DOI:10.1021/jf9021916

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Simultaneous Determination of Melamine and 5-Hydroxymethylfurfural in Milk by Capillary Electrophoresis with Diode Array Detection

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This article describes the development of a simple analytical approach for the simultaneous determination of melamine and 5-hydroxymethylfurfural (HMF) in milk samples using capillary electrophoresis (CE) with diode array detection (DAD) for the first time. Ultraviolet absorption at wavelengths of 214 and 280 nm was applied for the detection of melamine and HMF, respectively. Milk samples were extracted with 1% trichloroacetic acid using a high-speed blender and ultrasonication. After centrifugation and filtration, the extract was analyzed by CE-DAD directly. Micellar electrokinetic capillary chromatography was employed as the separation mode by adding sodium dodecyl sulfate (SDS) to the electrolyte. Under optimal separation conditions, melamine, HMF, and interferents were well resolved. The linear dynamic ranges were 0.05−100 μg/mL for melamine (R^2 = 0.9996) and 0.1−100 μg/mL for HMF (R^2 = 0.9997). The assay detection limits were 0.047 μg/mL and 0.067 μg/mL for melamine and HMF, respectively. Satisfactory results were obtained for the assay recovery rate and repeatability. The proposed method was successfully applied for the analysis of melamine and HMF in real milk samples, and the results of melamine were comparable to those obtained using HPLC-UV reference method.

KEYWORDS: Melamine; 5-hydroxymethylfurfural; milk; capillary electrophoresis; diode array detection

INTRODUCTION

Recently, nitrogen-rich melamine (67% by mass) had been found to be adulterated to milk products and animal feed in order to increase the apparent protein content, as protein concentrations are typically measured by analysis of nitrogen. Ingestion of melamine may lead to kidney stones, renal failure, and other health problems. The 2007 Pet Food Recalls and the 2008 Chinese Milk Scandal have raised concerns. Intensive controls on melamine by national safety authorities, importers, producers, and other parties of the food industry all over the world are being conducted to protect human health. Therefore, there is an increasing need to perform melamine testing. Melamine in vegetable samples and pet foods is traditionally analyzed by high performance liquid chromatography (HPLC) (*1*). Recently, mass spectrometry coupled with gas chromatography (*2*), liquid chromatography (*3*, *4*), and capillary electrophoresis (CE) (*5*) were developed for the analysis of melamine in complex matrixes. Though mass spectrometry is an effective tool for molecule identification, it is more expensive compared with UV spectroscopy, and usually, solid phase extraction is required prior to analysis. In 2009, capillary zone electrophoresis (CZE) with diode array detection (DAD) was reported for melamine determination in liquid milk, yogurt, whole milk powder, fish feed, and fish at residue levels (*6*).

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5-Hydroxymethylfurfural (HMF) is an organic compound derived from dehydration of sugars and has been identified in a wide variety of heat-processed foods including milk, fruit juices, spirits, honey, etc. In recent years, the presence of HMF in foods has raised toxicological concerns: the compound and its similar derivatives (5-chloromethyl- and 5-sulfidemethylfurfural) have been shown to have cytotoxic (*7*), genotoxic (*8*), and tumoral effects (*9*, *10*). However, further studies suggest that HMF does not pose a serious health risk (*11*), but the subject is still a matter of debate. HMF is formed as an intermediate product during the Maillard reaction (*12*, *13*) and has been widely used as an indicator to assess the heat load of a thermal process in the dairy industry (*14*, *15*) and to distinguish heat-treated milk (pasteurized, direct, and indirect UHT, in-container sterilized, concentrated, and powdered) (*16*). In 1959, Keeney and Bassette developed a colorimetric method of determining HMF in dairy products (*17*). Since then, many works have been reported on the analysis of HMF in milk and milk products. Reversed-phase HPLC has been applied to measuring HMF in heat processed milk after separation on a C18 reversed-phase silica column (*16*, *18*). Morales and Jiménez-Pérez (*19*) applied capillary chromatography for measuring HMF directly in infant milk-based formulas by its absorption at 280 nm. Later, analysis of HMF in foods was demonstrated by using GC-MS (*20*) and liquid chromatography multistage mass spectrometry (*21*, *22*).

In this study, we examine the feasibility of simultaneous determination of melamine and HMF in milk samples using

 Published on Web 09/17/2009

capillary electrophoresis with diode array detection. SDS was used as the sieving matrix. The factors affecting the separation efficiency were examined. Under optimal separation conditions, melamine, HMF, and interferents were separated with distinctly different separation selectivity. Satisfactory results were obtained for assay linearity, detection limit, recovery rate, and repeatability. The proposed method was successfully applied for the analysis of melamine and HMF in 10 commercial milk samples, and the melamine results were comparable to those obtained using the HPLC-UV reference method.

MATERIALS AND METHODS

Apparatus and Reagents. Separation of melamine and HMF was carried out on a Beckman P/ACE MDQ capillary electrophoresis system (Fullerton, CA), equipped with a photodiode array detector capable of scanning the full range of wavelengths from 190 to 600 nm. In order to achieve good sensitivity and avoid interference, 214 and 280 nm were chosen as the wavelengths for the detection of melamine and HMF, respectively. Electropherograms were collected using 32 Karat 8.0 Software. Electrophoresis was performed in fused-silica capillaries of 75 μm i.d. and 375 μm o.d. (Polymicro Technologies, Phoenix, AZ, USA). All capillaries were 58.5 cm long, and the distance from the point of injection to the window of on-column detection was 50 cm. SG2 pH Meter (Mettler-Toledo instrument (Shanghai) Co., Ltd.) was used for pH measurement.

Sodium dodecyl sulfate (SDS), phosphoric acid, sodium tetraborate, boric acid, monosodium phosphate, disodium phosphate, trichloroacetic acetic acid (TCA), sodium hydroxide, hydrochloric acid, and melamine (>99%) were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). HMF and Tris-base were purchased from Sigma-Aldrich (St. Louis, MO). All of the chemicals were in analytical-reagent grade and used without further purification. Buffer solutions were prepared with ultrapure water supplied by a Milli-Q RG unit from Millipore (Bedford, MA).

Solution Preparation. Standard stock solutions of 1.0 mg/mL of melamine or HMF were prepared by dissolving 5 mg of melamine or HMF in 5 mL of 1% TCA solution. The stock solutions were kept at 4 °C until needed and used within a month. Diluted standard solutions of 0.05, 0.1, 0.2, 0.5, 2, 5, 20, and 100 μg/mL were prepared by diluting the standard stock solutions in 1% TCA solution. Unless otherwise stated, the electrophoresis buffer (running electrolyte) consisted of 15 mM monosodium phosphate, 15 mM disodium phosphate, and 80 mM SDS, with pH adjusted to 6.85. The running electrolyte was ultrasonicated for 10 min and filtered through a 0.45 μm membrane (Bandao Co., Ltd., Shanghai, China) before use. The buffer was kept at room temperature and used within two weeks.

Sample Preparation. Two liquid milk and seven powdered milk samples were collected (some of them had been cleared from the shop shelves of local supermarkets in September of 2008). Liquid milk was first dissolved in 4% TCA solution at a volumetric ratio of 1:1. Then, 2 mL of the dissolved liquid milk and 2 mL of water were added into a 50-mL polypropylene centrifuge tube and vortexed for 2 min. Powdered milk (2.0 g) was dissolved with 6 mL of 1% TCA solution, vortexed for 2 min, and made up to 10.0 mL with 1% TCA solution. The mixture was ultrasonicated for 10 min, vortexed for 2 min, and centrifuged at 12000 rpm for 4 min. The medial liquid layer was collected and then centrifuged at 12000 rpm for 4 min. The medial liquid layer was collected and filtered through a 0.45 μm membrane. The filtrate was injected directly for capillary electrophoresis analysis.

Capillary Electrophoresis Conditions. The new capillary was conditioned prior to use by washing sequentially with methanol for 5 min, water for 3 min, 0.1 M HCl for 10 min, water for 5 min, 1.0 M NaOH for 15 min, water for 5 min, and then the running electrolyte for 20 min. Between each run, the capillary was rinsed with running electrolyte for 5 min. After all analyses on a day, the capillary was washed with 0.1 M NaOH for 5 min, water for 5 min, and the running electrolyte for 5 min. Sample introduction was performed by applying a pressure of 0.5 psi for 5 s. The electrophoretic separation was carried out with a total analysis time of 18 min at a constant voltage of +15 kV and at a thermostatted temperature of 25 °C.

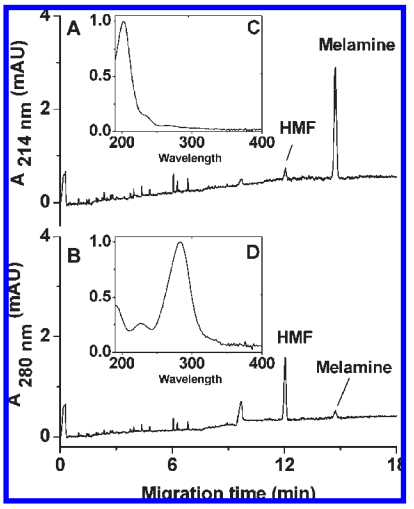

Figure 1. Electropherograms of the mixture of 5 μg/mL melamine and 5 μg/mL HMF standard solutions. (**A**) Electropherogram detected at 214 nm; (**B**) electropherogram detected at 280 nm) (**C**) ultraviolet absorption spectrum of melamine; (**D**) ultraviolet absorption spectrum of HMF. Conditions: separation solution, 15 mM monosodium phosphate, 15 mM disodium phosphate, and 80 mM SDS, pH 6.85; applied voltage, 15 kV; sample injection, 0.5 psi × 5 s.

RESULTS AND DISCUSSION

It has been reported that good separation can be obtained for melamine detection by simple capillary zone electrophoresis (*6*). However, HMF did not migrate out of the capillary even after a prolonged separation time at CZE mode, and the separation of melamine and HMF from the interferents was unsatisfactory in our experiment. Therefore, SDS was introduced into the electrolyte, and micellar electrokinetic capillary chromatography was employed for improved separation efficiency.

Detection Wavelength Selection. In order to find the optimal detection wavelengths, the UV−vis absorption spectra of melamine and HMF were screened in the range of 190−400 nm with a diode array detector. The results showed that the maximum absorption wavelengths of melamine and HMF were located at 202 and 280 nm, respectively. However, to avoid the interferences at short wavelength from other components coextracted from real milk samples, the detection wavelengths were set at 214 and 280 nm for melamine and HMF, respectively. As shown in **Figure 1**, capillary electrophoresis coupled with DAD detection allows good differentiation of melamine and HMF. Melamine and HMF can be identified by their migration times and their ultraviolet absorption spectra. The quantification was determined with an external standard calibration approach using peak area.

Sample Extraction Optimization. Melamine is a weak base (pK_b = 8), a polar compound, slightly soluble in water at room temperature and readily dissolved in most organic solvents or hot water. HMF is highly water soluble at room temperature. Generally, melamine and HMF can be extracted with polar organic solvents, buffer solution, or the mixture solutions of organic agent and water (*6*, *23*). Ding et al. investigated the extraction efficiency of different solvents for melamine in plant origin protein powders (*24*). 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 the present study, 1% TCA solution and 1% acetic acid solution were tested to precipitate proteins and to dissociate the target analytes from the sample matrix. Preliminary experiments showed that

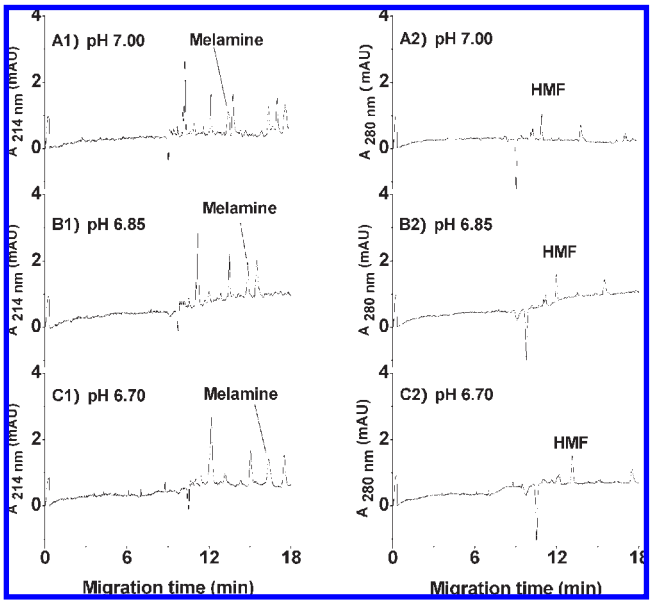

Figure 2. Effect of running buffer pH on the separation efficiency of melamine and HMF in a liquid milk sample spiked with 1 μg/mL of both melamine and HMF. (**A**) pH 7.00; (**B**) pH 6.85; (**C**) pH 6.70. A1, B1, and C1: electropherograms detected at 214 nm. A2, B2, and C2: electropherograms detected at 280 nm. Conditions: separation solution, 15 mM monosodium phosphate, 15 mM disodium phosphate, and 80 mM SDS. Other conditions were the same as those described in **Figure 1**.

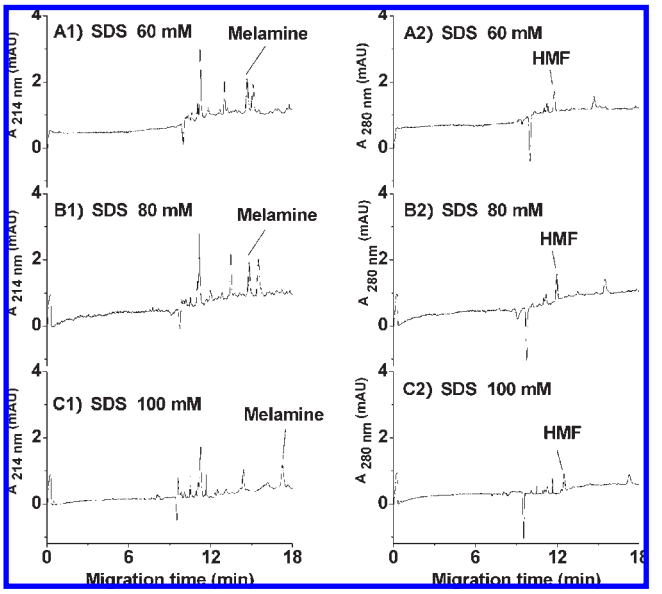

Figure 3. Effect of SDS concentration in the micellar system. (**A**) 60 mM SDS; (**B**) 80 mM SDS; (**C**) 100 mM SDS. A1, B1, and C1: electropherograms detected at 214 nm. A2, B2, and C2: electropherograms detected at 280 nm. Conditions: separation solution, 15 mM monosodium phosphate and 15 mM disodium phosphate, pH 6.85. Other conditions were the same as those described in **Figure 1**.

TCA yielded more efficient protein precipitation and higher recoveries for both melamine and HMF. Therefore, 1% TCA solution was chosen as the extractant in the following experiments. Besides, ultrasonic extraction and boiling water bath extraction were also compared. The mixture of milk sample and TCA solution was ultrasonicated for 10 min or heated with a boiling water bath for 10 min. Both approaches exhibited comparable performance in protein precipitation and analytes recovery. As ultrasonic extraction features a simple and safe operation, it was chosen as the optimum extraction method.

Separation Condition Optimization. The effects of carrier electrolyte composition, pH value, and SDS concentration on signal intensity and separation efficiency of melamine and HMF were investigated. Liquid milk was chosen as the model sample for separation optimization. A liquid milk sample dissolved in 1% TCA was spiked with 1 μg/mL of both melamine and HMF prior to the extraction process. Three different aqueous buffers were tested: Tris buffer (Tris-H_3PO_4), borate buffer ($H_3BO_3-Na_4B_2O_7$), and phosphate buffer ($Na_2HPO_4-NaH_2PO_4$). When the Tris buffer solution was used (pH 5.8, 6.0, 6.2, 6.4, 6.6, and 6.9), the signal of melamine was very weak, which could be ascribed to the hydrogen bonding interaction between melamine and Tris. The borate buffer solution could not provide extensive range of pH (only 7.4−9.0), and within this limited pH range, extracted components from milk samples could be not be baseline separated. The phosphate buffer solution yielded good separation and signal-to-noise ratio for both melamine and HMF among all three kinds of separation buffers. Therefore, the phosphate buffer was chosen as the running buffer.

Besides the running buffer composition, its pH value is also an important variable because of its effect on the electro-osmotic flow (EOF) as well as the net charge of melamine and HMF. The effect of the running buffer pH on the CE separation was investigated in the pH range of 6.70−7.00 (6.70, 6.85, and 7.00) with results displayed in **Figure 2**. As we can see, quite a few peaks showed up after 18 min of separation for the liquid milk sample. By comparing both the UV−vis absorption spectra and the migration time of standard compounds along with the standard addition method, the peaks for melamine and HMF were identified. It was found that the peaks of melamine and other extraction components were barely baseline resolved at pH 7.00. When the pH was decreased to 6.85, the separation between melamine and the nearby interferent was significantly improved. But with further decrease of pH to 6.70, the melamine peak was tailing, and the peak width was broadened along with prolonged migration time due to the low EOF. However, fewer peaks showed up when the separation was monitored at 280 nm, and the resolution of the HMF peak hardly changed with pH variation. Therefore, pH 6.85 provided a balance between good resolution and short analysis time.

In order to identify the most appropriate separation conditions, the concentration of SDS in the micellar system was investigated. During the separation, the micelles of SDS migrate to the anode which could interact with the samples in a chromatographic manner through both hydrophobic and electrostatic interactions. **Figure 3** presents the effect of SDS concentration on the separation efficiency of melamine and HMF. As revealed in **Figure 3**, the peaks of melamine and the interferent were barely baseline resolved at 60 mM SDS. When the SDS concentration was increased to 80 mM, the separations of both melamine and HMF from their interferents were improved. But with the further increase of SDS concentration to 100 mM, the separation time increased with no improvement in resolution; meanwhile, the detection sensitivity diminished for both melamine and HMF. Therefore, 80 mM SDS was chosen for subsequent analyses.

The influence of separation voltage on the EOF and the migration duration was also investigated. The increase of applied voltage led to shorter migration times and sharper peaks for melamine and HMF. As expected, on increasing the applied voltage there was an increase in electro-osmotic flow, leading to shorter analyses times. However, higher applied voltage was accompanied with higher current and increased Joule heating. Therefore, the optimal separation voltage was set at 15 kV.

Linearity, Detection Limit, and Reproducibility. The detection limit and quantification limit for the CE-DAD assay were defined

Table 1. Detection Limit, Quantification Limit, and Linear Dynamic Range of the CE-DAD Assay for Melamine and HMF Analysis

compound	detection limit (μg/mL)	quantification limit (μg/mL)	liquid milk quantification limit (μg/mL)	powdered milk quantification limit (μg/g)	correlation coefficient (R^2)	linear range (μg/mL)
melamine	0.047	0.05	0.20	0.25	0.9996	0.05−100
HMF	0.067	0.10	0.40	0.50	0.9997	0.10−100

Table 2. Repeatability of Migration Time and Peak Area for Melamine and HMF Analyzed by CE-DAD

	compound	migration time	peak area
intraday (n = 5)	melamine	2.25%	2.58%
	HMF	1.69%	3.53%
interday (n = 5)	melamine	4.30%	4.61%
	HMF	4.23%	5.42%

Table 3. Recovery Rate for Melamine and HMF Detection in Milk Samples by CE-DAD

compound	spiked concentration	average recovery	RSD (%) (n = 4)
	1.0 μg/mL	97.02%	3.09%
	5.0 μg/mL	94.85%	4.20%
HMF	1.0 μg/mL	99.20%	3.11%
	5.0 μg/mL	95.26%	2.85%

as $s_b + 3s$, and $s_b + 10s$, respectively, where s_b is the average signal of 10 blank injections, and s denotes the standard deviation. Calibration curves were obtained from six different concentrations of the mixture of melamine and HMF standard solutions. Each sample was injected in triplicate. Sample quantification limit was calculated by taking into account the 4-fold (liquid milk) and 5-fold (powdered milk) dilutions in the process of sample extraction. The measured detection limit, quantification limit, sample quantification limit, correlation coefficient, and linear dynamic range of the calibration plots for melamine and HMF are listed in **Table 1**. As the international maximum residue limits (MRL) for melamine in milk products were set as 1.0−2.5 mg/kg (*25*), the sample quantification limits of 0.2 μg/mL for liquid milk and 0.25 μg/g for powdered milk provided by the CE-DAD approach are well suited for the analysis of melamine content in milk products. As for HMF analysis, the sample quantification limits of 0.40 μg/mL for liquid milk and 0.50 μg/g for powdered milk provided by CE-DAD are comparable with that of 2.5 μmol/L or 0.325 μg/mL obtained with similar micellar electrokinetic capillary chromatography methods (*19*).

The reproducibility study comprised intraday and interday assays. The intraday analysis was conducted by sample pretreatment and CE analysis of the same milk sample five times on the same day, and the interday repeatability was determined by sample pretreatment and CE analysis of the same milk sample on five different days. **Table 2** shows the precision data of the migration time and the peak area for the milk samples analyzed. The relative standard deviations (RSD) of intraday and interday analysis were ⩽2.25% and ⩽4.30% for the migration time and ⩽3.53% and ⩽5.42% for the milk concentration for melamine and HMF, respectively.

Recoveries and Applications. To determine the recovery of the system, two different concentrations (1.0, 5.0 μg/mL) of melamine and HMF standard mixture were added to a liquid milk sample dissolved in 1% TCA prior to extraction. The recovery rates are reported in **Table 3**. The average recoveries were 95.94% for melamine and 97.23% for HMF, respectively, and the RSD was less than 5% for both melamine and HMF.

Ten commercial milk samples in liquid and powdered form were analyzed using the optimized method. After an extraction procedure with 1% TCA buffer using a high-speed blender, ultrasonication, centrifugation and filtration, the filtrate was injected into the capillary for analysis. **Figure 4** shows the representative electrophoretic profiles of a powdered milk sample extraction and the same sample spiked with 5 μg/mL of both melamine and HMF. As we can see, a strong melamine peak and a weak HMF peak were detected for the sample. On the basis of the calibration curves generated from peak area values of melamine and HMF standard solutions and taking into account the 5-fold dilutions in the process of sample extraction, the melamine and HMF contents in the original sample were calculated to be 33.84 μg/mL and 1.59 μg/mL, respectively (sample #4 in **Table 4**). The melamine and HMF contents detected by CE-DAD, melamine results obtained using the HPLC-UV reference method of China (*23*) by Quanzhou Inspection and Quarantine Bureau of China, and the compositions (protein, lipid, and carbohydrate) declared on the labels of the 10 commercial milk samples are given in **Table 4**. The concentration units used for melamine and HMF detection are in μg/mL and μg/g for liquid and powdered milk samples, respectively. As we can see, slightly higher values were obtained with the CE approach. Linear fit of the data obtained using CE-DAD and HPLC-UV methods yields a correlation coefficient R^2 of 0.99485, indicating that the quantification difference between the two methods originates from a systematic error. The discrepancies between CE-DAD and HPLC-UV may be due to the different sample pretreatment processes. Since an extra solid phase extraction process in addition to TCA extraction was carried out prior to HPLC injection, this may account for the relatively low recovery rate of the HPLC-UV method for melamine detection (80% by Quanzhou Inspection and Quarantine Bureau of China).

Let us take a close look at the melamine concentrations detected for the 10 commercial milk samples collected in September of 2008. Sample #1 is a fruit juice milk for kids, and melamine was not detected using both CE-DAD and HPLC methods. Sample #2 is sample #1 spiked with 10 μg/mL melamine, and

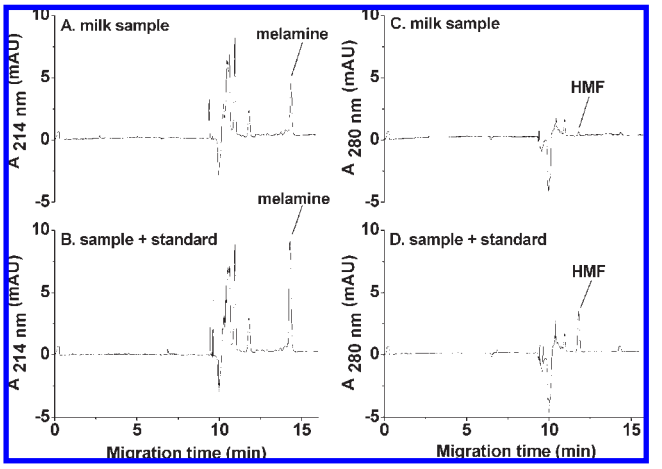

Figure 4. Representative electropherograms of a powdered milk extraction and the same sample spiked with 5 μg/mL of both melamine and HMF. (**A**,**B**) electropherograms obtained at 214 nm; (**C**,**D**) electropherograms obtained at 280 nm. Other conditions were the same as those described in in **Figure 1**.

Table 4. Detected Melamine and HMF Contents of the Ten Commercial Milk Samples along with Their Compositions

	compositions as declared on the label, g/100 g and g/100 mL for powdered and liquid milk samples, respectively			melamine		HMF
sample	protein	lipid	carbohydrate	CE-DAD	HPLC[a]	CE-DAD
1 (fruit juice milk for kids)	≥1.0	≥1.0	≥5.0	n.d.[b]	n.d.	0.59 μg/mL
2 (#1 spiked with 10 μg/mL melamine)	≥1.0	≥1.0	≥5.0	10.26 μg/mL	8.32 μg/mL	0.54 μg/mL
3 (powdered milk)	27.5	18.5	50.0	n.d.	n.d.	n.d.
4 (liquid milk, same brand as #3)	2.4	2.8	6.9	33.84 μg/mL	26.99 μg/mL	1.59 μg/mL
5 (powered infant milk)	17.5	21.8	50.0	20.10 μg/g	16.95 μg/g	2.25 μg/g
6 (powdered infant milk, same brand as #5, different batch)	17.5	21.8	50.0	23.63 μg/g	18.18 μg/g	1.27 μg/g
7 (powdered infant milk, imported from New Zealand)	18.8	23.5	51.1	n.d.	n.d.	2.10 μg/g
8 (powdered infant milk, imported from Netherland)	18.0	22.0	54.0	n.d.	n.d.	0.60 μg/g
9 (powdered infant milk)	11.8	25.9	57.2	1.32 μg/g	1.02 μg/g	n.d.
10 (powdered soy milk)	>15	>8	<75	31.73 μg/g	26.80 μg/g	0.91 μg/g

[a] The HPLC results of melamine were provided by Quanzhou Inspection and Quarantine Bureau of China, and the mean of recovery rates was 80%. [b] n.d.: not detected, i.e., below the sample quantification limit.

the detected melamine concentration by the CE-DAD approach is within the assay precision range, and good recovery was obtained. Sample #3 is a powdered milk, and melamine was not detected. Sample #4 (liquid milk) is from the same Chinese brand as sample #3, but the detected melamine value was relatively high. Samples #5 and #6 are powdered infant milk from the same Chinese brand but of different batches, and relatively high melamine contents were detected in both samples. However, no melamine was detected in samples #7 and #8 (powdered infant milk samples imported from New Zealand and Netherlands, respectively). Marginal melamine value was detected in sample #9 (powdered infant milk). It is surprising that melamine was also detected in powdered soy milk (sample #10).

HMF has been used in various foods as an indicator of the severity of heat treatment and also of the length and conditions of storage (*15*). For the 10 commercial milk samples analyzed, the HMF concentrations of two powdered milk samples (#3 and #9) were below 0.50 μg/g and could not be accurately quantified. For the other eight samples, the detected HMF concentrations varied from 0.54 μg/mL to 2.25 μg/g. As infant milk is heat-treated to guarantee its safety and to prolong its storage life, the measured HMF concentrations for infant milk were between 0.6 μg/g to 2.25 μg/g, which are comparable with those reported in the literature in powdered and liquid infant milk formulas (*15*). In summary, CE-DAD can provide simultaneous analysis of melamine and HMF in milk samples with satisfactory results. This method potentially offers a rapid and reliable method for routine analysis of melamine and HMF in milk samples.

ACKNOWLEDGMENT

We thank Quanzhou Inspection and Quarantine Bureau of China for melamine detection using the HPLC-UV reference method.

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Received June 26, 2009. Revised manuscript received August 29, 2009. Accepted September 3, 2009. This work was supported by the Program for New Century Excellent Talents in University (NCET-07-0729) and by the Scientific Research Foundation for Returned Overseas Chinese Scholars, State Education Ministry (SRF for ROCS, SEM) for which we are most grateful.