Determination of Melamine Residue in Liquid Milk by Capillary Electrophoresis with Solid-Phase Extraction

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Abstract

A novel solid-phase extraction–capillary electrophoresis (SPE–CE) method was developed for the determination of melamine residue in liquid milk. The conditions of SPE and CE were investigated and optimized. A 1% trichloroacetic acid plus 2.2% lead acetate solution were used for the extraction of analyte and the removal of protein. A Cleanert PCX SPE cartridges column was used for clean up. The 50 mM sodium dihydrogenphosphate running buffer (pH adjusted to 3.2 with citric acid) was used as a running buffer. The linearity is satisfactory in the range of 0.8–100 µg/mL with a correlation coefficient of 0.9998. Under the optimal conditions, the method limit of detection (LOD) and method limit of quantification were 0.12 mg/kg and 0.37 mg/kg, respectively. The recovery of melamine from different liquid milk samples was in the range of 89.5–98.5% with a relative standard deviation of 1.8–3.5%. The intra- and inter-day assay precision was 2.8% (n = 6) and 4.1% for five days, respectively. The developed method has been applied successfully for the determination of melamine residue in liquid milk samples. The results obtained by the proposed method agree with those obtained by high-performance liquid chromatographic method. The proposed method enables the quantitative determination of melamine residues at levels as low as 0.37 mg/kg in different liquid milk.

Introduction

Some foods generated from animals contain residues of melamine because these animals were fed melamine-contaminated feeds. In 2008, melamine has caused the death of certain infants in China, who had drunk milk containing this compound. Certain liquid milk and milk powder were adulterated with melamine to increase their total nitrogen concentration. Melamine might cause urolithiasis and bladder cancer (1). It is important to monitor melamine in raw milk and milk products.

A series of analytical techniques are available to determine melamine in a variety of pet foods, animal feeds, and human foods. High-performance liquid chromatography (HPLC) is more attractive than gas chromatography because no preliminary derivatization procedures are required. HPLC has been used for the determination of melamine in wheat gluten (2), feedstuff (3), and vegetables (4) with a limit of detection (LOD) of 65 µg/L, 5 mg/kg, and 0.5 mg/kg, respectively. The solid-phase extraction and high-performance liquid chromatographic (HPLC) method was presented for the determination of melamine in pet food with the limit of quantification (LOQ) of 0.5 mg/kg (5). A simple HPLC method was developed for the determination of melamine and its degradation products in rice protein concentrate with the decision limit of 65 mg/kg and detection capability of 75 mg/kg for melamine (6). A new method for the determination of melamine residue in plant origin protein powders was developed using HPLC–diode array detection (HPLC–DAD) for preliminary screening of the samples for melamine with an LOQ of 10 mg/kg and HPLC–mass spectrometry (MS)–MS was used in the confirmatory of melamine with an LOQ of 0.5 mg/kg (7). LC–MS–MS is the principal analytical method for detection and quantification of melamine in pet food and animal feed with low LOD of 10–1.7 µg/kg (8–12).

Analysis of melamine in human foods is very important work for health safety. Recently, China government adopted 2.5 mg/kg as a maximum residue limit (MRL) of melamine in liquid milk. HPLC method was adopted as the internal standard for determination of melamine in feeds and in raw milk and dairy products with LOQ 2 mg/kg (13,14). To our knowledge, there are few research reports for the determination of melamine in liquid milk and milk products. An HPLC method was described for the determination of melamine in milk powder and milk with an LOQ of 1 mg/kg (15,16). Yan et al.(17) presented a hydrophilic interaction chromatography–MS–MS method for the determination of melamine residue in raw milk and dairy products with an LOD of 0.05 mg/kg.

Like HPLC, capillary electrophoresis (CE) has been successfully coupled to MS detection for the analysis of a wide range of significantly different types of compounds. Von Brocke et al. reviewed the advances in capillary electrophoresis-electrospray ionization–MS (18). An effective method for the determination of the major components of (methoxymethyl)melamine resins with a quantitative analysis of nonreactive melamine by CE using electrospray ionization-MS was presented (19). Improved analysis of melamine–

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formaldehyde resins by capillary zone electrophoresis–MS using ion-trap and quadrupole-time-of-flight mass spectrometers was presented (20). Recently, a high performance capillary electrophoresis (HPCE) method was established for the separation of melamine in milk and milk powder with LOQs of 0.5 mg/kg in milk and 1.0 mg/kg in milk powder (21). An approach to determine melamine in dairy products, fish feed, and fish at residue levels using capillary zone electrophoresis (CZE) with DAD was described, and 0.25 and 0.5 mg/kg melamine in liquid samples and solid samples can be reliably detected, respectively (22). A new method for the determination of melamine in milk powder samples using CZE with either UV or MS detection is presented. This method allowed the determination of melamine in milk powder down to a level of 0.50 mg/kg using UV detection and 0.25 mg/kg when ESI quadrupole/TOF-MS was employed for detection (23).

In this work a sensitive SPE–CE method was developed for determination of melamine in different liquid milks. The proposed method has lower LOQ than HPLC method (2–4,6,7,13–16), SPE-HPLC (5), and CE-UV detection method (21,23) and allowed the determination of melamine in different liquid milk samples down to a level of 0.37 mg/kg. The proposed method was compared with HPLC method without significant difference for the determined results. This method is sensitive, reliable, and accurate and has been applied successfully for routine determination of melamine in raw milk, semi-finished milk, and flavored milk samples.

Experimental

Chemicals and reagents

Cleanert PCX cartridges (3 mL/60 mg) were obtained from Beijing Agela Technologies Company (Beijing, China). An individual stock standard solution, 1000 µg/mL, was prepared by dissolving the melamine (analytical grade, > 99.5%) in a mixture of methanol and water (1:4, v/v), and was stable for at least one month when stored at 4°C. A fresh working standard solution was prepared daily by diluting the stock solution with a mixture of methanol and water (1:4, v/v) for different studies. The solution was filtered through a 0.45-µm microporous membrane of mixed cellulose ester. The acetonitrile (chromatographic grade) was filtered through a 0.22-µm microporous membrane of polyvinylidene fluoride before used. A 50 mM NaH_2PO_4 buffer solution (pH adjusted to 3.2 with citric acid) was used as an electrolyte for CE separation. All the reagents were of analytical grade except for additional illustration. Doubly distilled water was obtained from quartz distillation apparatus.

Instrument

All experiments were performed with an Agilent 3D CE system (Agilent, Waldbronn, Germany) equipped with air-cooling and a DAD (200–400 nm). Data were collected with the Agilent Chemstation version A.10.02 chromatographic data system. A 48.5 cm (40.0 cm × 50 µm i.d.) uncoated fused-silica capillary from Agilent was utilized. A TGL-16M centrifuge (Xiangyi Centrifuge Co., Ltd., Hunan, China) and PHS-3C pH meter (Shanghai Precision & Scientific Instrument Co. Ltd., Shanghai, China) were used in sample treatment.

The HPLC equipment was a Shimadzu HPLC system (Kyoto, Japan) with a binary pump, a gradient controller (SCL-10Avp), an on-line-degasser (DGU-12A), a column thermostat (CTO-10Avp), and a DAD (SPD-M10Avp). CLASS-VP workstation was used as the data acquisition system. The analytical column was a ZY1104 C_{18} column (150 × 4.6 mm i.d., 5 µm, Shimadzu). After the C_{18} column was conditioned with a mobile phase of 0.01 M sodium *n*-heptanesulfonate–acetonitrile (83:17, v/v, pH 2.7) at 1 mL/min at 30°C, a 20-µL volume of sample solution was injected in the column and then eluted with the mobile phase. The linear equation describing the relationship between melamine concentration and its peak area was determined by least-squares weighted for UV detection at 235 nm. Quantification was carried out by using matrix-matched standards calibration for comparing with CE method.

Sample extraction and clean-up

A 50 mL of 1% trichloroacetic acid solution and 2 mL 2.2% lead acetate solution were added to 5 g of milk in order to eliminate protein and extracte analyte. The mixture was placed in an ultrasonic cleaner for 20 min to mix well, standing for 2 min. Then a 30-mL volume of mixed solution was centrifuged for 10 min at 10,000 rpm. A 10 mL of the supernatant was applied to a Cleanert PCX cartridge (3 mL/60mg, Agela Technologies Company of China, Beijing, China), which had been previously conditioned with 3 mL of methanol and 3 mL of water. SPE cartridge was washed in turn with 5 mL of water and 5 mL of methanol. Melamine was eluted with 6 mL of 25% ammonia solution–methanol (1:20, v/v). The eluate was evaporated to dryness at 50°C under a stream of nitrogen, and the residue was re-dissolved in 0.5 mL of methanol–water (1:4, v/v). Then the solution was filtered through a 0.25-µm microporous membrane of mixed cellulose ester for CE and HPLC analysis.

Electrophoresis conditions

At the beginning of each day, the capillary was conditioned with 1 M NaOH solution for 3 min, water for 3 min, and the 50 mM running buffer for 4 min in order to equilibrate the capillary and minimize hysteresis effects. The 50 mM sodium dihydrogenphosphate running buffer (pH adjusted to 3.2 with citric acid) was used for separation of melamine. Sample introduction was made at the positive side using the pressure of 5 kPa for 8 s. The high-voltage power supply was set to 22 kV. Capillary temperature was kept at 20°C, and the content of melamine in liquid milk samples was determined at 225 nm using matrix-matched standards calibration curve (concentration points at 0.25, 1.0, 2.0, 4.0, 8.0, 10 mg/kg).

Results and Discussion

Extraction and clean-up

Melamine is generally extracted with polar organic solvents,

buffer solution, or the mixture solutions of organic reagent and water. For the extraction of melamine from matrix, several reagents were used, such as water for vegetable materials with a recovery of 70.2–80.2% (4), acidic acetonitrile for catfish, trout, tilapia, salmon, and shrimp with a recovery of 54.9–74.8% (9), and 50% acetonitrile for porcine muscle tissue with a recovery of 83% (10), as well as a 50:50 acetonitrile–water and hydrochloric acid for catfish tissue with a recovery of 76.3% (24). In our initial test, trichloroacetic acid solution was chosen to precipitate proteins and to dissociate the target analyte from the milk sample matrix. However, the solid fat layer was above the aqueous layer after centrifuging, which made it difficult to obtain the aqueous layer by decantation. Trichloroacetic acid plus lead acetate were used for the extraction of melamine and the removal of protein from feed (13). In this work, by using 50 mL of 1% trichloroacetic acid plus 2 mL 2.2% lead acetate for the extraction of the analyte and the removal of protein from liquid milk samples, higher extraction efficiencies (85.5–95.5%) of melamine were achieved. Therefore, 1% trichloroacetic acid plus 2 mL 2.2% lead acetate was shown as the optimum extractant.

After centrifugation of the eluate, the obtained supernatant with acidity was applied for clean-up. If the supernatant was used directly for CE analysis, the recovery and sensitivity for melamine would be decreased. Use of Oasis MCX cartridges as SPE column and mixed solution containing 5% methanol and 3% ammonia solution as eluting reagent were applied for clean-up feed matrix prior to HPLC with the recovery of 84.1–89.9% for melamine (5). Cleanert PCX is a cation-exchange SPE column with both cation exchange and reversed-phase adsorption mechanisms. It could extract polar and non-polar compounds with better effect for clean-up to milk matrix. Melamine is a cation compound with weak alkaline (pKa = 8) and is only slightly soluble in water. Under acidic condition the analytes in the supernatant are protonated and retained by the cation exchange material, and under alkaline condition the analytes are neutral and are not retained. To get effective clean-up purpose, the effect of supernatant flow rate, composition, and volume of both washing reagent and eluting reagent on clean-up was investigated by finally observing electropherograms. The optimal conditions were obtained as follows: flow rate of supernatant was 1 mL/min, then melamine-adsorbed SPE cartridges was washed in turn with 5 mL of water and 5 mL of methanol, then finally melamine in the cartridges was eluted with 6 mL of 25% ammonia solution–methanol (1:20, v/v). The high recovery of 89.5–98.5% for melamine was achieved.

The eluate from SPE cartridges was evaporated to dryness at 50°C under a stream of nitrogen. The obtained residue was redissolved with a methanol–water solution (1:4, v/v), the lower volume of which would increase the concentration factor of melamine. The use of 0.5 mL of methanol–water (1:4, v/v) for dissolving the residue could need the request for CE sampling with higher concentration factor for melamine.

Choice of detection wavelength

The detection wavelength was examined in the range of 200-400 nm using 1 mg/mL melamine solution and sodium dihydrogenphosphate–citric acid buffer. Electropherograms of melamine are shown in Figure 1.

The spectrum of melamine consists mainly of three absorption peaks; the height and area of the first absorption peak at 225 nm are higher then that of the other absorption peaks. So a 225 nm was selected as optimal wavelength for the detection of melamine.

Effect of running buffer

A series of buffers were investigated and compared under same concentration, pH value, separation voltage, and sampling time to obtain effective separation and higher sensitivity. The results are shown in Figure 2.

Compared with other buffers, higher peak height, lower baseline noise, and higher stability as well as good peak phase were observed using sodium dihydrogenphosphate–citric acid as a running buffer, so it was selected as a running buffer in further tests.

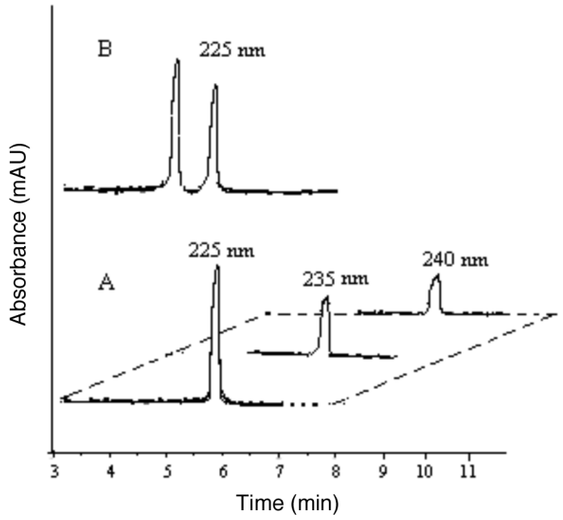

Figure 1. Electropherograms of melamine under different wavelengths. (A) Standard solution and (B) real spiked sample solution.

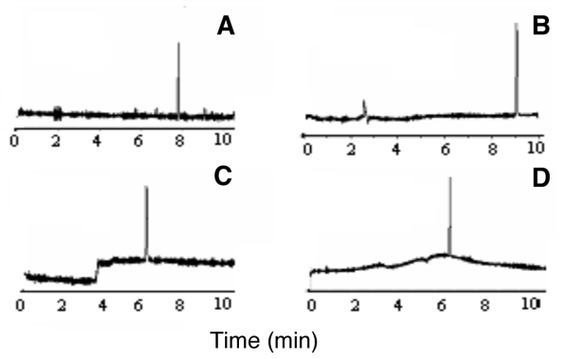

Figure 2. Electropherograms of melamine using different buffers. (A) sodium dihydrogenphosphate; (B) sodium dihydrogenphosphate–citric acid; (C) acetic acid–acetic sodium; (D) citric acid–citric sodium; melamine: 10 µg/mL.

The effects of different concentrations (40, 50, 60, 70, and 80 mM) of sodium dihydrogenphosphate (pH adjusted to 2.8 with citric acid) on migration time were tested. The result showed that the migration time and peak area were increased gently with the increase of buffer concentration, but higher concentration would inference separation. So a 50 mM buffer was used in this work.

The melamine (pKa = 8) could be protonated under acidic condition with positive electric charge. The effect of pH value from 2.8 to 4.0 on separation of melamine was investigated with 50 mM sodium dihydrogenphosphate (pH adjusted with citric acid) as a running buffer, as shown in Figure 3.

It is indicated that the sensitivity increased with decreasing pH, and the migration time shorted with increasing pH. However, higher baseline noise was observed when pH was lower than 3.2, and a shoulder peak and tailing were observed when pH = 4.0. The melamine could be protonated completely at conditions of low pH. In addition, the negative charge density in the inner wall of the capillary decreases at conditions of low pH; therefore, the electrostatic interaction between analytes, especially that of proteins in sample matrix and the inner wall of the capillary, would reduce. Thus, the peak dispersion and asymmetry can be minimized (25). Therefore, as a compromise consideration among resolution, sensitivity, baseline noise, and migration time, the pH 3.2 buffer was used for CE separation.

Effect of separation voltage and temperature

The effect of different voltages (12–27 kV) on separation of melamine in liquid milk was investigated. The high-voltage power supply influences migration speed of melamine, which influences separation effect. An electrophoresis current increases with the increase of voltage so that migration time decreases, but Joule heat increases and produces radial temperature gradient and decreases separation efficiency. The height of the peak of melamine at 12 kV is lower 20% than that at 22 kV. The peak could not be separated absolutely from the peak of impurity in milk sample when voltages were beyond 27 kV. A 22 kV voltage was selected for capillary electrophoresis in this work with higher column efficiency and shorter analytical time.

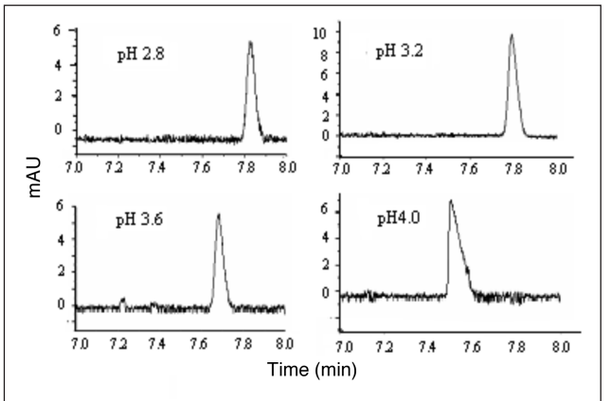

Figure 3. Effect of pH on separation of melamine. Conditions: 50 mM sodium dihydrogenphosphate–citric acid; column temperature: 25°C; separation voltage: 15 kV; sampling at 5 kPa for 4s; melamine: 10 µg/mL.

The effect of the capillary temperature (15–30°C) on separation was investigated. The result showed that migration time increased with the decrease of capillary temperature. When the temperature was set at 15°C, the peak shape of melamine was bad. When the temperature was set at 30°C, the migration time was shorted, but peak height was decreased. So column temperature was set at 20°C with migration time of 4.8 min and without peak dispersion and asymmetry.

Specificity

Under optimized conditions the precision (relative standard deviation, RSD) of migration time for six replicates of the same test solution of a quality control milk sample with 1 mg/kg level was 0.52%. Electropherograms of standard solution and spiked milk sample solution are shown in Figure 4. The peak for melamine in standard solution was observed. A peak for impurity in real spiked sample solution was observed, which could be separated completely with the peak for melamine. The source of the impurity peak needs to be examined further. No interfering peak was observed at the retention time of melamine. Quantification was carried out by using matrix-matched standards calibration.

Linearity and detection limit

Under the optimal conditions, the linearity for analysis of melamine was evaluated with the measured peak area of the standard solution with similar matrix to the test solution against their concentrations. The obtained linear regression equation was as follows: A = 0.4143 + 1.2415C, where A is peak area of spiked standards based on three parallel mea-

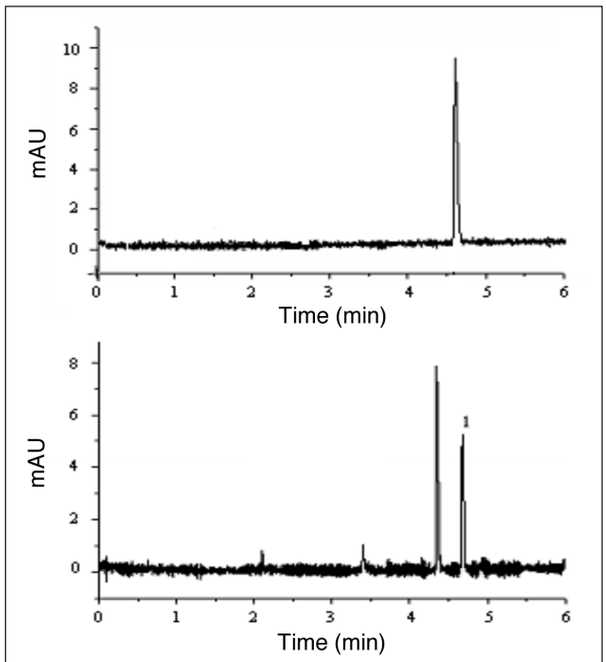

Figure 4. Electropherograms of standard solution and liquid milk sample under the optimal conditions. Conditions: column temperature: 25°C; separation voltage: 22 kV; sampling at 5 kPa for 4s; 50 mM sodium dihydrogenphosphate–citric acid(pH 3.2); melamine: 10 µg/mL.

surements and C is the concentration (µg/mL) of spiked standards for melamine. The linearity was proved to be satisfactory in the range of 0.8–100 µg/mL, including seven concentration points (0.8, 2.0, 5.0, 10, 25, 70, 100 µg/mL) with a correlation coefficient of 0.9998.

The LOD was determined as the sample concentration that produces a peak with a height three times the level of the baseline noise, and the LOQ was calculated as the sample concentration that produces a peak with a height 10 times the ratio of signal to noise (26). The instrument LOD value for melamine was 0.20 µg/ mL, and the instrument LOQ value was 0.63 µg/mL. For a 0.5-mL final test solution prepared from 5 g sample, the method LOD and the method LOQ were 0.12 mg/kg and 0.37 mg/kg, respectively. The LOD value for this method is lower than that for the reported HPLC (2–4,6,7,15,16) and SPE-HPLC (5,13,14) as well as CE methods (21,23), and slightly lower that for CE method (22) and CE-ESI quadrupole-TOF-MS (23). The proposed method enables the quantitative determination of melamine residues at levels as low as 0.37 mg/kg in different liquid milk.

Liquid milk analysis

Under optimized conditions, the proposed method was used for the determination of melamine in different liquid milk samples. At the same time recovery experiments were carried out by adding the melamine standard in real milk sample followed by extraction, clean-up, and CE analysis. The obtained content of melamine in some liquid milk samples and recovery are given in Table I along with the result determined by HPLC.

Among these liquid milk samples, a positive milk sample was discovered, which was from melamine contaminants fortified. For other raw milk, semi-finished milk, and flavored milk samples the melamine contents were below 2.5 mg/kg, which was in accordance with the quality requested by the Chinese government. The recovery of melamine from different milk samples was in the range of 89.5–98.5% with an RSD of 1.8–3.5%. The intra- and inter-day assay precision (RSD) (n = 6) was 2.8% and 4.1% for five days, respectively. The results obtained by the proposed method agree with those obtained by HPLC method. Statistical analysis of the results using Student t-test and the variance ratio F-test showed no significant difference at p = 0.05 between the performance of the two methods as regards to accuracy and precision.

Table I. Determination of Melamine in Liquid Milk Samples

		This method			HPLC		
Sample	Content (mg/kg)	Added (mg/kg)	Found (mg/kg)	%Recovery	%RSD (n = 3)	Content (mg/kg)	%RSD (n = 3)
Raw milk	2.1	2.0	3.90	90.0	2.5	2.09	2.4
	2.2	3.0	5.13	97.7	2.0	2.19	2.3
Semi-finished milk	1.6	2.0	3.57	98.5	2..5	1.67	3.0
	2.0	2.0	3.85	92.5	2.3	1.89	2.5
Flavored milk	3.8	3.0	6.70	96.7	1.8	3.75	2 3
	1.8	2.0	3.68	94.0	2.4	1.87	2.6
	1.8	2.0	3.66	93.0	2.5	1.69	2.8
	1.5	2.0	3.29	89.5	2.8	1.74	3.0
	0.5	0.4	0. 88	95.0	3.5	0.53	3.2

Conclusions

A novel SPE–CE method was developed for the determination of melamine residue in liquid milk. The proposed method is sensitive, reliable, and accurate and enables the quantitative determination of melamine residues at levels as low as 0.37 mg/kg in different liquid milk.

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