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Determination of melamine in milk powder by capillary electrophoresis

Žana Černova,

Ina Razmislevičienė,

Audrius Padarauskas*

Department of Analytical and Environmental Chemistry, Vilnius University, Naugarduko 24, LT-03225 Vilnius, Lithuania

Capillary electrophoretic (CE) technique was developed for a rapid determination of melamine. The optimized separations were carried out in 50 mmol/l tris(hydroxymethyl) aminomethane-phosphate electrolyte (pH 2.5) using a capillary coated with a poly(diallyldimethylammonium chloride)/poly(sodium-4-styrenesulfonate) bilayer and direct UV detection at 214 nm. The calibration curve was linear in the concentration range 0.2–10.0 mg/l for melamine with correlation coefficients higher than 0.9997. The detection limit was 0.06 mg/l. The proposed method was applied for a rapid determination of melamine in milk powder samples. The recovery tests established for real samples were within the range 93.5–104%.

Key words: melamine, capillary electrophoresis, milk powder

INTRODUCTION

Melamine (1,3,5-triazine-2,4,6-triamine; MEL) is a nitrogen-containing compound (Fig. 1) used in the production of plastics, flame retardants and melamine–formaldehyde resins for surface coatings, laminates and adhesives, but it is not approved as an ingredient in food [1]. However, due to the high nitrogen content (66% by mass), MEL is deliberately added to food or food-related products because protein content is usually estimated by determining the nitrogen content, and the added MEL can boost the nitrogen content of the products so as to make them appear to have more protein and to reduce the costs. In 2008, several companies in China were implicated in a scandal involving the milk and infant food formula which had been adulterated with melamine, leading to kidney stones and other renal failures, especially among young children. Nearly 300,000 people had become ill, with more than 12 800 hospitalizations and four infant deaths. Determination of melamine in food, and particularly in dairy products for children, is therefore of great importance to ensure food safety.

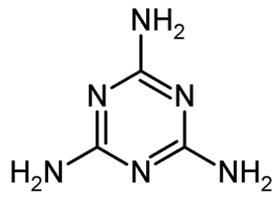

Fig. 1. Structure of melamine

High-performance liquid chromatography (HPLC) is a commonly used technique for determination of melamine in a wide variety of samples, such as soil [2], animal feeds [3], plant matter [4] and food products [5, 6]. However, most of the HPLC methods employ a mass spectrometer (MS) as a detector, which is very expensive compared with conventional UV detectors. Moreover, for HPLC methods, additional sample clean-up procedures are often required.

The analysis of complex biological samples requires the highest level of separation selectivity control and column efficiency. In the case of charged analytes, capillary electrophoresis (CE) provides an excellent alternative to HPLC. The main advantages of CE over HPLC include low solvent consumption, high speed and efficiency, simplicity and reduced sample preparation time. Several comprehensive surveys of CE methods developed for determination of ionic compounds in a wide variety of matrices have been published [7–9]. Recently, Klampfl et al. [10] analyzed melamine by CE utilizing UV and MS detectors but the run time was not particularly fast (15 min).

The aim of this work was to develop a rapid and simple CE method for determination of melamine in milk powder.

* Corresponding author. E-mail: audrius.padarauskas@chf.vu.lt

EXPERIMENTAL

Separations were performed on a P/ACE 2100 apparatus (Beckman Instruments Inc., Fullerton, CA, USA) equipped with a UV detector with wavelength filters (200, 214, 230 and 254 nm). Fused silica capillary (Polymicro Technology, Phoenix, AZ, USA) of 75 μm I.D. and 57 cm total length (50 cm to the detector) was used. Samples were introduced in the hydrodynamic mode by overpressure ($3.43 \cdot 10^3$ Pa). System Gold software (Beckman Instruments Inc.) was used for data acquisition. Direct UV detection was employed at 214 nm. All experiments were conducted at 25 °C using a liquid thermostated capillary cartridge.

Melamine (≥99.5%), poly(diallyldimethylammonium chloride) (PDDAC, 20 wt. % in water, average molecular weight ~100000–200000), poly(sodium-4-styrenesulfonate) (PSS, average molecular mass ~70000) and tris(hydroxymethyl)aminomethane (Tris) were purchased from Sigma-Aldrich (Milwaukee. WI, USA) and used as received. Ethylenediamine was purchased from Merck (Darmstadt, Germany). All other reagents were of analytical-reagent grade and obtained from Sigma-Aldrich.

All electrolyte and standard solutions were prepared using double-distilled degassed water. Stock standard solution (100 mg/l) of MEL was prepared in 0.01 mol/l HCl. Working standard solutions were prepared by appropriate dilution with water. Carrier electrolytes were prepared by neutralization of 0.05 mol/l H_3PO_4 solution with Tris to pH 2.5.

All electrolyte and sample solutions were filtered through a 0.2 μm membrane filter. Each new fused-silica capillary was flushed with 1 mol/l NaOH for 10 min and then with deionized water for 10 min. The first layer of the polymer was deposited by flushing the capillary with 0.1% (w/v) PDADMA, prepared in 0.5 mol/l NaCl, for 5 min followed by a 5 min water rinse. Next, the capillary was flushed with 0.1% (w/v) PSS, prepared in 0.5 mol/l NaCl, for 5 min and water for 2 min. Finally, the capillary was flushed with the carrier electrolyte for 2 min. Between all electrophoretic separations the capillary was rinsed with carrier electrolyte for 2 min.

Milk powder samples were obtained from a local supermarket and were prepared according to a slightly modified procedure described in [10]. In brief, an aliquot of 0.5 g of the milk powder sample was dissolved in 5 ml of 0.01 mol/l HCl. Then, an aliquot of 5 ml of dichlormetane was added, the solution was vortexed for 1 min and centrifuged at 5000 rpm for 5 min. The aqueous phase was diluted with 5 ml acetonitrile, vortexed for 1 min and centrifuged for 5 min. The supernatant was evaporated to about 1 ml under a stream of nitrogen (50 °C), diluted to 5 ml with a carrier electrolyte, filtered and analyzed.

RESULTS AND DISCUSSION

In order to obtain a well-shaped and symmetrical peak, the mobility of the electrolyte cation should match the mobility of the analyte as closely as possible [11, 12]. In addition, buffering the electrolyte is essential for reproducible and rugged separations [13]. This factor should be especially important in the CE analysis of a weak base such as melamine. In order to obtain a high efficiency and pH stability within a short time of analysis, the electrolyte nature and pH were optimized.

To examine the influence of different electrolyte co-ions on the separation performance, ethylenediamine, triethanolamine and Tris cations were compared. All experiments were performed in an electrolyte containing 50 mmol/l of H_3PO_4 neutralized with an appropriate base to pH 3.0. Phosphate exhibits buffering properties in the pH range 1.2–3.2, is transparent in the UV range and is well suitable for the preparation of CE electrolytes. Figure 2 compares the electropherograms obtained for the MEL standard in the electrolytes studied. One can see that the highest peak efficiency and the shortest migration time for MEL using Tris co-ion

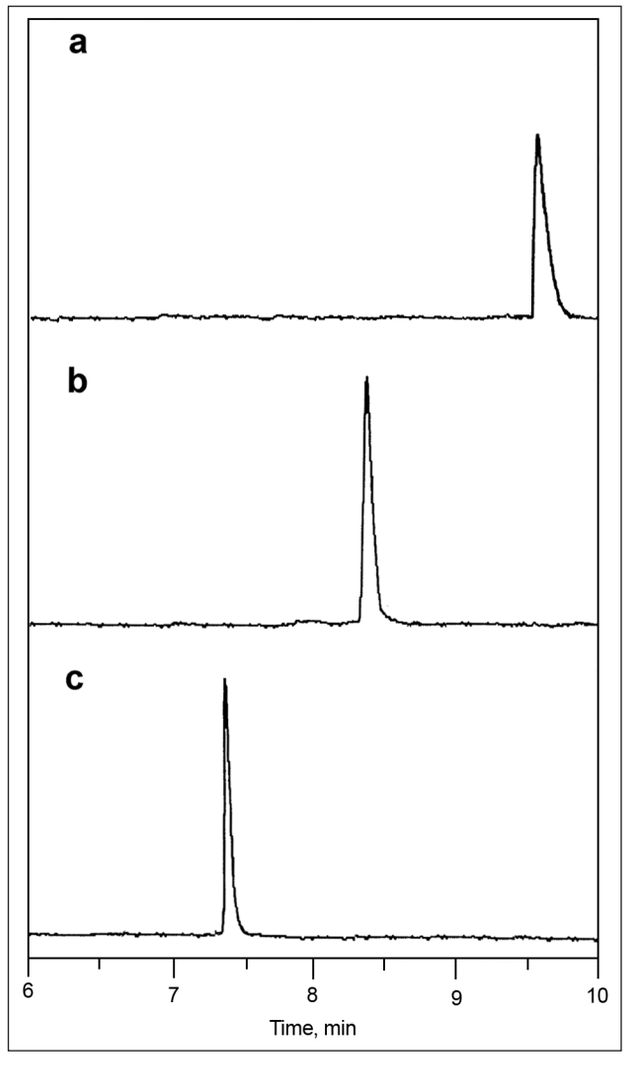

Fig. 2. Effect of electrolyte co-ion on the peak shape and migration time of melamine. Electrolyte, 50 mmol/l of H_3PO_4 neutralized to pH 3 with (a) ethylenediamine, (b) triethanolamine and (c) Tris. Voltage, 30 kV. Direct UV detection at 214 nm

were obtained. Based on these results, Tris-phosphate was chosen as a carrier electrolyte. However, due to a very slow electroosmotic flow (EOF) in the acidic electrolyte, the total run time (about 7.5 min) was too high for a fast analysis. The rate of the EOF strongly depends on the pH of the electrophoresis medium and decreases rapidly below pH 6, resulting in a longer time of analysis. Elimination of the pH-dependency of the EOF requires a chemical modification of the capillary surface. The most commonly used approach is a bilayer capillary coating [14]. The two-stage process involves initially flushing the capillary with electrolyte containing the polycation. The multiply charged polycations coat the entire capillary wall, making it strongly positively charged. The capillary is then flushed with a buffer containing the polyanion. The polyanions adsorb to the positively charged layer and form a highly negatively charged layer which is insensitive to pH changes, resulting in a strong and constant cathodal EOF. In the present work, the capillary coated with a poly(diallyldimethylammonium) / poly(styrenesulfonate) bilayer was tested for the separation of melamine. Such capillary coatings have been successfully employed for to modify EOF velocity in CE separation of inorganic ions and organic molecules [15–18]. The complete capillary coating protocol is given in Experimental. Using a capillary coated with a PDDAC / PSS bilayer, the separation time was reduced

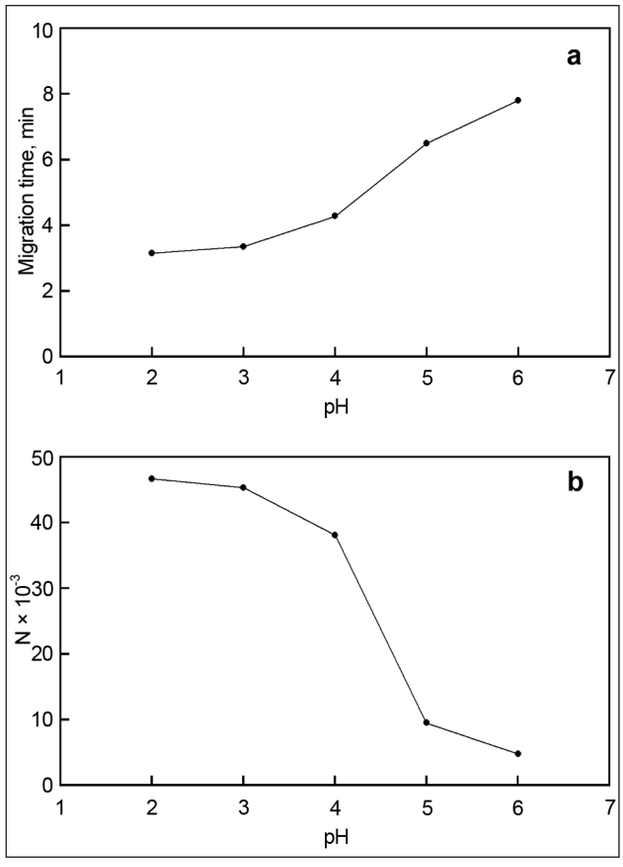

Fig. 3. Effect of electrolyte pH on the migration time (a) and peak efficiency (b) of melamine. Electrolyte, 50 mmol/l of H_3PO_4 neutralized with Tris to desired pH

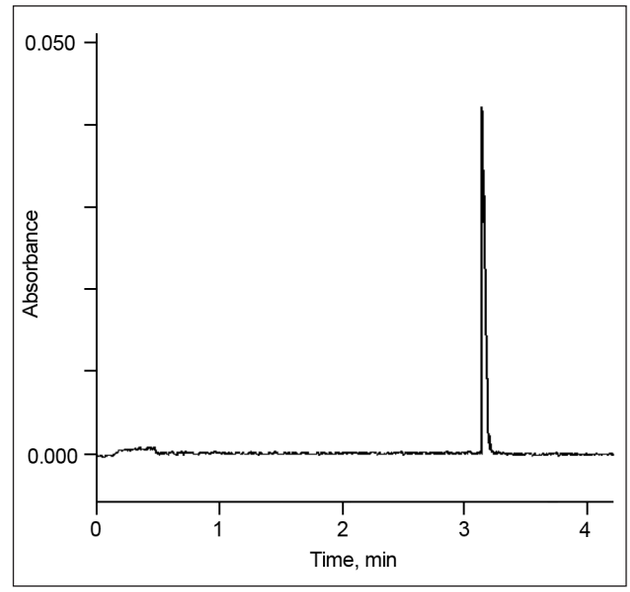

Fig. 4. Electropherogram of a standard melamine solution (10 mg/l) using a capillary coated with poly(diallyldimethylammonium)/poly(styrenesulfonate) bilayer. Electrolyte, 50 mmol/l of H_3PO_4 neutralized with Tris to pH 2.5. Other conditions as in Fig. 2

by a factor of about 2.4, while the peak efficiency remained quite satisfactory.

In CE, ionic analyte separation is based on both their charge and size. For a weakly basic MEL (pK_a = 5), its mobility is pH-dependent because the protonation of the analyte is controlled by electrolyte pH. The effect of electrolyte pH on the migration time and peak efficiency (theoretical plate number, N) of MEL is summarized in Fig. 3. As expected, due to the protonation of MEL at pH values near and below its pK_a, the net charge and, consequently, the mobility of MEL cation increases, resulting in a shorter migration time and a better peak efficiency. The best separation performance was observed in the pH range 2–3. Taking the buffering capacity into account, pH 2.5 was considered to be the best value for the carrier electrolyte. The electropherogram obtained under optimum conditions for a standard MEL solution is shown in Fig. 4. As one can see, a significantly better separation performance in respect to peak efficiency and separation time was obtained for the coated capillary.

Once the optimized conditions were selected, the method was validated with respect to the following parameters: linearity of the calibration curve, limit of detection, limit of quantification, precision and accuracy.

The linearity of the method was tested by preparing a calibration curve for each analyte with six points. The test concentration range was 0.2 to 10 mg/l, and each concentration level was injected (12 s) three times. The assay showed a linearity with a relative standard deviation (RSD) ≤4.1% for the relative responses (peak area divided by concentration) obtained in the concentration range tested and the correlation coefficient >0.9997 found for both analytes. The intercept

was not significantly different from zero. In addition, it was checked whether at the lower limit of the linear range, defined as the method limit of quantification, the analytical performances were satisfactory. Consequently, for the first concentration (0.2 mg/l) of the linear range, the signal-to-noise ratio was measured and the RSD of the peak area was calculated with five replicates. The signal-to-noise ratio was 13 and the repeatability RSD 7.8%. The detection limit (three times the baseline noise) was 0.06 mg/l. These data confirm the suitability of the proposed method for its application to real samples.

Method accuracy was determined by measuring repeatability and intermediate precision (between-day precision). In order to determine the repeatability of the method, six replicate injections of the standards at three MEL concentration levels (0.2, 0.5 and 1 mg/l) were carried out. The intermediate accuracy was evaluated over three days by performing six successive injections daily. A relatively little dispersion was observed for migration times because both repeatability and intermediate accuracy were less than 1.8%. For peak areas, the repeatability was in the range 2.3–4.8%, whereas intermediate precision ranged from 3.9 to 6.3% depending on the concentrations.

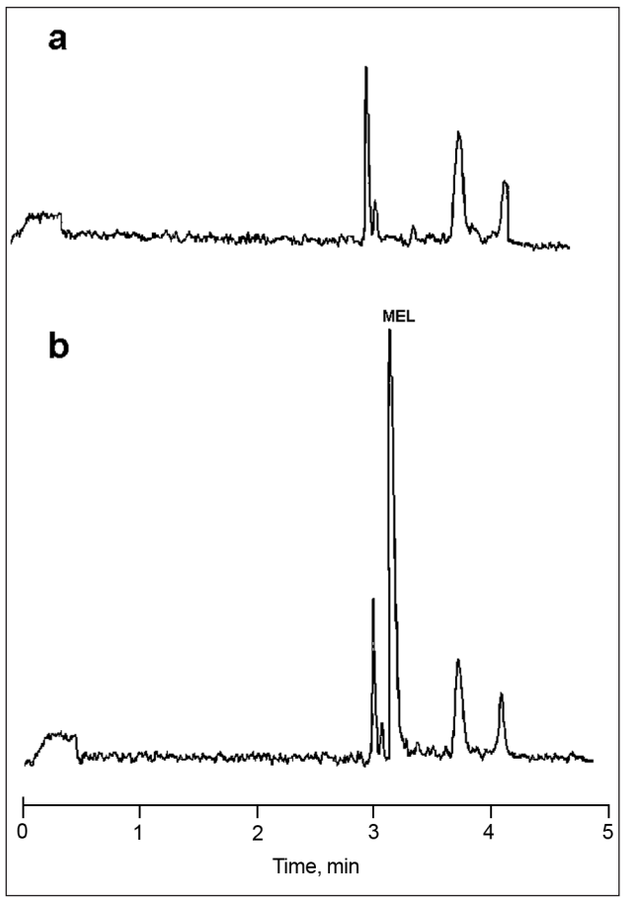

Fig. 5. Electropherograms of milk powder sample (a) and milk powder sample fortified at 2 mg/kg of melamine standard (b)

To evaluate the proposed CE system for real samples, it was used to determine MEL in milk powder samples. Among the 10 samples obtained from local supermarkets, none were found positive for MEL. Figure 5 shows the electropherograms obtained for a non-fortified milk powder sample (a) and the same sample fortified at 2 mg/kg of MEL (b). To evaluate the accuracy of the method, a recovery study was carried out with two samples, and the results are summarized in Table. As one can see, the concentrations found are generally in good agreement with the added concentrations, with recoveries between 93.5 and 104%. These results suggest that interferences by the other matrix components are not significant and the CE conditions are suitable to obtain an adequate accuracy of the method.

Table. **MEL recovery test results for two milk powder samples (n = 3)**

Sample No	Added, mg/kg	Found, mg/kg	Recovery, %
1	2.00	1.87	93.5
	5.00	4.83	96.6
2	2.00	2.08	104
	5.00	4.90	98.0

In comparison with GC and HPLC techniques, the CE method has a minimal set-up time, a lower sample matrix dependence, needs lower costs and reagent consumption and gives better separation efficiencies in a shorter time of analysis.

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Žana Černova, Ina Razmislevičienė, Audrius Padarauskas

MELAMINO NUSTATYMAS PIENO MILTELIUOSE KAPILIARINĖS ELEKTROFOREZĖS METODU

S a n t r a u k a

Optimizuotas kapiliarinės elektroforezės metodas melaminui nustatyti. Atskiriama dvigubu poli(dialildimetilamonio chlorido)/poli(natrio-4-stirensulfonato) sluoksniu padengtame kapiliare 50 mmol/l tris(hidroksimetil)aminometano fosfato elektrolite (pH 2,5, 214 nm) detektuojant. Kalibracinė kreivė yra tiesinė 0,2–10,0 mg/l melamino koncentracijų intervale, koreliacijos koeficientas ≥0,9997, aptikimo riba – 0,06 mg/l. Metodas pritaikytas melaminui nustatyti pieno milteliuose. Analičių išgavos iš realių mėginių siekia 93,5–104 %.