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Received July 26, 2010
Revised November 16, 2010
Accepted November 16, 2010

Research Article

Determination of cypromazine and its metabolite melamine in milk by cation-selective exhaustive injection and sweeping-capillary micellar electrokinetic chromatography

In this study, we described a high-sensitive on-line preconcentration method for cypromazine (CYP) and melamine (MEL) analysis using cation-selective exhaustive injection (CSEI) combined with sweeping-MEKC. The optimum conditions of on-line concentration and separation were discussed. The BGE contained 100 mM SDS, 50 mM phosphoric acid (pH = 2.0) and 15% acetonitrile (v/v). The sample was injected at 10 kV for 600 s, separated at −20 kV, and detected at 210 nm. The sensitivity enhancements were 6222 for CYP and 9179 for MEL. The linear dynamic ranges were 0.4−25 ng/mL for CYP ($r = 0.9995$) and 0.2−12 ng/mL for MEL ($r = 0.9991$). The LODs (signal-to-noise ratio, 3) were 43.7 and 23.4 pg/mL for CYP and MEL, respectively. The proposed method was applied to analyze CYP and MEL in dairy products pretreated using off-line SPE to minimize the influence of the matrix. The recoveries of CYP and MEL were satisfactory (ca. 74–83%). The experimental results suggest that the CSEI-sweeping-MEKC method is feasible for the application to simultaneously detect trace levels of CYP and its metabolite MEL in real milk samples.

Keywords: Cation-selective exhaustive injection-sweeping-MEKC / Cypromazine / Melamine / Milk / On-line preconcentration
DOI 10.1002/jssc.201000559

1 Introduction

Cypromazine (CYP), *N*-cyclopropyl-1,3,5-triazine-2,4,6-triamine (Fig. 1A), is a triazine insect growth regulator used as a foliar spray to control leaf miners in vegetables, mushrooms, potatoes and ornamentals. It is also used to control Diptera larvae in chicken manure by feeding to the poultry or treating the breeding sites and control flies on animals. Like other triazine derivative pesticides, CYP is highly effective in pesticide control but it is also toxic to humans and the environment. In addition, it can metabolize via dealkylation reactions in both plants and animals and undergo environmental degradation to form melamine (MEL) [1]. MEL (1,3,5-triazine-2,4,6-triamine) (Fig. 1B) is an industrial chemical commonly used in the manufacture of plastics, flame retardants and other materials. Due to the high amount of nitrogen in MEL, it was deliberately added to the food or the animal feed for increasing the apparent protein content which is usually estimated by determining the nitrogen content [2]. Although MEL has a low toxicity in mammals ($LD_{50} = 3161$ mg/kg) (http://www.inchem.org/documents/sids/sids/108781.pdf.), recent studies have reported that MEL is able to form an insoluble salt, may precipitate in kidneys, and cause renal functional failure [2, 3]. As a result, the 2007 Pet Food Recalls and the 2008 Chinese Milk Scandal have attracted worldwide attention. Subsequently, MEL was detected in food and feed products in 47 countries. Intensive regulatory control and inspection on MEL by national safety authorities, importers, producers and other parties of the food industry all over the world are being conducted to protect public health. Due to the widespread use of MEL in applications involving contact with food, trace amounts of MEL may be found in food. On one hand, MEL may be found in the food from illegal additive. On the other hand, MEL may also enter the food chain indirectly through animal feeds that have been treated with CYP containing MEL. Recently, there have been reports of MEL findings in milk, egg and soya products, which may have originated from the animal feed and carried over into the food. However, these

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Abbreviations: BGS, background solution; **CSEI,** cation-selective exhaustive injection; **CYP,** cypromazine; **HCB,** high-conductivity buffer; **MEL,** melamine; **MeOH,** methanol

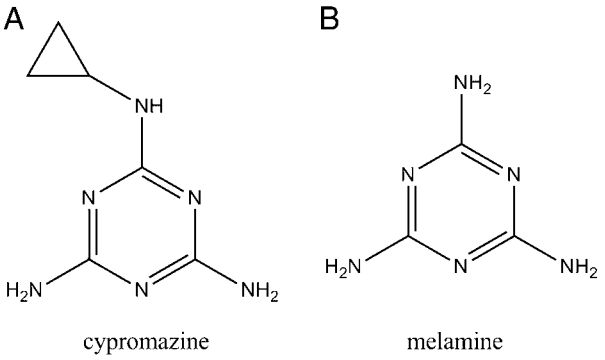

Figure 1. The chemical structures of CYP and MEL.

occurrences of MEL have not yet been distinguished between the intentional adulteration of food or feed and residues from the legitimate use of CYP. Therefore, there is an increasing need to simultaneously analyze trace levels of MEL and CYP in different matrices where the pesticide may be used.

Various analytical approaches of sample preparation and determination of MEL or CYP residues in biological samples have been reported, including GC [4], HPLC [5–9], ELISA [10], Raman spectroscopy [11–13], LC-MS [2, 9, 14–18] and GC-MS [6, 16, 19–22]. Literature reviews showed that there were only few methods which can simultaneously determine MEL and CYP. CYP residue and its metabolite, MEL, in Chinese cabbage and soil has been analyzed by gas chromatography with nitrogen-phosphorus detection (GC-NPD) [4] or mass selective detection (GC-MSD) [6]. Likewise, CYP and MEL in milk, eggs, chicken and tilapia muscle samples have been analyzed by GC-MS [20, 23]. Methods using high-performance liquid chromatography with ultraviolet detection (HPLC-UV), photodiode array detection (HPLC-DAD) or mass spectrometry (LC-MS) have also been developed to analyze CYP and MEL in animal-derived food samples [7, 8, 14, 15]. Among them, GC-MS methods showed good sensitivity and selectivity, but precolumn derivatizations or complicated pretreatments were always inevitable. LC-MS method could combine the high separation efficiency of HPLC with the low detection limits and high confidence in identifications of MS, which is now the principal analytical method used by the US Food and Drug Administration. However, its high consumed cost and high cost of equipment and personnel were not suitable for ordinary analytical laboratories.

As an analytical tool, CE has many advantages including rapid analyses, high separation efficiencies, low consumption of solvent and minimal sample requirements. However, poor concentration sensitivity of CE with UV detection due to the small sample injection volume and the short optical path length limited the use of CE. Therefore, using powerful detectors and developing sample preconcentration techniques is of necessity for expanding its application [24–28]. Nielen et al. and Cook et al. used capillary zone electrophoresis-mass spectrometry (CZE-MS) to analyze MEL resins with good sensitivity [29, 30], but MS instruments are much more expensive in comparison to conventional UV detectors. Recently, Yan et al. and Xia et al. used CZE with DAD detector to analyze MEL and some other related compounds in food products and pet feed [31, 32]. Chen and Yan used micellar electrokinetic chromatography (MEKC) with DAD detector to analyze MEL and 5-hydroxymethylfurfural in milk samples [33]. However, the sensitivity of the CZE-DAD and MEKC-DAD methods was unsatisfactory. On-line concentration approaches have been successfully coupled to UV detection for the analysis of MEL and related compounds. A cation-selective injection (CSI)-MEKC and sweeping-MEKC method as well as a transient isotachophoretic stacking CE method have been reported to trace the MEL contaminant in milk products and tableware samples [34–36]. To our knowledge, the use of an on-line concentration technique in combination with CE for simultaneous determination of MEL and CYP has not been reported.

The aim of this study is to establish a sensitive and feasible CE method for the analysis of MEL and CYP in dairy products. The solid-phase extraction (SPE) was used for sample pretreatment, and cation-selective exhaustive injection (CSEI)-sweep-MEKC method was applied for on-line stacking and separation. Several separation parameters, including nonmicellar separation buffer, the concentrations of sodium dodecyl sulfate (SDS) and organic modifier, the injection length of the high-conductivity buffer (HCB), water plug, the methanol (MeOH) in sample matrix and the injection time of the sample were investigated. We also compared the sensitivity enhancements using CSEI-sweeping-MEKC with sweeping-MEKC and normal MEKC. The proposed method was successfully applied to the analysis of CYP and its metabolite MEL in commercial milk samples.

2 Materials and methods

2.1 Chemicals and reagents

CYP standard (99.0%) was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany) and MEL standard (99%) was obtained from Sigma-Aldrich (Shanghai, China). Acetonitrile (ACN) and MeOH were of HPLC grade from Sinopharm Chemical Reagent (Shanghai, China). All the other solvents and reagents were of analytical grade and used without further purification. Sodium dihydrogen phosphate, phosphoric acid, trichloroacetic acid (TCA) and chloroform were products of Tianjin Chemical Reagent Factory (Tianjin, China). Hydrochloric acid, sodium hydroxide, ammonium hydroxide, isopropanol (IPA), trichloromethane and SDS were purchased from Sinopharm Chemical Reagent. Water was purified through a Milli-Q water system (Millipore, Milford, MA, USA).

2.2 Apparatus

All experiments were performed with a Beckman P/ACE MDQ CE system (Beckman, Fullerton, CA, USA), equipped with a diode-array detector (190–600 nm) and a liquid-cooling device. To achieve highest sensitivity for CYP and MEL, 210 nm was chosen as the best wavelength. An uncoated fused silica capillary tube (60 cm total length, 50 cm length to detector, 50 μm id) (Yongnian, China) was used throughout. The capillary tube was assembled in the cartridge format. Instrument control, data acquisition and data analysis were carried out using 32 Karat software (version 7.0). Before separation, the new capillary was preconditioned sequentially with MeOH (10 min), 1 M hydrochloric acid (10 min), deionized water (5 min), 1 M sodium hydroxide (10 min) and then deionized water again (5 min). Under optimal conditions, the nonmicellar BGE consisted of 50 mM phosphoric acid (pH 2.0) containing 15% v/v ACN. The HCB solution was 200 mM phosphoric acid (pH 1.8). The micellar BGE comprised 100 mM SDS in 50 mM phosphoric acid (pH 2.0) containing 15% v/v ACN. Between runs, the capillary was flushed sequentially with MeOH (3 min), water (7 min) and nonmicellar BGE (5 min) in the CSEI-sweeping-MEKC mode or with micellar BGE (5 min) in the MEKC and sweeping-MEKC modes. The sample was extracted in a KH-250DB Ultrasonic cleaner (Hechuang, Kunshan, China) and centrifuged in a TDL80-2Blow-speed centrifuge (Anke, Shanghai, China).

2.3 Preparing standards

A stock standard solution (1 mg/mL) of CYP was prepared by dissolving 10 mg of the product in 10 mL of MeOH. Standard stock solution containing 1 mg/mL MEL was prepared by dissolving 10 mg of MEL in 10 mL of 50% v/v MeOH aqueous solution. The solutions were stable for at least 2 months and stored in the dark at 4°C. The working standard was prepared by diluting stock solution to various concentrations in 80% v/v MeOH. Stock solutions of 0.5 M SDS and 10% m/m TCA were prepared every 2 wk in purified water. Nonmicellar background solutions (nonmicellar BGSs) were prepared by dilution of 0.5 M phosphoric acid stock solution and additives in water. Micellar BGSs were prepared by dilution of the SDS stock solution, 0.5 M phosphoric acid stock solution and additives in water. The nonmicellar and micellar BGSs were prepared every day to prevent repeatability problems. The pH was adjusted to desired values with 1 M phosphoric acid and was measured using a pH meter (PHS-3C). The buffers and sample solutions were filtered through 0.45 μm membrane filters before CE analysis.

2.4 Milk sample preparation

Liquid milk and milk powder were purchased from local supermarkets in Wuhan. The procedure for the sample preparation was similar to the previous reports [3, 23], and is described below. For liquid milk, 2 mL of 10% m/m TCA, 6 mL of deionized water and 1 mL of chloroform were added into each 15 mL polypropylene centrifuge tube containing 2 mL of liquid sample. The samples were vortex mixed for 30 s, ultrasonically extracted for 20 min and then centrifuged at 8000 rpm for 10 min. The supernatants were filtered with a 0.45 μm filter and then ready for SPE. For milk powder, 2.0 g of sample was added to a 15 mL centrifuge tube. In each tube, 1 mL of 10% TCA, 9 mL of deionized water and 1 mL of chloroform was added in sequence. Then the following operations, including mixing, extracting, centrifuging and filtering, were performed in the same way as for the liquid sample.

Bond Elut Plexa PCX cartridges were obtained from Varian (Palo Alto, CA, USA) for SPE. The cartridge (3 cc/60 mg) was fitted with a vacuum manifold (Varian, Vac Elut, USA) and conditioned with 3 mL of MeOH followed by 3 mL deionized water. The sample extracts were then added to the SPE columns and passed through the cartridge at a flow rate of 5 mL/min. After the sample effused completely, the cartridge was washed with 3 mL of water and 3 mL of MeOH, respectively. Vacuum continued to be applied until all the liquid had eluted and the SPE cartridge was completely dry. Finally, the residues were eluted by gravity from the SPE columns with 6 mL 5% v/v ammonium hydroxide in MeOH into a 10 mL glass vials. The eluate was then evaporated to dryness at 40°C under a stream of nitrogen, and the dry residue was redissolved by adding a proper amount of MeOH (80% v/v) solution to ensure that the final extracts were dissolved in the same solvent as the analytical standards.

2.5 Method procedures

The MEKC, sweeping-MEKC and CSEI-sweeping-MEKC methods were similar to that reported by Su et al [37]. The capillary is first filled with nonmicellar BGS at low pH (pH 2.0) to suppress the EOF. In the MEKC procedure, samples were pressure-injected at 3.45 kPa for 4 s. The separation proceeded with the micellar BGE and a negative applied potential (−20 kV). In the case of sweeping-MEKC, a large volume of analytes (6.9 kPa, 60 s) is injected into a capillary and a negative voltage (−20 kV) was applied. This procedure permits the SDS-anionic surfactant micelles (in the inlet reservoir) to enter the sample zone. Thus, the samples were swept and concentrated near the junction between the sample solution and the BGS. In the case of CSEI-sweeping-MEKC, the capillary was first filled with phosphate buffer (50 mM, pH 2.0) containing 15% v/v ACN, followed by the injection of a HCB (200 mM phosphate, 6.9 kPa for 99.9 s). After sample injection (10 kV for 600 s), phosphate buffer (50 mM, pH 2.0) containing 15% v/v ACN and 100 mM SDS was used as the sweeping buffer, and a voltage of −20 kV was applied. The negative voltage allowed the entry of negatively charged micelles from the inlet vial into the

capillary to sweep and stack the analytes into narrow bands. The separation was also performed under this mode.

3 Results and discussion

3.1 Sample extraction

Considering the molecule polarity of MEL and CYP, sample extraction is performed with polar solvents, buffer solution or the mixture solution [6, 7, 15]. In our study, TCA, MeOH, water and ACN were tested to precipitate proteins and to dissociate the target analytes from the sample matrix. We found TCA yielded more efficient protein precipitation. Therefore, TCA was chosen as the extractant in the following experiments. Three different concentrations (1, 2 and 5% m/m) of TCA were used to investigate the effect of the recovery for CYP and MEL in milk samples. The best extraction efficiency was found at 2% TCA. Aside from centrifugation and filtration, SPE is most often incorporated into methods for further sample clean-up after extraction. CYP and MEL are highly polar basic compounds, being normally difficult to obtain enough retention in a C18 column to separate analytes from salts and polar matrix components, which could interfere with them by decreasing the recovery [15]. In order to improve retention for CYP and MEL, a polymeric cation exchange SPE could be used. However, the solid fat layer was above the aqueous layer after centrifuging, which made it difficult to pass through the cartridge. 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.

3.2 Optimizing conditions for separation using CSEI-sweeping-MEKC

The CSEI-Sweeping-MEKC mode might be affected by various factors, including nonmicellar separation buffer, sweeping buffer, HCB, water plug, the MeOH in sample matrix and sample injection time. Each of these conditions was examined in order to obtain the best separation and enhancement efficiencies.

3.2.1 Choosing the separation buffer

To optimize the separation buffer, the phosphate concentration (50–100 mM) and pH (2–3.2) and amounts of different organic modifiers (0–20% v/v) were varied. It was found that the better separation was obtained when the separation buffer with the tested concentrations and pH values containing 15% ACN was used. The organic solvent was the most important factor influencing the separation efficiency of the analytes. We examined the effects of ACN, MeOH and IPA in a range of concentrations (0–20% v/v) and ACN appeared to be the best choice. The electropherograms are shown in Fig. 2 from which it can be seen that the addition of MeOH in buffer can obtain the baseline separation; however, the peak shape of MEL is worse than that with ACN. Figure 2 also shows the effects of ACN concentrations on the separation of MEL and CYP. It shows that the interferences of matrix can be eliminated and the baseline separation could be achieved in the buffer containing 15% ACN. In the range of ACN percentage from 0 to 20%, the migration time increased and an improvement in separation could be observed. If concentrations less than 10% ACN are used, the peak time

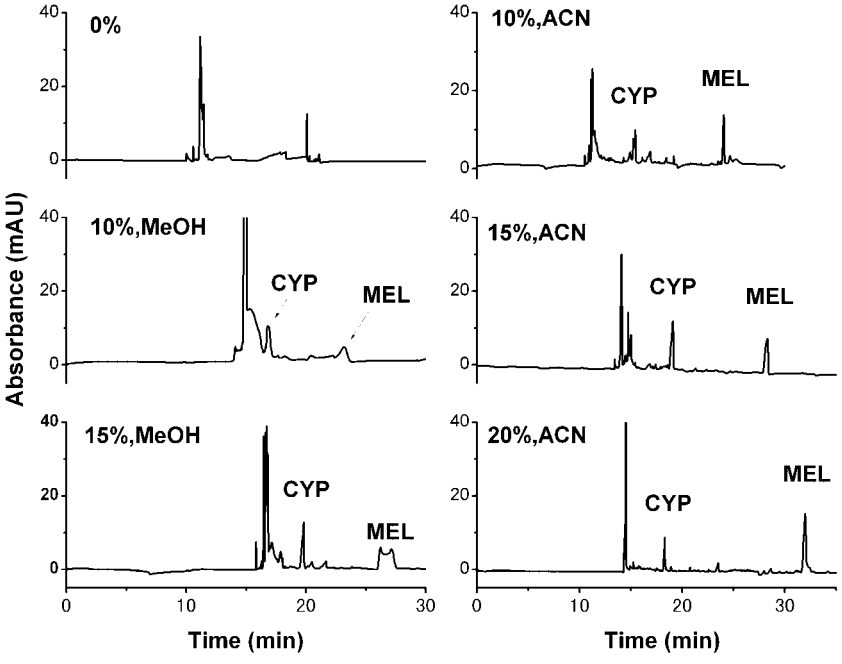

Figure 2. Effect of volume fraction of MeOH or ACN in nonmicellar separation buffer on the separation of analytes. Conditions: separation buffer, 50 mM phosphate (pH 2.0) with MeOH or ACN; HCB, 200 mM phosphate, 6.9 kPa for 99.9 s; sweeping buffer, 100 mM SDS in 50 mM phosphate (pH 2.0) contains MeOH or ACN; applied voltage, −20 kV (detector at anode side); uncoated fused silica capillary, 40 cm (effective length) × 50 μm id; sample size, electrokinetic injection 10 kV, 600 s; wavelength, 210 nm. Sample concentrations: MEL 10 ng/mL; CYP 20 ng/mL.

of MEL shortened, and may be interfered by the matrix peaks. When the concentration of ACN was further increased to 20%, MEL absorption peak was not detected within the 40 min running time. The separation buffer was selected as a phosphate buffer (50 mM, pH 2.0) containing 15% ACN.

3.2.2 Effect of the HCB zone and the SDS concentration

The effects of the injection length of HCB (200 mM phosphate) (none, 6.9 kPa for 50 s, 6.9 kPa for 99.9 s and 6.9 kPa for 180 s) on stacking of the analytes were tested. As seen in the electropherograms (Fig. 3), peak shape of MEL was improved obviously with the increasing of the length of the HCB zone. However, peak time of MEL exceeded 30 min when the injection of HCB was 6.9 kPa for 180 s. Therefore, we selected the injection of 6.9 kPa for 99.9 s as the length of HCB in this study. A few studies have suggested that inserting a plug of water after injecting the HCB zone can improve the sample stacking as a result of the different electric field strengths accelerating the cations into the capillary [25, 38, 39]. In our studies, different injection times of water from 0 to 5 s at 3.45 kPa were tested. The results indicated that the procedures did not have any obvious effect. Therefore, we did not inject water in our subsequent experiments.

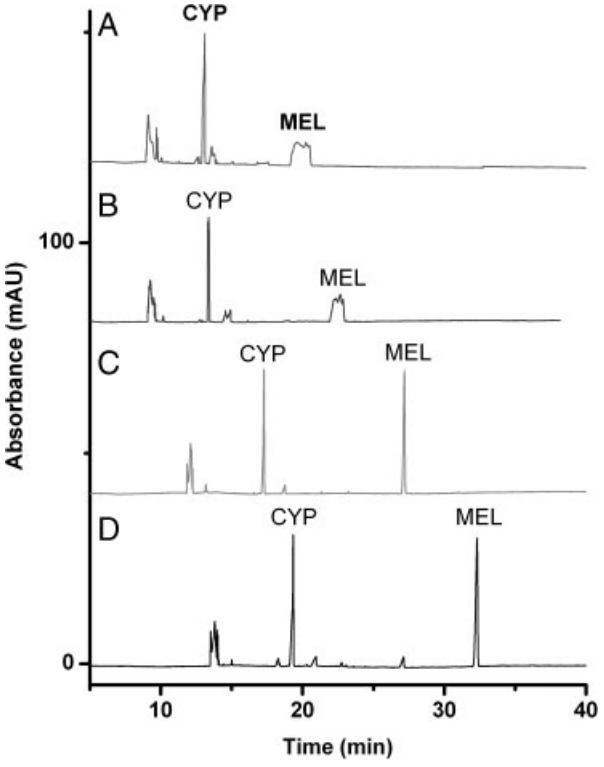

Figure 3. Effect of injection length of HCB on CSEI-sweeping-MEKC analysis. (A) 6.9 kPa, 30 s; (B) 6.9 kPa, 60 s; (C) 6.9 kPa, 99.9 s; (D) 6.9 kPa, 180 s. For other CE conditions, see Fig. 2.

We tested different levels of SDS (50–125 mM) in phosphate (50 mM, pH 2.0) as the sweeping buffer. The effect of the concentration of SDS on separation is investigated (data not shown). Peak time of MEL decreased obviously with the increasing of SDS concentration. However, the high concentration of SDS leads to clustered absorption peaks and lowered resolution. When 125 mM SDS was employed, the peak of CYP was disturbed by system peaks. To get a compromise between peak resolution and separation time, 100 mM SDS was adopted for the further experiments.

3.2.3 Effect of MeOH in sample matrix

The dissolution of analytes in water-miscible organic solvents (such as ACN and acetone) as compared to water has been shown to enhance on-line sample stacking in CE based on electrokinetic injection [40, 41]. The effects of ACN, MeOH and water in the sample matrix on the detection sensitivity of the system based on electrokinetic injection were tested. The results (data not shown) indicated that the presence of ACN and MeOH in sample matrix markedly enhanced the stacking ability, and of the three levels of ACN and MeOH that were tested, the inclusion of 80% v/v ACN provided the best improvement. However, the current with high content of ACN is not stable as that with the same content of MeOH. Therefore, 80% MeOH in the sample matrix was employed further attempts to investigate.

3.2.4 Effect of the injection time of the sample

It is known that the length of the stacked sample zone was dependent on the injection time during field-amplified sample injection (FASI), and a longer sample injection time should result in a longer sample zone and a higher stacking effect. In order to examine the effects of electrokinetic injection, different injection times (300–900 s at 10 kV) were tested. The peak intensity of two analytes was obviously increased when injection time was increased from 300 to 600 s, but no increase significantly in peak intensity was observed when the injection time went beyond 600 s and the separation became worse (data not shown). Therefore, we found that a sampling time of 600 s at 10 kV provided the optimal resolution and maximum peak enhancement.

3.3 Comparing MEKC, sweeping-MEKC and CSEI-sweeping-MEKC

A comparison of the conventional MEKC, sweeping-MEKC and CSEI-sweeping-MEKC method is demonstrated in the electropherograms as shown in Fig. 4. The sensitivity of the CSEI-sweeping-MEKC method relative to the conventional MEKC was improved by a factor between 6222 and 9179; relative to sweeping-MEKC, the improvement factor was between 635 and 823. The above results demonstrated that the proposed CSEI-sweeping-MEKC indeed improved

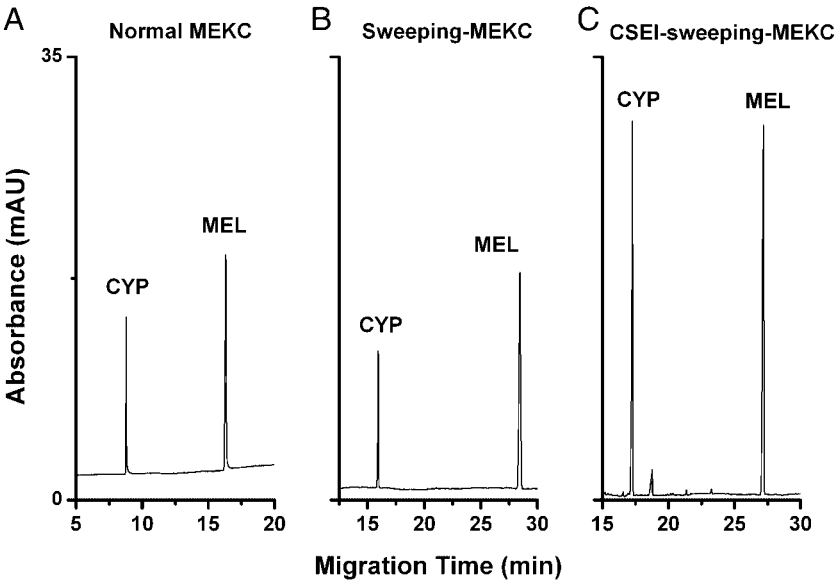

Figure 4. Comparison of normal MEKC, sweeping-MEKC and CSEI-sweeping-MEKC analyses. (A) Normal MEKC conditions: separation buffer; 100 mM SDS in 50 mM phosphoric acid (pH 2.0) containing 15% v/v ACN; sample concentration, 60 μg/mL in water; injection length, 1.6 mm. (B) Sweeping-MEKC conditions: separation buffer, 100 mM SDS in 50 mM phosphoric acid (pH 2.0) containing 15% v/v ACN; sample concentration, 5 μg/mL in water; injection length, 50 mm. (C) CSEI-sweeping-MEKC conditions: HCB, 200 mM phosphate, 6.9 kPa for 99.9 s; other conditions were the same as those employed to obtain Fig. 3.

Table 1. Ranges of linearity, calibration curves, coefficients of correlation (*r*), LODs and values of RSD for CYP and MEL using CSEI-sweeping-MEKC analytical methods

	CYP	MEL
Range of linearity (ng/mL)	0.4–25	0.2–12
Calibration curve	$Y = 3.846898 + 9.6440506X$	$Y = 8.11235 + 30.79811X$
Coefficient of correlation	0.9995	0.9991
LOD ($S/N = 3$, ng/mL)	0.0437	0.0234
Intra-day ($n = 5$)		
RSD of migration time (%)	3.06	3.28
Inter-day ($n = 3$)	3.31	4.04
Intra-day ($n = 5$)		
RSD of peak area (%)	6.38	7.91
Inter-day ($n = 3$)	8.91	9.67

markedly the detecting sensitivity compared to conventional MEKC and sweeping-MEKC.

3.4 Method validations

To evaluate the practical applicability of the proposed method, linearity, LOD and repeatability were measured for MEL and CYP in standard solutions under optimized conditions. Calibration curves were obtained from six different concentrations of the mixture of MEL and CYP standard solutions. Each sample was injected in triplicate. The measured detection limit, correlation coefficient and linear dynamic range of the calibration plots for MEL and CYP are listed in Table 1. The linearity between peak area and the concentrations was investigated in the range of 0.2–12.00 ng/mL for MEL and 0.4–25.00 ng/mL for CYP. The results indicate that an excellent linear relationship was attainable over the concentration range studied with a correlation coefficient of 0.9991 for MEL and 0.9995 for CYP, respectively. The LOD, calculated for an S/N of 3, was 23.4 pg/mL for MEL and 43.7 pg/mL for CYP. Precision was evaluated in terms of repeatability. The repeatability of the presented method was determined with a standard solution at concentration levels of 0.5, 2 and 5 ng/mL for CYP and MEL. The results, expressed as relative standard deviation (RSD) of migration time and peak areas, are also summarized in Table 1. The results indicated that the proposed CSEI-sweeping-MEKC method provided good performances for the analyses of CYP and its metabolite MEL.

3.5 Separating and determining CYP and its metabolite MEL in dairy products

Biological samples have complexity that makes them difficult subjects for analysis. To avoid the large matrix

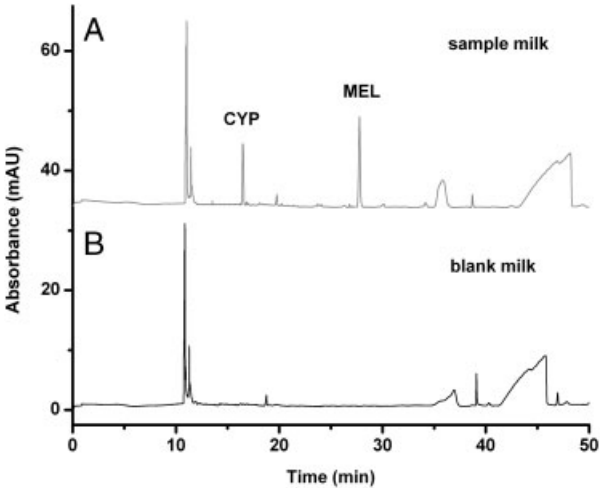

Figure 5. CSEI-sweeping-MEKC electropherograms of SPE extracted (A) blank and (B) spiked milk samples. Analyses were performed according to the optimized conditions of the CSEI-sweeping-MEKC method; sample concentrations: MEL, 6 ng/mL; CYP, 12 ng/mL.

effect that results from using real samples, we combined SPE with CSEI-sweeping-MEKC to determine the levels of CYP and its metabolite MEL in liquid milk and whole milk powder. The peaks were identified by comparison of the migration time of CYP and MEL in real samples with that of MEL and CYP standard and by spiking the MEL and CYP to the sample solutions as well as by the maximum absorption wavelength. Figure 5 shows that after we pretreated blank liquid milk through SPE, we could not detect MEL and CYP. When we spiked the MEL and CYP into the blank samples prior to performing the SPE process, we can determine the presence of CYP and its metabolite MEL in liquid milk samples without other impurities interfering with the CSEI process. Under the optimized SPE conditions, the quantitative results and the recoveries of the method, which were determined by adding different amounts of CYP and MEL to the sample matrix before sample pretreatment, are listed in Table 2. The average total recoveries of CYP in liquid milk and whole milk powder were 76.9% with RSD ranging from 12.3 to 15.3%, whereas the average recoveries of MEL were 79.3% with RSD ranging from 11.8 to 14.2%, respectively. The LOQs ($S/N = 10$) of CYP and MEL in light of our work was 0.86 and 0.42 ng/mL. These results demonstrated that the proposed CSEI-sweeping-MEKC method combining SPE indeed possesses a relatively high detecting sensitivity to analyze the CYP and its metabolite MEL in dairy products.

4 Concluding remarks

A highly sensitive method based on CSEI-sweeping-MEKC combining SPE has been developed and validated for the analysis of CYP and MEL. The method allows the simultaneous determination of CYP and MEL with minimum instrumentation needs, analysis time and cost. It can be used efficiently in conjunction with on-line concentration techniques. More than 6000-fold enhancement in detection sensitivity for the two compounds was demonstrated when using CSEI-sweeping-MEKC (relative to MEKC). The LODs of CYP and MEL were as low as 23.4 and 43.7 pg/mL for standards. We successfully applied the CSEI-sweeping-MEKC method, in conjunction with SPE, to the analysis of these two compounds in dairy products. This developed analytical method functioned with acceptable repeatability. Therefore, this method should be useful for the determination of trace amounts of the CYP and its metabolite MEL in dairy products.

Table 2. Results for the determination of the CYP and MEL in milk sample extracts

Sample	Compound	Orig amount	Added (ng/mL)	Found (ng/mL)	Recovery[a)] (%)	RSD[b)] (%)
Liquid milk	CYP	–[c)]	10	7.44	74.4	14.5
			20	15.62	78.1	12.3
	MEL		5	3.87	77.4	13.5
			10	8.04	80.4	11.8
Milk powder	CYP	–	10	7.69	76.9	15.3
			20	15.64	78.2	14.7
	MEL		5	3.84	76.8	14.2
			10	8.25	82.5	12.7

a) The recovery was the mean recovery of three recovery experiments at the same level.
b) The RSD was calculated from three recovery experiments at the same level.
c) Not detectable.

The authors gratefully acknowledge the support for this research by National Natural Science Foundation of China (20975042) and Nature Science foundation key project from Hubei Province of China (2008CDA080).

The authors have declared no conflict of interest.

5 References

[1] Cook, A. M., Huetter, R., *J. Agric. Food Chem.* 1981, *29*, 1135–1143.

[2] Andersen, W. C., Turnipseed, S. B., Karbiwnyk, C. M., Clark, S. B., Madson, M. R., Gieseker, C. A., Miller, R. A., Rummel, N. G., Reimschuessel, R., *J. Agric. Food Chem.* 2008, *56*, 4340–4347.

[3] Yan, N., Zhou, L., Zhu, Z. F., Chen, X. G., *J. Agric. Food Chem.* 2009, *57*, 807–811.

[4] Bardalaye, P. C., Wheeler, W. B., Meister, C. W., *J. Assoc. Off. Anal. Chem.* 1987, *70*, 455–457.

[5] Cabras, P., Meloni, M., Spanedda, L., *J. Chromatogr. A* 1990, *505*, 413–416.

[6] Yokley, R. A., Mayer, L. C., Rezaaiyan, R., Manuli, M. E., Cheung, M. W., *J. Agric. Food Chem.* 2000, *48*, 3352–3358.

[7] Wei, R., Wang, R., Zeng, Q., Chen, M., Liu, T., *J. Chromatogr. Sci.* 2009, *47*, 581–584.

[8] Chou, S. S., Hwang, D. F., Lee, H. F., *J. Food Drug Anal.* 2003, *11*, 290–295.

[9] Kim, B., Perkins, L. B., Bushway, R. J., Nesbit, S., Fan, T., Sheridan, R., Greene, V., *J. Assoc. Off. Anal. Chem. Int.* 2008, *91*, 408–413.

[10] Garber, E. A. E., *J. Food Prot.* 2008, *71*, 590–594.

[11] Okazaki, S., Hiramatsu, M., Gonmori, K., Suzuki, O., Tu, A., *Forensic Toxicol.* 2009, *27*, 94–97.

[12] Lin, M., He, L., Awika, J., Yang, L., Ledoux, D. R., Li, H., Mustapha, A., *J. Food Sci.* 2008, *73*, 129–134.

[13] Armenta, S., Quintás, G., Garrigues, S., de la Guardia, M., *Anal. Chim. Acta* 2004, *524*, 257–264.

[14] Qian, M. R., Zhang, H., He, H. M., Wang, X. Y., Chen, Z. M., Wu, L. Q., *Chin. J. Anal. Chem.* 2009, *37*, 902–906.

[15] Sancho, J. V., Ibáñez, M., Grimalt, S., Pozo, Ó. J., Hernández, F., *Anal. Chim. Acta* 2005, *530*, 237–243.

[16] Xia, X., Ding, S., Li, X., Gong, X., Zhang, S., Jiang, H., Li, J., Shen, J., *Anal. Chim. Acta* 2009, *651*, 196–200.

[17] Filigenzi, M. S., Tor, E. R., Poppenga, R. H., Aston, L. A., Puschner, B., *Rapid Commun. Mass Spectrom.* 2007, *21*, 4027–4032.

[18] Filigenzi, M. S., Puschner, B., Aston, L. S., Poppenga, R. H., *J. Agric. Food Chem.* 2008, *56*, 7593–7599.

[19] Toth, J. P., Bardalaye, P. C., *J. Chromatogr.* 1987, *408*, 335–340.

[20] Zhu, X., Wang, S., Liu, Q., Xu, Q., Xu, S., Chen, H., *J. Agric. Food Chem.* 2009, *57*, 11075–11080.

[21] Li, J., Qi, H. Y., Shi, Y. P., *J. Chromatogr. A* 2009, *1216*, 5467–5471.

[22] Miao, H., Fan, S., Wu, Y. N., Zhang, L., Zhou, P. P., Li, J. G., Chen, H. J., Zhao, Y. F., *Biomed. Environ. Sci.* 2009, *22*, 87–94.

[23] Zhu, X., Liu, Q., Li, D., Wang, S., Wang, X., *Se Pu* 2009, *27*, 401–405.

[24] Craig, D. B., Wong, J. C. Y., Dovichi, N. J., *Anal. Chem.* 1996, *68*, 697–700.

[25] Quirino, J. P., Terabe, S., *Anal. Chem.* 2000, *72*, 1023–1030.

[26] Malá, Z., Šlampová, A., Gebauer, P., Boček, P., *Electrophoresis* 2009, *30*, 215–229.

[27] Kartsova, L. A., Bessonova, E. A., *J. Anal. Chem.* 2009, *64*, 326–337.

[28] Simpson Jr, S. L., Quirino, J. P., Terabe, S., *J. Chromatogr. A* 2008, *1184*, 504–541.

[29] Cook, H. A., Klampfl, C. W., Buchberger, W., *Electrophoresis* 2005, *26*, 1576–1583.

[30] Nielen, M. W. F., van de Ven, H. J. F. M., *Rapid Commun. Mass Spectrom.* 1996, *10*, 74–81.

[31] Yan, N., Zhou, L., Zhu, Z. F., Chen, X. G., *J. Agric. Food Chem.* 2009, *57*, 807–811.

[32] Xia, J. G., Zhou, N. Y., Liu, Y. J., Chen, B., Wu, Y. N., Yao, S. Z., *Food Control* 2010, *21*, 912–918.

[33] Chen, Z., Yan, X., *J. Agric. Food Chem.* 2009, *57*, 8742–8747.

[34] Tsai, I. L., Sun, S. W., Liao, H. W., Lin, S. C., Kuo, C. H., *J. Chromatogr. A* 2009, *1216*, 8296–8303.

[35] Hsu, Y. F., Chen, K. T., Liu, Y. W., Hsieh, S. H., Huang, H. Y., *Anal. Chim. Acta* 2010, *673*, 206–211.

[36] Wang, X., Chen, Y., *J. Chromatogr. A* 2009, *1216*, 7324–7328.

[37] Su, H. L., Feng, L. I., Jen, H. P., Hsieh, Y. Z., *Electrophoresis* 2008, *29*, 4270–4276.

[38] Jen, H. P., Tsai, Y. C., Su, H. L., Hsieh, Y. Z., *J. Chromatogr. A* 2006, *1111*, 159–165.

[39] Meng, P. J., Fang, N., Wang, M., Liu, H. W., Chen, D. D. Y., *Electrophoresis* 2006, *27*, 3210–3217.

[40] Shihabi, Z. K., *J. Chromatogr. A* 2005, *1066*, 205–210.

[41] Li, Q., Huie, C. W., *Electrophoresis* 2006, *27*, 4219–4229.

