

The remarkable characteristics of micellar solutions are significantly helpful for the sample preparation step for clinical,[70] environmental,[71] as well as for liquid[72] and solid[73] food analysis. Most of the hydrophobic and other non-water-soluble compounds are solubilized. Besides, micelles tend to bind proteins and other macromolecules provoking their denaturation. Therefore, they are barely retained and harmless eluted near the dead time, rather than precipitating into the column. After dilution in a micellar solution and filtration, the sample can be directly injected without extraction and cleanup steps. In this manner, the experimental procedure is strongly expedited, and less instrumentation and chemicals are used.[65]

4. Method Validation

4.1. Development and optimization of the analytical methods

Several analytical procedures based on MLC to quantify melamine in several matrices with quite different physical properties and chemical composition, such as milk[54], dietetic supplements[55], water,[57] body fluids,[58] and swine kidney,[56] have been previously described. These methods were optimized by selecting the main experimental conditions (sample preparation, column type, pH, kind and amount of surfactant, organic solvent and absorbance wavelength) to obtain a resolved peak of melamine, a normal-shaped peak with high efficiency, short analysis time, sufficient sensitivity to reach expected levels, high recovery, and low variability of the experimental results. Although these methods aim to determine the same compound, the optimization strategy was adapted to each matrix. Here, we summarize the development of these analytical procedures.

4.2. Sample preparation

As previously indicated, the use of micellar mobile phases allows the quantitative injection of liquid samples. For solid samples, a previous solid/liquid extraction is mandatory to recover the analyte in a solution. Despite the different composition of the studied matrices, the use of micellar solvents and mobile phases allows the treatment of dissimilar samples in a similar method. In general terms, liquid samples (milk and dietetic supplements, water, biological fluids, and water) were diluted in a micellar solution of 0.05 M sodium dodecyl sulfate (SDS) solution phosphate-buffered at pH 3, filtered and directly injected into the chromatographic system.[54,55,57,58] The powdered milk and dietetic supplements were reconstituted in water as recommended by the supplier and then processed as liquid samples.[54,55] Conversely, swine kidney (1 g) was solid/liquid extracted by ultrasonication with 2 mL methanol and then diluted with 3 mL of the same micellar solution.[56]

The filtration is essential to avoid the introduction of particles and nonsoluble compounds in the chromatographic system, which can provoke column clogging, stationary

phase deterioration, and increase of the pressure. The dilution in micellar media is needed to previously denature proteins and solubilize them together with other nonsoluble compounds.[70,73] The surfactant, buffer, and pH were set at the same values as in the mobile phase (Sections Selection of the surfactant and Selection of the pH).

The dilution ratio was optimized on the basis of avoiding the early blocking of the filter membrane, and then obtaining a solution enough representative of the whole sample, without excessively diminishing the sensitivity. The results are shown in Table 1.

Table 1. Optimized conditions for sample treatment and chromatographic run.

Matrix	Dilution ratio(v/v)	[SDS] (M) in mobile phase	[1-propanol] (%, v/v) in mobile phase	Melamine retention time (min)	References
Milk	1/10	0.05	7.5	9.3	55
Dietetic Suplements	1/10	0.15	0	8.1	56
Swine Kidney	1/5	0.11	7.5	6.0	57
Plasma	1/5	0.2	0	6.3	58
Urine	1/5	0.2	0	6.3	58

Waste and drinking water do not need previous dilution because they contain a low amount of particles.[57] Conversely, milk and dietetic supplements have a huge amount of suspended substances, such as proteins, fats, and oligosaccharides, so that a 10-fold dilution is required before the filtration.[54,55] Plasma and urine contain less concentration of proteins and fats than milk, then a fivefold dilution was enough.[58] Swine kidney analysis requires a different approach because it is a solid matrix with a high content of proteins, fats, cholesterol,

vitamins, electrolytes, and secondary metabolites. Moreover, melamine can be found in both the inner and outer layer of the particles. Therefore, the solid/liquid extraction with an organic solvent and ultrasonication was mandatory to recover melamine, together with other soluble substances and suspended particles, in an injectable solution. This extract was finally diluted in the micellar solution to a final ratio of 1/5 to facilitate the filtration and work with a nonvolatile solvent close to the mobile phase (Table 1).[54–58]

4.3. Chromatographic conditions

In all cases, the stationary phase was in a column with a hydrophobic C18 coating (reverse phase): 150 x 4.6 mm2 i.d., pore size 100 Å, and particle size 5 μm; the most widely used column in MLC to resolve basic compounds.[64] The mobile phase runs at a constant flow rate of 1 mL/min, and 20 μL of each processed solution was injected using an autosampler device.

Absorbance was selected for detection. The spectrophotometric properties of a solute are enhanced in a micellar environment, resulting in an increase of the wavelength and the absorptivity.[67] Therefore, the optimal wavelength was performed by taking the absorption spectrum of melamine in the 200–300 nm range, during the chromatographic analysis of a standard solution by MLC. Therefore, the optimization was performed at the same conditions as applied to the real samples. As the maximum signal-to-noise ratio was found at 210 nm, the detection of absorption wavelength was set at this value.[54]

4.3.1. Selection of the surfactant

The surfactants more used in MLC are SDS, cetyltrimethylammonium bromide (CTAB), polyoxyethylene 23 dodecyl ether (Brij-35R), and *p*-octyl benzene polyoxyethylene 9.5 alcohol (Triton™ X-100). These surfactants are described in Table 2.[62] The ionic surfactants are provided as salts, which totally dissociate in water. The Krafft point (for ionic surfactants) and the cloudy point (for nonionic surfactants) are the temperatures at which the

solubility of the surfactant equals the CMC and the surfactant forms a separate cloudy phase, respectively.

Sodium dodecyl sulfate was selected for the analysis because of its practical performances: it is an easily manageable powdered solid, harmless, biodegradable, relatively inexpensive, with adequate CMC, Krafft point under laboratory temperature, highly soluble in water, low viscosity of aqueous solutions, and a relatively low increase in system pressure and ability to denature and dissolve proteins. In fact, SDS can be considered as the surfactant of choice for MLC, and it has been selected in at least 90% of the analytical reports.[62,66]

In a C18 stationary phase, the hydrocarbon tail is bonded by hydrophobic interaction with the immobilized hydrocarbon chain, whereas the sulfate group is oriented outward. Therefore, an anionic outer hydrophilic layer appears, which affects the penetration depth of the solutes and provides some ion exchangeability, as in ion-pairing HPLC. Otherwise, the hydrophobicity of the inner alkyl-bonded phase is enhanced.[66]

The quantity of surfactant adsorbed on the stationary phase has important implications regarding the retention behavior of the solutes. Several studies have shown that this value rapidly increases with the concentration of SDS in the mobile phase and remains constant beyond 10 mM (saturation point) [69]. MLC analysis must be conducted using a saturated stationary phase to keep constant the extent of the modifications induced by the use of a surfactant. Otherwise, the use of excessively high concentrations of SDS is not recommended because of the high viscosity of the mobile phase, the excessive diminishing of the efficiency, and the increase of the background noise in the detector [62]. Therefore, the working range was restricted from 50 mM (several times the CMC) to 200 mM [61,62].

Table 2. Main parameters (in aqueous solution) of the surfactants most used in MLC.

	SDS	CTAB	Brij-35	Triton X-100
Charge formula	Anionic	Cationic	Nonionic	Nonionic
Hydrophobic tail	Linear hydrocarbon	Linear hydrocarbon	Linear hydrocarbon	Branched hydrocarbon–phenyl
Polar head	Sulphate	Quaternary amine	Polyethylene oxide chain	Polyethylene oxide chain
Molecular weight (g/mol)	288.4	364.5	1225	625
CMC (mM)	8.1	0.83	0.06	0.3
Aggregation number at 25°C	62	90	41	140
Partial specific volume (L/mol)	0.246	0.364	1.12	0.743
Krafft point (°C)	16	26	-	-
Cloudy point at 1–6% solution (°C)	-	-	100	64

SDS: sodium dodecyl sulfate; CTAB: cetyltrimethylammonium bromide.

4.3.2. Selection of the pH

Because of the basic activity of melamine, the pH must be optimized and fixed to avoid the oscillation of analyte properties during the elution. In C18-bonded silica columns, the working range pH is from 2.0 to 9.5. However, the use of even slightly basic and extreme acidic solutions is not recommended because it causes a slow and continuous degradation of the stationary phase.[75] Average values of pH (around 5.0) were also discarded as the two forms of melamine coexist. Therefore, the study was restricted to 3 and 7. A phosphate buffer was selected to fix the pH as it works in the 2–3 and 6–8 range.

According to its low value of Po/w = -1.4, melamine is a quite polar compound. Therefore, it would barely interact with an SDS-modified C18 stationary phase by means of hydrophobic interaction. Adequate retention would be only acquired by the electrostatic interaction between a positively charged melamine and the negative charge of the modified stationary phase.[76] This was confirmed experimentally as melamine elutes at the dead time (≈ 1.0 min) at pH 7, while, at pH 3, a workable retention time (<20 min) was obtained. Consequently, the optimal mobile phase pH was set to 3.[54]

4.3.3. Selection of the organic solvent

Because of its polarity, melamine elutes at a useful retention time using pure aqueous mobile phases.[54] The addition of an organic solvent was investigated to regulate the retention time and improve the efficiency. Finally, 1-propanol was selected because of its average hydrophobicity: 1-butanol and 1-pentanol are too hydrophobic, and acetonitrile, methanol, and ethanol require a higher proportion to give the same effect as 1-propanol.[62,67]

The presence of 1-propanol affects the micellization process. The selected alcohol solvates the sulfate group and reduces its electrostatic repulsion between the monomers, facilitating the formation of the micelle and diminishing the CMC.[62] Otherwise, the interaction of the organic solvents with SDS micelles increases their solubility and reduces their evaporation rate.[65] The maximal concentration of 1-propanol compatible with the existence of the micelles is 15%. Therefore, the studies were conducted between 2.5 and 12.5% of 1-propanol.[61,62]

4.3.4. Optimization of the SDS/1-propanol concentrations

The amount of SDS and 1-propanol must be optimized to elute melamine without interferences at the minimum analysis time. The possible interfering compounds are not the same for the studied matrices, according to their different chemical composition. Therefore, the challenge to establish the optimal concentration of SDS/1-propanol was separately undertaken.[54–58]

In all cases, hybrid mobile phases containing SDS/1-propanol and pure SDS micellar mobile phases were tested. From the preliminary studies, it was found that the elution strength and the efficiency increase at higher proportions of 1-propanol as expected in MLC. Otherwise, the retention time and efficiency decreased at higher values of SDS concentration. This behavior points to an attractive interaction between melamine and the SDS micelles, probably by electrostatics.[62,68]

The optimization was separately performed for each matrix. A large number of peaks were detected near melamine peak in milk, dietetic supplements, swine kidney, wastewater, and urine. For each matrix, the SDS/1-propanol amounts were selected on the basis of

maximizing the resolution between melamine and the interfering compounds eluting immediately before (P1) and after (P2) melamine (these compounds were not identified and were different for each matrix). An interpretative strategy was applied with the aid of a mathematical model based on several mechanistic equations, which relate the chromatographic behavior of a solute to the SDS concentration and 1-propanol proportion in the mobile phase, by maintaining the other chromatographic parameters at a constant value. This chemometric model is based on the three-phase partitioning theory.[69] Two separate mechanistic models were constructed for hybrid and pure mobile phases, respectively, and then the data obtained for these mobile phases were independently processed. The retention factor (k) and the peak shape were modeled by Eqs. (1) and (2), respectively:[69]

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

$$h(t) = H_0 e^{-0.5\left(\frac{t-t_R}{s_0+s_1(t-t_R)}\right)^2} \quad (2)$$

The symbol k means the retention factor; K_{AS}, the partition constant of the analyte between the modified column and the bulk mobile phase times the volume phase ratio; K_{AM}, the partition constant of the analyte between the micelle and the bulk mobile phase; K_{AS} and K_{MD} refer to the modification of K_{AS} and K_{AM}, respectively, induced by the organic solvent; $h(t)$, the height of the detector signal due to the elution of the compound; and H_0, the maximal peak height. H_0 was taken as default value as it depends on the concentration and the sensitivity.[69] The constant s_0 quantifies the broadness of the Gaussian curve and $s1$, the distortion of the normal model.[69] These parameters can be calculated as indicated in.[77]

For each compound (melamine, P1, and P2), these two equations were adjusted using the MICHROM software,[78] taking the experimental values of k, N, and B/A obtained by the following mobile phases:

– Hybrid mobile phases: SDS (M)/1-propanol (%, v/v): 0.05/2, 0.05/12.5, 0.1/7.5, 0.15/2.5, and 0.15/12.5%. They were selected by a full factorial design plus the central value: the combination of the maximal and minimal concentration recommended for SDS and 1-propanol in MLC and the intermediate concentrations.[61,62]

– Pure micellar mobile phases: SDS (M): 0.05, 0.10, 0.15, and 0.20 M. Four values between the minimal and maximal working concentrations of MLC.[61,62]

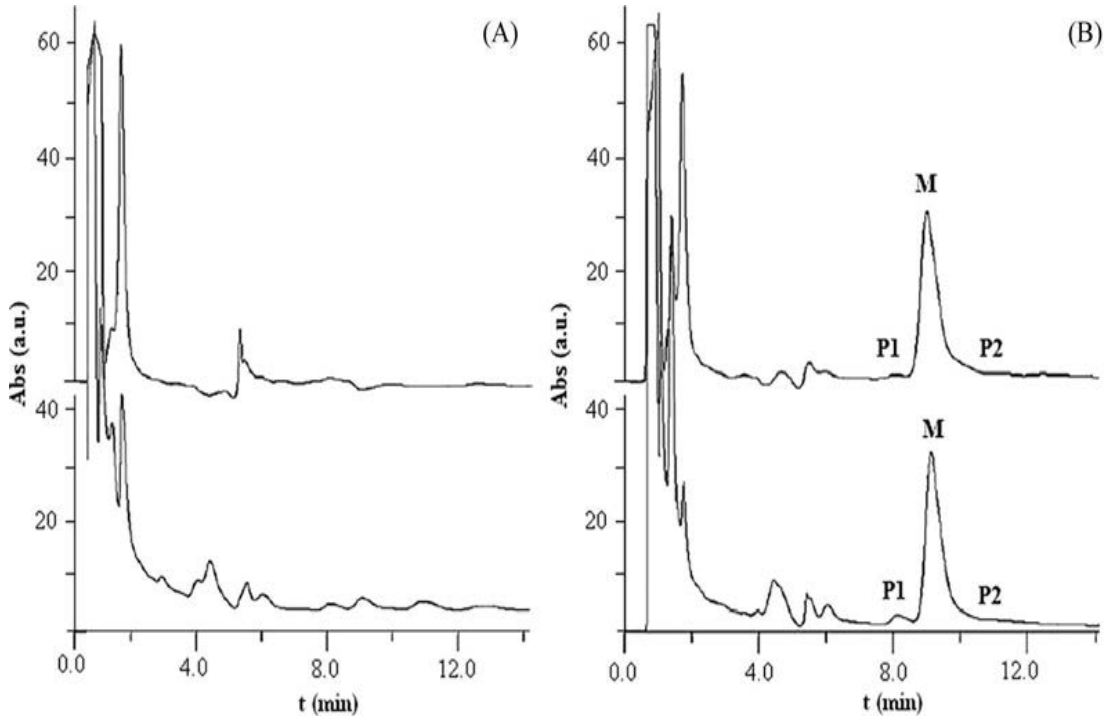


Figure 1. Chromatograms of (a) blank milk and (b) milk samples—2 mg/L melamine spiked. The top chromatograms correspond to Nutriben continuación" (Alter Group, Seville, Spain) and "Blemil Plus 2A" (Laboratorios Ordesa, Sant Boi de Llobregat, Spain). Both were powdered milk for infants supplied by a local chemist.

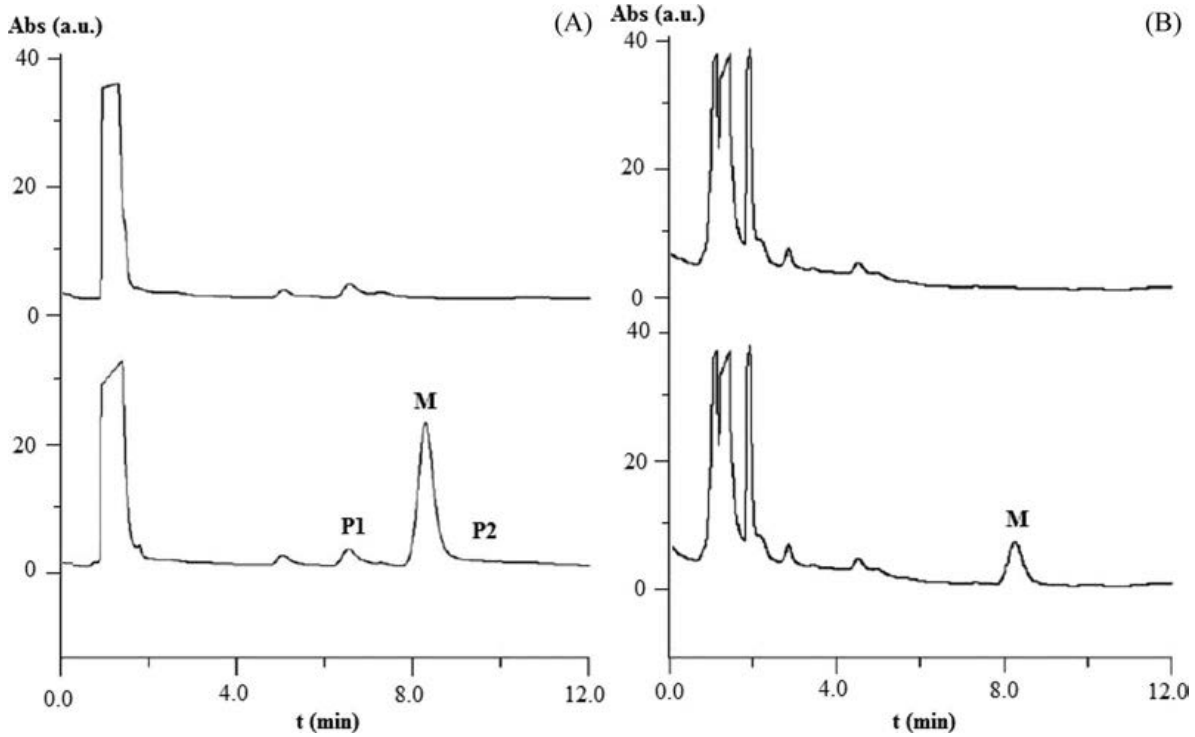


Figure 2. Chromatograms corresponding to the following powdered dietetic supplements provided by a local chemist: (top) PediaSure Vanilla (Abbott Laboratories Spain, Madrid) for infants and (bottom) Nutrinovex Endurance (Grupo Farmacéutico de Levante, Castelló, Spain) for adults. In both cases (a) and (b) refer to blank matrix and 2-mg/L melamine spiked sample.

Once fitted, the equations are able to predict the chromatographic behavior of these substances at intermediate values by interpolation. The theoretical values of *k, N*, and B/A obtained for the three analytes, at each SDS/1-propanol concentration, were combined to calculate the individual (r_{ij}) and global resolution (Z), following the valley-peak and the unnormalized product, respectively.[69] Besides, the equations can be used to draw simulated chromatograms, and then the analyst can visualize the theoretical changes of retention time and peak shape at slight variations of SDS and 1-propanol concentration. The optimal conditions were selected on the basis of obtaining the maximum resolution at an analysis time as short as possible but far enough from the front of the chromatogram. The results can be seen in Table 1. The error in the prediction of the retention factor was <4%. The chromatograms obtained by the analysis of melamine-spiked milk, dietetic supplements, swine kidney, urine, and wastewater samples can be seen in Figures 1–5, respectively. In all figures, P1 and P2 represent the peaks appearing immediately before and after melamine, respectively. The obtained chromatograms show dissimilar shape because of the differences in the optimal mobile phase and the chemical composition of the matrix.

Unlike the other studied samples, plasma matrix (Figure 6) and drinking water chromatograms show a clean baseline without any peak at >4 min at any SDS/1-propanol combination. Then all the studied mobile phases can be used without overlapping risk. For plasma samples, the same chromatographic conditions as urine were suggested to allow the simultaneous analysis of both body fluids by clinical laboratory for bioanalytical purposes.[58] Drinking water was analyzed using the same mobile phase as wastewater, due to the similitude of both matrices, to enable the study of aqueous samples using the same chromatographic program.[57]

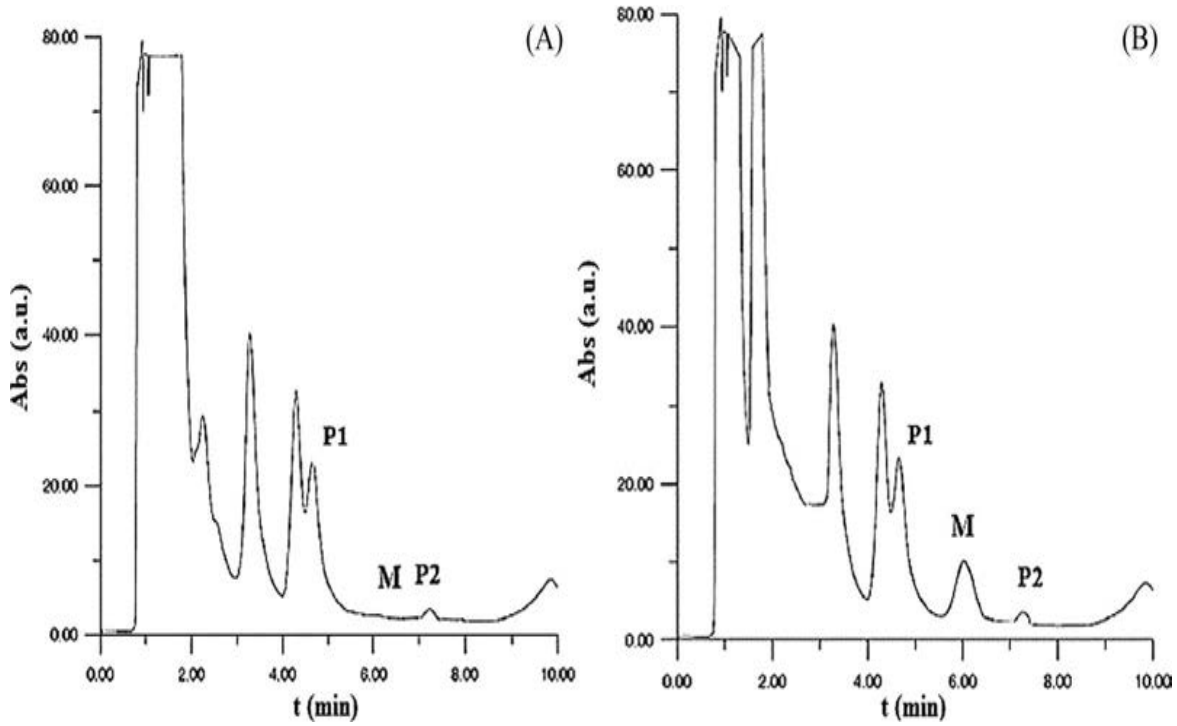
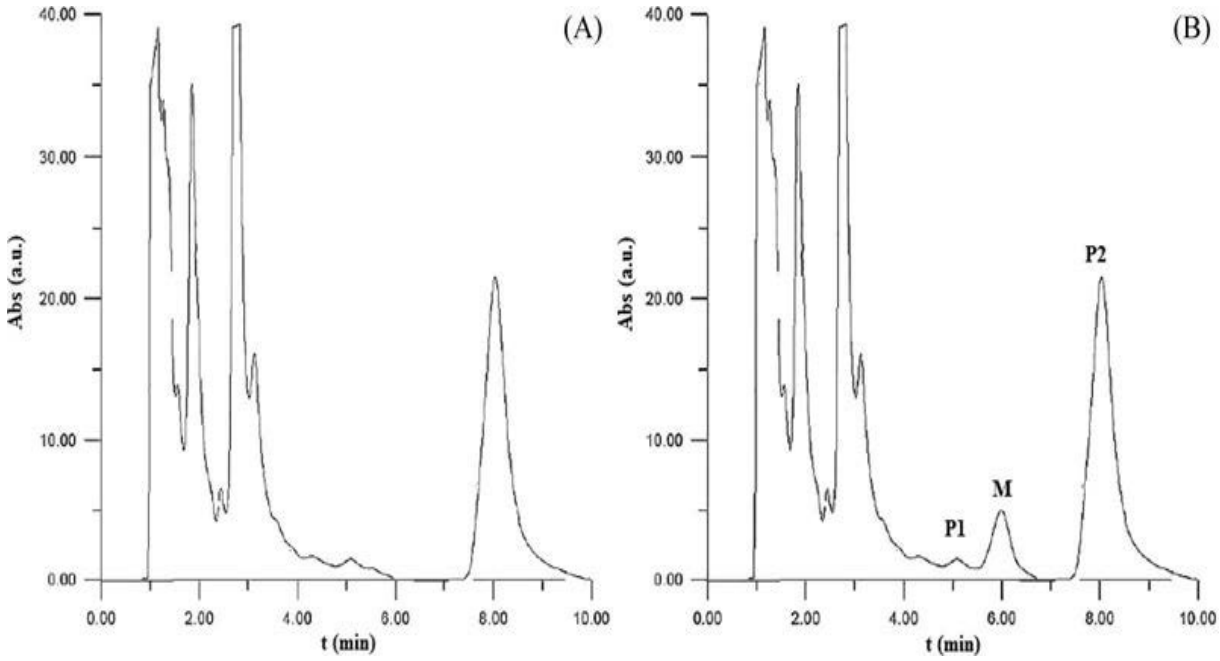


Figure 3. The chromatogram obtained by the analysis of swine kidney sample: (a) blank and (b) spiked with 0.3 mg/kg (M) of melamine.



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506 **Figure 4**. Chromatograms corresponding to the analysis of urine: (a) blank and (b) spiked with 2.5 mg/L melamine.

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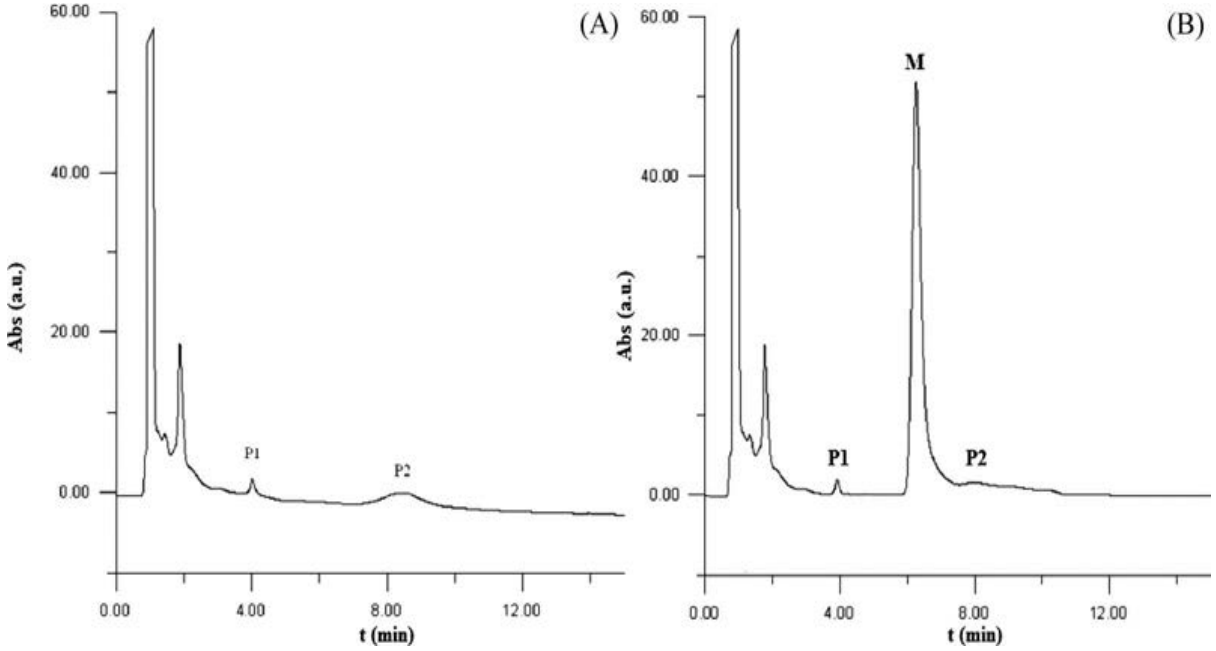

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513 **Figure 5.** Chromatograms obtained by the analysis of output wastewater from a chemical company which produces Nylon and
514 other organic compounds: (a) blank and (b) after spiking by 1 mg/L of melamine.

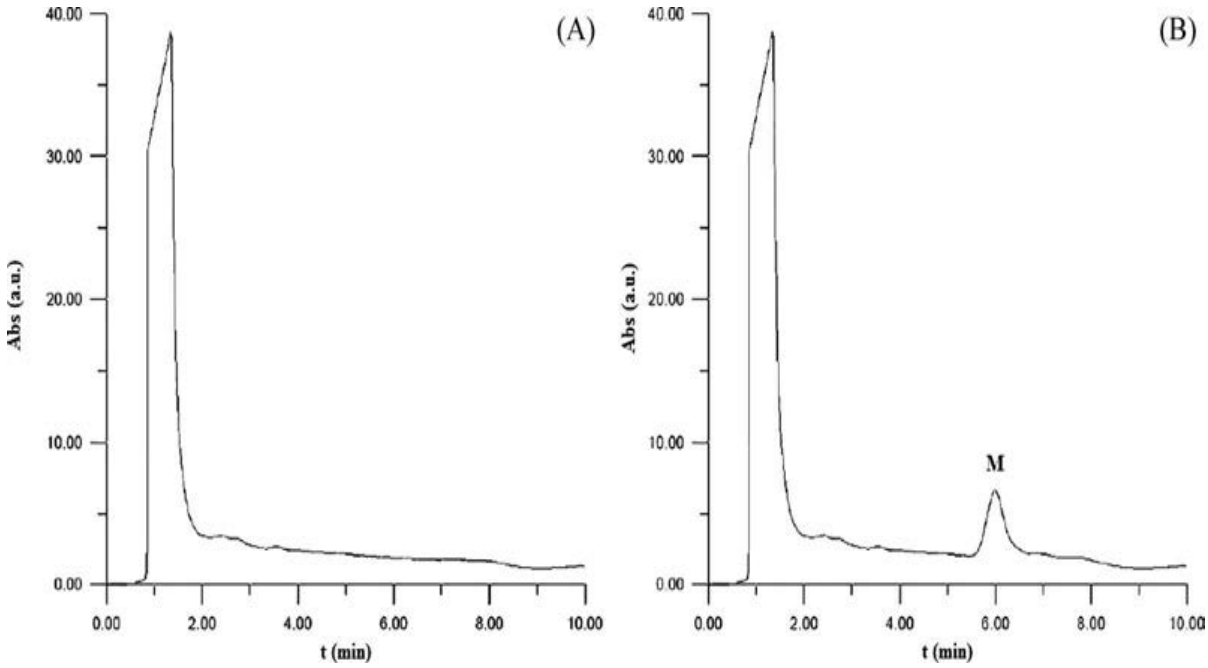

Figure 6. Chromatograms obtained by the analysis of plasma: (a) blank and (b) spiked with 2.5 mg/L of melamine.

4.4. Validation of the procedures

The presented analytical procedures were separately and successfully validated, as part of their development, following the FDA (body fluids, water and swine kidney)[79] and EU Decision 2002/957/EC (milk and dietetic supplement)[80] guidelines. The studied validation parameters were selectivity, sensitivity, the limit of detection (LOD), the limit of quantification (LOQ), calibration range, linearity, intra- and interday accuracy and precision, and robustness. A detailed description of each validation parameter [74] can be found in Table 3 (calibration and sensitivity), 4 (accuracy and precision), and 5 (robustness), respectively. The validation was performed using spiked samples, and the concentration values refer to the quantity in the unprocessed sample, not in the injected solution.

Table 3. Comparison of the sensitivity and linearity for melamine detection of the considered analytical methods (melamine concentration in mg/L or mg/kg, for liquid or solid matrices, respectively).

Matrix	LOD	LOQ	Calibration Range	Linearity (r^2)	References
Milk	0.05	0.2	0.20-100	0.9990	[54]
Dietetic Supplements	0.09	0.2	0.20-100	0.9996	[55]
Swine Kidney	0.1	0.3	0.3-30	0.9997	[56]
Water	0.013	0.03	0.03-5	0.9998	[57]
Plasma	0.05	0.25	0.25-25	0.9995	[58]
Urine	0.05	0.25	0.25-25	0.9996	

5. Discussion and comparison of the analytical procedures

In the studied matrices, melamine was successfully identified, the peak area was measured without overlapping, and reliably quantified thus assessing the reliability of the procedures and their applicability to real and commercial samples. The similarities and the differences of the described methods, in terms of sample treatment, chromatographic conditions, and validation results, are deeply discussed. In general, we can see that similar points are related to the analyte, while the different parameters are those depending on the composition of the matrices.

5.1. Chromatographic conditions and selectivity

548 The main chromatographic conditions, such as stationary phase, surfactant, pH, and
549 absorbance wavelength, are the same in all works because they have been uniquely selected
550 for their ability to provide an adequate resolution, retention time and sensitivity. The SDS/1-
551 propanol concentrations were different because they were optimized considering the specific
552 substances of the matrix. The retention time of melamine was relatively short and similar in all
553 cases (6.0–9.3 min; Table 1).[54–58]

554 In all cases, no compounds appear at the window time of melamine. For plasma and
555 drinking water, no other peaks were observed beyond >4 min, and then no confusion in the
556 identification of melamine was possible. For the other matrices, the optimized chromatographic
557 conditions allow avoiding coelution between melamine and the two nearest compounds P1 and
558 P2. In milk, these compounds elute at -1.2 min and +1.7 min; in dietetic supplements, at -2 min
559 and +1 min; in urine, at -0.9 min and þ2.0 min; in wastewater, -2.2 min and +1.9 min; and in
560 swine kidney, -1.5 min and +1.1 min, taking as reference the retention time of melamine. In

561 four cases, the peaks were eluted far enough from melamine and no overlapping was detected.
562 Therefore, a similar adequate specificity was obtained for the studied matrices.

563

564 *5.2. Calibration range*

565 The minimal level of the calibration curve was taken by visual appreciation, whereas
566 the maximal concentration level was established as the higher concentration expected in this
567 kind of sample. Therefore, the studied calibration interval for each matrix was selected as the
568 working range of practical applications, and adequate linearity (r2 >0.9990) was found (Table
569 3).[54–58]

570 The method for analysis of water was developed to cover low concentrations (0.03–5
571 mg/L). Indeed, contaminated wastewater from industry or crops is normally diluted in
572 irrigation ditches, rivers, and barrages, resulting in microgram per liter levels. Otherwise,
573 drinking water is not expected to contain melamine, as it is supposed to have undergone several

574 strong purification processes.[57] For milk and dietetic supplements, the calibration range
575 covers a wide interval of concentration levels (0.2–100 mg/L). Indeed, we expect moderate/

elevated concentrations of melamine in adulterated samples because melamine is deliberately added to increase the price of the product.[54,55] In fact, milk samples with a high concentration of melamine (up to 3300 mg/L) were found during China incident in 2008.[5] The upper calibration range was inferior for swine kidney, plasma, and urine, as the expected concentration in a practical case is significantly lower than in foodstuff, as, after ingestion, melamine is rapidly absorbed, distributed, and eliminated, without bioaccumulation. This prevents melamine from getting a too high concentration in body tissues and fluids. A high level is only obtained either immediately after the intoxication or by continuous ingestion of adulterated food.[13,27]

5.3. Sensitivity

The sensitivity (Table 3) was sufficient to detect melamine at the expected concentration in each kind of sample. As the measured absorbance wavelength and the injected volume were the same for all procedures, the differences in sensitivity were due to the sample pretreatment and the chemical composition. In addition, they are able to detect noncompliant samples, which is extremely useful for quality control purposes.[54–58]

The limits of detection and quantification were lower for water samples because no dilution was applied in the experimental procedure.[57] The other matrices show similar values of sensitivity. Indeed, urine, plasma, and swine kidney use a lower dilution ratio than milk and dietetic supplements, but the biological fluids and the methanolic extract of swine kidney contain a higher amount of solubilized compounds. These compounds are not removed by the filtration and produce a higher noise in the baseline.

5.4. Accuracy, precision and robustness

601 The values of accuracy, precision (Table 4), and robustness (Table 5) were similar for
602 all the methods. Indeed, the sample pretreatment is comparable and the matrix effects are
603 negligible because melamine is effectively separated from the matrix compounds during the
604 chromatographic run. Besides, they fit the requirements of the validation guidelines. Therefore,
605 the methods provide reliable quantitative values of melamine concentration, and their
606 performances barely change through time and with slight variations of the experimental
607 running conditions.[54–58]

608

609 **Table 4**. Comparison of the accuracy (calculated concentration/true concentration, %), precision (R.S.D., %), and recovery
610 (%) for melamine quantification for each studied matrix.

		Intraday		**Interday**		
Matrix	**Evaluated Levels**	**Accuracy**	**Precicion**	**Accuracy**	**Precicion**	**References**
Milk	9	85.0-106	< 7.6	90.2-105.9	< 9.7	[54]
Dietetic Supplements	4	85.8-114.3	< 12.4	95.0-101.3	< 10.5	[55]
Swine Kidney	4	91.7-103.6	< 7.2	91.0-103.3	< 7.6	[56]
Water	4	87.8-104.1	< 14.8	93.3-101.6	< 13.4	[57]
Plasma	4	85.7-103.7	< 10.2	94.8-103.6	< 9.1	[58]
Urine	4	92.2-103.8	< 9.3	93.6-103.5	< 9.1	

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Table 5. Variation (RSD, %) of retention time and peak area at slight changes in the main chromatographic conditions.

	SDS		1-Propanol		pH		Flow Rate		References
Matrix	t_R	A	t_R	A	t_R	A	t_R	A	
Milk	7.4	2.7	1.3	4.5	1.8	5.0	5.2	2.0	[54]
Dietetic Supplements	4.8	2.3	-	-	7.2	5.0	4.7	1.6	[55]
Swine Kidney	1.6	4.1	2.7	5.0	2.0	3.5	5.2	4.2	[56]
Water	1.7	2.9	1.8	4.0	1.2	9.0	5.1	5.1	[57]
Plasma	2.7	4.6	-	-	0.22	7.1	4.9	7.1	[58]
Urine	2.7	6.7	-	-		4.6	5.0	4.5	

SDS: sodium dodecyl sulfate. n = 3.

5.5. Advantages of the procedures

The here-presented procedures show significant benefits over those based on hydro-organic HPLC, which disadvantages in the sample preparation have been described in Section "Basic description of micellar liquid chromatography".[2,31,32]

The main feature of these MLC assays is, undoubtedly, the strong simplification of the sample preparation, which can be performed in 2–3 min. Effectively, the strong solubilization power of micellar solutions allows the direct injection of the samples, even from the diverse origin, without risk for the column, after a simple dilution and filtration. Therefore, tedious and time-consuming extraction or cleanup steps or complex and expensive online systems for sample purification, normally used in hydro-organic RP–HPLC, are avoided. As the sample is quantitatively introduced in the chromatographic system, the addition of an internal standard is not needed. The participation of the operator, the number of steps, the complexity of the experimental handling, and then the potential sources of variance are minimized. This reduces the probability of analyte loss, either by side reactions, low yielding in the cleanup step, or

operational or instrumental error, thus increasing the sample throughput and the reproducibility of the method.

The use of micellar solutions also improves the separation performances. Proteins and other molecules are barely retained because of their strong affinity with the SDS micelles. Besides, the modification of the stationary phase, due to the adsorption of the anionic surfactant, improves the retention of the protonated melamine, and then its elution can be delayed until most of the possible interfering compounds have been removed. The use of an isocratic mode, instead of a gradient, allows the successive injection without stabilization time, thus reducing the effective duration of the chromatographic run and the production of waste.

These MLC procedures hold important practical advantages. The used solutions contain innocuous and biodegradable reagents (SDS, phosphate buffer, and salts), and only a small amount of toxic, flammable, and volatile organic solvent: <2 mL of methanol per sample in the dilution solution and <7.5% of 1-propanol in the mobile phase. These values are significantly less than usually required in hydro-organic RP–HPLC. In addition, the alcohol becomes less dangerous in micellar solutions, as its volatility decreases. Therefore, the operator is barely exposed to dangerous chemicals, and the produced waste contains a minimum proportion of pollutants. This fits the current trend in analytical chemistry. Besides, the work and expenditure related to waste segregation and treatment are reduced.[81]

A large number of samples per day can be processed using these procedures due to the short global analysis time and easy experimental work. Therefore, they are particularly useful in laboratories receiving many samples which must be quickly analyzed, such as clinical, food safety, and environmental ones. Besides, the methods only require basic apparatus and instrumentation, and a small number of inexpensive reagents, and then the analysis of the samples can be performed at a reasonable price, which is interesting in the current context of economic crisis.

6. Conclusions

In the present review, we prove the applicability of MLC for the determination of melamine in several kinds of samples, which must be controlled to evaluate and manage the impact of this compound on the consumer's health: milk, dietetic supplements, swine kidney, plasma, urine, as well as drinking, and wastewater. The strategy for the development and validation of the analytical assays, as well as the finally obtained results, has been described. We have also outlined and explained the similarities and differences between them. Besides, the advantage of using MLC over hydro-organic RP–HPLC has been discussed.

The main feature of the procedures is their simplicity, so that it consists of a one-step assay based on dilution and direct injection, in spite of the diversity and complexity of the studied matrices. Only the solid sample had to be liquid extracted before the dilution. The experimental and chromatographic conditions were the same for all methods, except the dilution ratio and the SDS/1-propanol concentration, which was adapted to the chemical composition of each sample. In all cases, melamine was resolved from the endogenous compounds of the matrix in <12 min with an adequate peak shape and sufficient sensitivity. In general, the procedures and their corresponding performances were found quite similar, and the differences were because of the dissimilar chemical composition of the sample. The methods were validated in spiked samples, following the requirements of official validation guidelines, and then successfully applied to practical cases. No significant differences in terms of selectivity, linearity, accuracy, precision, and robustness were found between the studied matrices. However, the water analysis method shows higher sensitivity and its calibration range cover a lower concentration interval, while these parameters were similar for the other matrices.

According to the validation results, the methods are able to reliably detect and quantify melamine at the permitted levels for dairy products, as well as to detect slight melamine intoxication through the biological fluids or industrial spills in environmental water. Besides, the here-described analytical procedures were relatively inexpensive, easy-to-handle, rapid, safe, eco-friendly, accessible, efficient, reliable, and useful for routine analysis. According to the practical aspects and the validation results, MLC can be considered as an attractive and alternative to be implemented by analytical laboratories devoted to the global preventing and managing of the melamine menace.

5. Conflict of interest

The authors state that there is no commercial/financial conflict of interest.

6. Acknowledgements and funding

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