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# Determination of Melamine in Dairy Products, Fish Feed, and Fish by Capillary Zone Electrophoresis with Diode Array Detection

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This paper describes an approach to determine melamine (MEL) in liquid milk, yogurt, whole milk powder, fish feed, and fish at residue levels using capillary zone electrophoresis with diode array detection (CZE-DAD) for the first time. Suspicious samples were extracted with 1% trichloroacetic acid while 1 mL of chloroform was used to precipitate fat in the real samples. After centrifuging and filtering, the extract was analyzed by CZE-DAD directly. By investigating the variables of extraction, separation, and detection in detail, the entire analytical procedure including sample preparation could be completed within 30 min. The limits of detection and quantitation for MEL were found to be 0.01 and 0.05 $\mu$g mL$^{-1}$, respectively. The proposed method was successfully applied for the analysis of MEL in dairy products, fish feed, and fish with total recoveries ranging from 93 to 104%.

**KEYWORDS: Melamine; dairy products; fish feed; fish; capillary zone electrophoresis−diode array detection**

## INTRODUCTION

In September 2008, infant formulas that were illegally adulterated with melamine (MEL) led to health problems for thousands of infants in China. This accident captured attention from all over the world. Yet this is not the first time the compound has been detected in food. In the spring of 2007, MEL-contaminated pet food sickened or killed thousands of cats and dogs in the United States (*1*). MEL is used to make plastics, fertilizer, and other products and is not approved as an ingredient in food. However, nitrogen-rich MEL was deliberately added to the food or the 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. Unfortunately, MEL can result in the formation of insoluble MEL cyanurate crystals in kidneys, thus causing renal failure (*2*−*4*). Therefore, there is an urgent need to establish a simple and effective method for the analysis of MEL.

Gas chromatography (*5*, *6*), liquid chromatography (*7*−*17*), and capillary electrophoresis (CE) (*18*) have been developed for the analysis of MEL. Gas chromatography and liquid chromatography have been used to determine MEL in lots of matrices, such as soil (*5*), animal feeds and grains (*6*−*8*), animal tissues (*9*−*13*), plant matter (*14*, *15*), and raw milk and dairy products (*17*). While CE was used to determine MEL only in melamine resins, the migration time of MEL was 20 min (*18*). Most of the above methods employed a mass spectrometer as a detector, which is an effective tool for structural determination; however, it is more expensive compared with UV spectroscopy (*19*).

With procedures based on the results of these previous reports, this paper, for the first time, presents an approach using capillary zone electrophoresis with diode array detection (CZE-DAD) to determine MEL in dairy products, fish feed, and fish. The presented approach is simple, efficient, sensitive, and rapid. This approach utilizes the strong polarity of MEL to achieve the baseline separation of MEL and interferent in simple CZE mode. It also employs the excellent ultraviolet absorption of MEL to determine MEL at residue levels (1.0−2.5 mg/kg) (*20*) sensitively. To assess its applicability, the proposed method was applied for the analysis of MEL in liquid milk, yogurt, whole milk powder, fish feed, and fish.

## MATERIALS AND METHODS

**Apparatus and Materials.** All capillary electropherograms were recorded on a Beckman P/ACE MDQ system (Fullerton, CA), equipped with a diode array UV detector (190−600 nm). To achieve highest sensitivity for MEL, 206 nm was chosen as the best wavelength. Data acquisition and instrument control were carried out using 32 Karat software (version 7.0). Electrophoresis was performed in fused silica capillaries of 75 $\mu$m i.d. and 365 $\mu$m o.d. obtained from Yongnian Ruifeng Chromatogram Equipment (Yongnian, China). All capillaries were 50.2 cm long, having an effective length of 40 cm, and were thermostated at 25 °C. The sample was extracted in a KH-250 DB ultrasonic cleaner (Hechuang, Kunshan, China) and centrifuged in a TDL80-2B low-speed centrifuge (Anke, Shanghai, China).

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All solvents and reagents were of analytical grade and used without further purification. Standard of MEL was obtained from J&K Chemical Ltd. (Beijing, China). Sodium dihydrogen phosphate, phosphoric acid, trichloroacetic acid (TCA), and chloroform were products of Tianjin Chemical Reagent Factory (Tianjin, China). Deionized water was used throughout.

**Solutions Preparation.** Ten percent TCA was prepared by dissolving 10 g of TCA in water to a final volume of 100 mL. Standard stock solution containing 400 $\mu$g/mL MEL was prepared by dissolving 4.0 mg of MEL in 10 mL of 20% methanol aqueous solution. Low concentration of MEL in 1% TCA solution was prepared by diluting the standard stock solution and 10% TCA with water.

The separation buffer was composed of 30 mM $NaH_2PO_4$ (pH 3.2). It was obtained by diluting the stock solutions of 400 mM $NaH_2PO_4$ and adjusted accurately to the desired pH value with 1.0 or 0.1 M $H_3PO_4$. All solutions were filtered with a 0.45 $\mu$m filter before use.

**Sample Preparation.** Liquid milk, yogurt, whole milk powder, fish feed, and fish were purchased from local supermarkets. For liquid milk or yogurt, 1 mL of 10% TCA, 7 mL of deionized water, and 1 mL of chloroform were well mixed with 2 mL of liquid sample in a 15-mL polypropylene centrifuge tube. The sample was ultrasonically extracted for 10 min and centrifuged at 4000 rpm for 2 min. Then the supernatants were filtered with a 0.45 $\mu$m filter and used for direct injection. Fish were homogenized in a commercial mincer and stored in a hermetic recipient at −20 °C until use. One gram of whole milk powder, fish feed, or homogenate of fish was accurately weighed. Then, 1 mL of 10% TCA, 9 mL of deionized water, and 1 mL of chloroform were added to the weighed sample in sequence. The following operations, including mixing, extracting, centrifuging, and filtering, were performed in the same way as for the liquid sample.

**CZE Conditions.** The new capillary was conditioned prior to use by flushing at 138 kPa sequentially with methanol for 10 min, water for 3 min, 1.0 M NaOH for 20 min, water for 3 min, and running buffer for 20 min and, finally, equilibrated at 25 kV with running buffer for 60 min. To ensure a good reproducibility, the capillary was rinsed at 138 kPa sequentially with deionized water (1 min), 0.5 M NaOH (1 min), deionized water (1 min), and running buffer (3 min) at the beginning of each run. Sample introduction was facilitated by applying a pressure of 3.45 kPa for 3 s. A constant voltage of 25 kV was applied during analysis.

## RESULTS AND DISCUSSION

**Selection of Detection Wavelength.** As diode array detection in CE could facilitate identifying the compounds and establishing the peak purity in the complex matrix (*21*), the detection wavelength was examined in the range of 190−400 nm. The results, which could be used to avoid the interference of other compounds in real samples, showed that the maximum absorption wavelength of MEL was located at 206 ± 2 nm. Therefore, the detection wavelength was set at 206 nm in subsequent experiments.

**Selection of Extraction Conditions.** MEL is generally extracted with polar organic solvents, buffer solution, or the mixture solutions of organic agent and water. Ding et al. investigated the extraction efficiency of different solvents for MEL in plant origin protein powders (*7*). The extraction recoveries of methanol, methanol/water (1:1 v/v), acetonitrile, acetonitrile/water (1:1 v/v), water, and 1% TCA solution were 32, 88, 27, 82, 85, and 86%, respectively. In this study, TCA solution was chosen to precipitate proteins and to dissociate the target analyte from the 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. To get the aqueous conveniently, 1 mL of chloroform was added to the tested samples so that the fat layer could be deposited to the bottom of the polypropylene centrifuge tube. Liquid milk was chosen as the model sample for investigating the effect of the concentration of TCA and the ratio of extractant volume to sample on extraction efficiency and sensitivity.

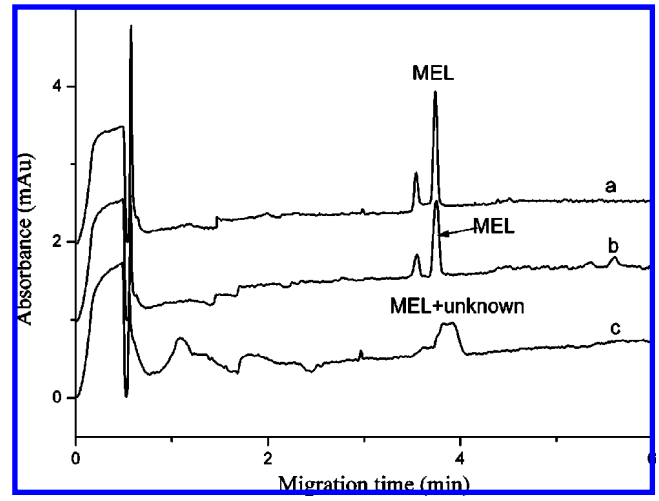


**Figure 1.** Influence of TCA concentration in extractant on peak shape. The liquid milk spiked with 2 $\mu$g mL$^{-1}$ MEL was extracted with (a) 1% TCA, (b) 2% TCA, and (c) 5% TCA. Detection wavelength, 206 nm; sample injection, 3.45 kPa × 3 s; background electrolyte, 20 mM $NaH_2PO_4$, pH 2.8 (see Sample Preparation for other extraction conditions).

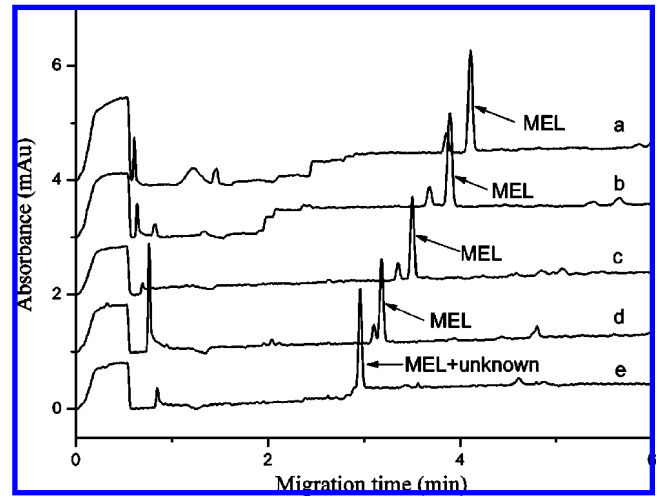


**Figure 2.** Effect of buffer pH in the range of 2.4−4.0. Background electrolyte, 20 mM $NaH_2PO_4$, (a) pH 2.4, (b) pH 2.8, (c) pH 3.2, (d) pH 3.6, and (e) pH 4.0. The liquid milk spiked with 2 $\mu$g mL$^{-1}$ MEL was extracted with 1% TCA. The other conditions were the same as in **Figure 1**.

The concentration of TCA was investigated in the range of 0.5−5%. It was found that the efficiency of protein precipitation was improved with increasing TCA concentration, but the results indicated that the peak was broadened (**Figure 1**, curves b and c) when the concentration of TCA was beyond 2%. The phenomenon of peak broadening was due to the high ionic strength of the sample matrix, which was caused by the added high concentration of TCA. Therefore, 1% TCA was chosen as the optimum extractant. In all of the following experiments, the real concentration of TCA in the extraction system would be always kept at 1% by diluting 10% TCA with liquid sample and water (for liquid samples) or only with water (for solid samples) during the extract operations.

Then, the ratio of extractant volume to the volume of liquid milk was investigated at 2:1, 5:1, 20:1, and 50:1, with a constant content of MEL in the liquid milk. The results showed that the protein could not be precipitated effectively when the ratio was

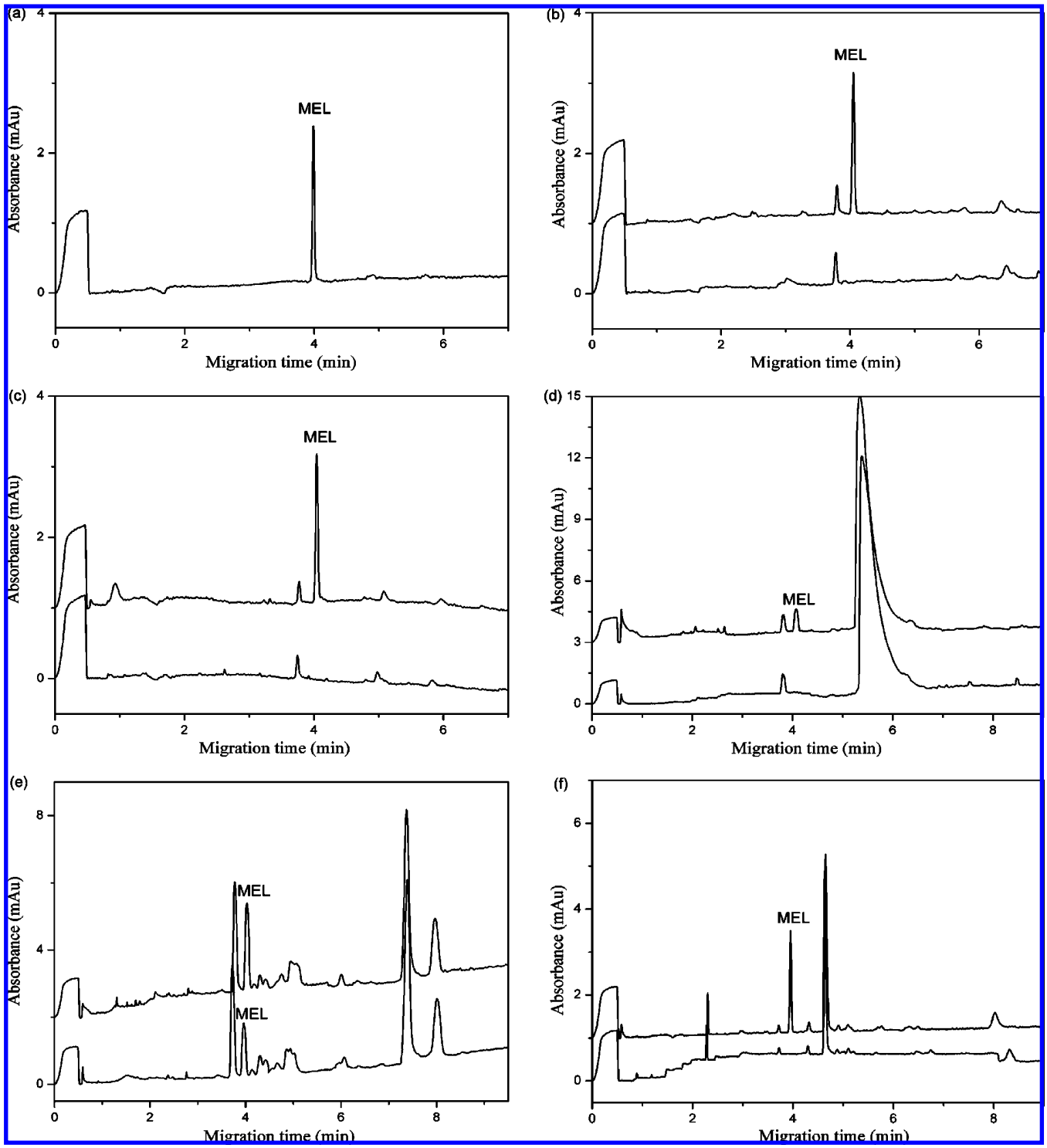


**Figure 3.** Typical electropherograms of the standard, sample, and spiked sample under optimum pretreatment conditions and separation conditions (30 mM $NaH_2PO_4$, pH 3.2): (**a**) standard of MEL in 1% TCA; (**b**) liquid milk and spiked liquid milk; (**c**) yogurt and spiked yogurt; (**d**) whole milk powder and spiked whole milk powder; (**e**) fish feed and spiked fish feed; (**f**) fish and spiked fish. The upper lines in panels **b**−**f** correspond to real samples spiked with 2 $\mu$g $mL^{-1}$ MEL.

set at 2:1 due to the phenomenon of peak broadening. With the ratio increase beyond 5:1, the peak width was narrow but the peak height reduced proportionately for the sample being diluted. Therefore, the ratio of extractant volume to sample was set at 5:1 for the extraction of MEL in liquid samples. However, the results indicated that the protein in solid samples could not be precipitated effectively when 5:1 (v/w) was used, whereas the 10:1 (v/w) could precipitate protein effectively. Therefore, the ratios of 5:1 (v/v) and 10:1 (v/w) were chosen for the extraction of MEL in liquid samples and solid samples, respectively.

In the literature about the analysis of MEL, solid phase extraction (SPE) was another commonly used method for sample preparation (*5*, *7*, *10*, *13*, *17*). It generally includes the procedures of conditioning, loading sample, rinsing, and elution. In addition, liquid extraction was utilized to extract the solid samples and complex liquid samples at first, and then SPE was used for cleanup or preconcentration (*5*, *7*, *10*, *17*). Generally,

**Table 1.** Figures of Merit of CZE Method for Determining MEL

| regression eq | | | | | RSD of migration time (%) | | RSD of peak area (%) | |
|---|---|---|---|---|---|---|---|---|
| slope | intercept | correl coeff | linear range ($\mu$g/mL) | LOD[a] ($\mu$g/mL) | intraday ($n = 5$) | interday ($n = 3$) | intraday ($n = 5$) | interday ($n = 3$) |
| $2.32 \times 10^3$ | $1.08 \times 10^2$ | 0.9995 | 0.05−10 | 0.01 | 0.36 | 0.70 | 1.67 | 3.97 |

[a] Detection limits corresponding to concentrations giving signal-to-noise ratio of 3.

the cleaner matrix obtained with SPE was more suitable for mass spectrometry. However, the recovery and the reproducibility of SPE were not so satisfactory. In the presented method, high recoveries ranging from 93 to 104% (see Applications and Recoveries) were obtained. Furthermore, the whole sample preparation procedure included sampling, mixing, ultrasonic extracting, centrifuging, and filtering, and all operations can be completed within 15 min. Therefore, the developed extraction method of short extraction time, simple operation, and good recovery was more suitable for practical application.

**Optimization of Separation Conditions.** In preliminary measurements, it was found that MEL and an interferent from the real samples migrated together when sodium tetraborate or sodium tetraborate containing sodium dodecyl sulfate was used as alkaline background electrolyte. Therefore, acidic buffer was used to protonate the amino groups and facilitate their separation as cations. $NaH_2PO_4$ buffer was chosen as background electrolyte, and the pH, which was adjusted with 1 or 0.1 M $H_3PO_4$, was investigated in the range of 2.4−4.0. The electropherograms presented in **Figure 2** clearly indicate that the resolution and sensitivity increased with decreasing 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 (*22*). However, higher baseline noise was observed when the pH was lower than 3.2. Accordingly, a value of 3.2 is recommended as a compromise consideration among resolution, sensitivity, baseline noise, and migration time.

The influence of buffer concentration ranging from 10 to 40 mM on separation was then studied by keeping a constant buffer pH of 3.2. The results implied that a value of 30 mM $NaH_2PO_4$ could enhance absorbance and meanwhile improve resolution. Accordingly, a solution of 30 mM $NaH_2PO_4$ at pH 3.2 is recommended as optimum background electrolyte for the determination of MEL. A representative electrophoregram of MEL standard under the optimal extraction, separation, and detection conditions is shown in **Figure 3a**.

**Linearity, Detection Limit, and Precision.** The method validation including linearity, limits of detection (LOD), and precision was carried out using MEL under the optimized conditions. Figures of merit are summarized in **Table 1**. The linearity between peak area ($\mu$AU × s) and the concentrations ($\mu$g mL$^{-1}$) was investigated in the range of 0.05−10.00 $\mu$g mL$^{-1}$ for MEL. The results indicate that an excellent linear relationship was attainable over the concentration range studied with a correlation coefficient of 0.9995 for MEL. The LOD, calculated for an S/N of 3, was 0.01 $\mu$g mL$^{-1}$ for MEL, indicating high sensitivity. Precision was evaluated in terms of reproducibility. The reproducibility of the presented method was determined with a standard solution at concentration levels of 0.5, 2, and 5 $\mu$g/mL for MEL. The results, expressed as relative standard deviation (RSD) of migration time and peak areas, are also given in **Table 1**. As can be observed, the RSDs of the migration times and peak areas were 0.36 and 1.67% (intraday, $n = 5$) and 0.70 and 3.97% (interday, $n = 3$), respectively, indicating good precision.

**Table 2.** Results for the Determination of the MEL in Sample Extracts

| sample | orig amount ($\mu$g/mL) | added ($\mu$g/mL) | found ($\mu$g/mL) | recovery (%) | RSD[a] (%) | av recovery (%) |
|---|---|---|---|---|---|---|
| liquid milk | —[b] | 0.5 | 0.54 | 108 | 3.44 | 102 |
| | | 2 | 2.02 | 101 | 1.73 | |
| | | 5 | 4.91 | 98 | 4.48 | |
| yogurt | — | 0.5 | 0.51 | 103 | 0.60 | 104 |
| | | 2 | 2.10 | 105 | 0.78 | |
| | | 5 | 5.25 | 105 | 1.86 | |
| milk powder | — | 0.5 | 0.50 | 100 | 2.75 | 100 |
| | | 2 | 2.00 | 100 | 2.42 | |
| | | 5 | 5.05 | 101 | 3.78 | |
| fish feed | 3.02 | 0.5 | 3.50 | 95 | 2.09 | 93 |
| | | 2 | 4.86 | 92 | 0.67 | |
| | | 5 | 7.67 | 93 | 3.99 | |
| fish | — | 0.5 | 0.47 | 94 | 0.97 | 104 |
| | | 2 | 2.13 | 106 | 2.47 | |
| | | 5 | 5.64 | 112 | 4.49 | |

[a] The relative standard derivation was calculated from three recovery data. [b] No detectable.

**Applications and Recoveries.** To assess its applicability, the proposed method was applied to the analysis of MEL in liquid milk, yogurt, whole milk powder, fish feed, and fish. The peaks were identified by comparison of the migration time of MEL in real samples with that of MEL standard and by spiking the MEL to the sample solutions as well as by the maximum absorption wavelength. Representative electropherograms for the analysis of standard and real samples are illustrated in **Figure 3**. The quantitative results and the recoveries of the method, which were determined by adding different amounts of MEL to the sample matrix before sample pretreatment, are listed in **Table 2**. It is shown that the average total recoveries of MEL ranged from 93 to 104%. The LOD, calculated for an S/N of 3, were $1.17 \times 10^{-2}$, $1.29 \times 10^{-2}$, $1.25 \times 10^{-2}$, $1.16 \times 10^{-2}$, and $9.78 \times 10^{-3}$ $\mu$g mL$^{-1}$ in the matrices of liquid milk, yogurt, whole milk powder, fish feed, and fish, respectively. The limit of quantitation (LOQ) was determined by measuring the lowest levels at which MEL can reliably be detected in the extract of tested matrices. MEL was spiked to the blank samples (the tested samples containing no MEL) such as liquid milk, yogurt, whole milk powder, and fish. The final concentration of MEL in the extract of these blank samples was 0.05 $\mu$g mL$^{-1}$. The results showed that the peaks of spiked 0.05 $\mu$g mL$^{-1}$ MEL in the tested matrices can be easily identified. Therefore, the LOQ of MEL in light of our work was 0.05 $\mu$g mL$^{-1}$. Taking into account the 5-fold (liquid samples) and 10-fold (solid samples) dilutions in the process of sample extraction, 0.25 and 0.5 $\mu$g g$^{-1}$ MEL in liquid samples and solid samples can be reliably detected, respectively. The results were satisfactory, and these data

supported the suitability of the proposed method for its application to real samples.

## ABBREVIATIONS USED

MEL, melamine; CZE-DAD, capillary zone electrophoresis with diode array detection; TCA, trichloroacetic acid; SPE, solid phase extraction.

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