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A novel mixed-mode solid phase extraction for simultaneous determination of melamine and cyanuric acid in food by hydrophilic interaction chromatography coupled to tandem mass chromatography

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ABSTRACT

Utilizing a solid phase extraction column (MCT) containing mixed hydrophilic functional gel and cation exchange sorbent, a sensitive and rapid HPLC–MS/MS method for simultaneously determining the residues of melamine (MEL) and cyanuric acid (CYA) in human foodstuffs was developed. MEL and CYA in egg, pork, liver, kidney and pork, shrimp, sausage casing, honey, soybean milk, soybean powder and dairy product were extracted using acetonitrile/water, defatted with hexane and isolated using MCT solid phase extraction column. The residues were separated upon a hydrophilic interaction liquid chromatography (HILIC) column and analyzed by electrospray ionization under negative–positive switched mode on a triplequadrupole mass spectrometry. The selected reaction monitoring was performed on $[M+H]^+$ of m/z 127.9 to provide the transition of 127 > 85 and 127 > 68 (MEL) while the $[M-H]^-$ of m/z 127.1 was selected as the precursor ion for CYA resulting in product ions m/z 85 and 42. Isotope labeled internal standard ($^{15}N_3$-MEL and $^{13}C_3$-CYA) and matrix-matched calibration were both used to observe the recovery to be 70.0–129.6% and 70.0–128.9% with RSD of 1.4–23.3% and 1.5–21.7% for MEL and CYA, respectively ($n = 6$). All the LODs and LOQs of MEL and CYA were less than 39.4 and 99.1 $\mu g\,kg^{-1}$, respectively, in 18 matrices, which were sensitive enough for quantitative analysis. This method has been proven effective in simultaneous determination of melamine and cyanuric acid when inspecting unknown and positive samples.

1. Introduction

Melamine (MEL) is a typical triazine compound with the chemical formula $C_3H_6N_6$ which is primarily used in the synthesis of melamine formaldehyde resins for the manufacture of laminates, plastics, coatings, commercial filters, glues or adhesives, and dishware and kitchenware [1–3]. Analogues (cyanuric acid, ammeline and ammelide) can be produced as impurities during the manufacturing process for MEL [4,5]. Because of its high nitrogen percentage of 66.7%, MEL was illegally added to food products in order to increase the apparent protein content. Chinese Sanlu infant formula powder contamination incident was a result of adulteration of melamine, which led to consumer's panic and distrust to China's dairy products and gave a great shock to Chinese dairy industry. According to the animal toxicological test, melamine imposes a potential risk to human health by combining with cyanuric acid (CYA) to form a complex, which could produce significant renal damage and crystals in nephrons [6]. A provisional maximum residue limit (MRL) for melamine was published as 1 and 2.5 mg/kg in baby formula and milk products, respectively, by Chinese Ministry of Public Health [7]. U.S. FDA also made the issues regarding Minimum Required Performance Limit (MRPL) of MEL and CYA within an interim method of LIB4422. The method limit of quantitation (LOQ) for MEL and CYA were: 25 and 50 μg/kg, respectively, for tissue and liquid formula and both 200 μg/kg for dry infant formula powder [8].

Up to now, biological screening approaches based on ELISA [9], chemiluminescence [10] and SERS [11] has been proven a rapid method, but only obtained semi-quantitative result. In order to gain more complete and accurate information about MEL and related compounds in food there have been various instrumental methods such as high-performance liquid chromatography (HPLC) [12–14], gas chromatography (GC) [15–17] using selective detection technique, and single and tandem mass spectrometry for analysis of MEL and related compounds in food of human consumption and feed of animal breeding. The small and polar nature of MEL and

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Table 1
Selective reaction monitoring transition and mass spectrometry parameters for MEL, CYA, $^{15}N_3$-MEL and $^{13}C_3$-CYA internal standard.

Compound	Ion mode	Precursor ion (Q1)	Product ion (Q3)	CE[a] (V)	DP[b] (V)	EP[c] (V)	CXP[d] (V)
MEL		127.1	85.5[e]	27	71	8	14
	Positive		68.4	43	71	11	10
$^{15}N_3$-MEL		130.1	87.4	27	61	8	14
CYA		127.9	42.0[e]	−14	−50	−10	−1
	Negative		85.0	−18	−50	−10	−1
$^{13}C_3$-CYA		130.9	43.0	−30	−45	−10	−1

[a] Collision energy.
[b] Collision energy declustering potential.
[c] Collision cell entrance potential.
[d] Collision cell exit potential.
[e] Quantitative transition.

CYA results that a further derivation procedure is needed for GC based method. As a result the majority of analysis method was HPLC method using polar reversed-phase column, cyano-columns, amino columns or normal C18 and C8 reversed-phase column with the mobile phase containing ion-pair reagent [11,18]. The most recent works employed hydrophilic interaction liquid chromatography (HILIC) to obtain a successfully simultaneous separation of MEL and CYA on the same column [6,19,20]. However, MEL belongs to weak basic compound with pK_a value of 8, the sample preparation mainly involved in a polar liquid extraction such as aqueous solution containing formic acid, acetic acid and phosphate buffer at pH 5 and acetonitrile or methanol, sometimes followed by further clean-up with solid phase extraction (SPE) based on cation exchange (e.g. Waters Oasis MCX) [8,20]. Whereas CYA is typical weak acid compounds with pK_a of 6, generally sample preparation of SPE is related with anion-exchange sorbents (e.g. Waters Oasis MAX) [8]. Other solid phase sorbents used to further process sample extracts include graphitized carbon phase and C18 for CYA and MEL, respectively [13,21]. Further literature survey revealed that there are few papers describing the simultaneous solid phase extraction of MEL and CYA. Hence public concern over MEL and CYA residues in foodstuffs has urged the development of a method for the simultaneous and comprehensive sample preparation.

The aim of present work was to develop a simple and rapid method based on mixed-mode solid phase SPE column containing hydrophilic functional silica gel and cation exchange sorbents for simultaneous extraction of MEL and CYA in food followed by determination of HILIC–MS/MS. The matrices of egg, pork, liver and kidney of pig, shrimp, honey, soybean milk, soybean powder and protein powder, milk and dairy products were used to verify the performance of method.

2. Materials and methods

2.1. Materials and reagents

Standards of MEL and CYA were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). ^{15}N labeled melamine isotope and ^{13}C labeled cyanuric acid isotope were obtained from Toronto and Witega, respectively. Acetonitrile, methanol, formic acid and ammonium acetate (HPLC grade) were obtained from Fluka (Buchs, Switzerland). The hydrophilic functional silica gel and cation exchange sorbent were both purchased from Anpel (Shanghai, China). A MilliQ ultrapure water system from Millipore (MA, USA) was used to prepare the HPLC-grade water for sample treatment and mobile phase. All other reagents used in the experiment were of analytical grade.

2.2. Instrumentation

A Shimadzu Prominenece UFLC (Shimadzu, Tokyo, Japan) liquid chromatography coupled to an AB API 4000 triple quadrupole tandem mass spectrometry (AB, CA, USA) was used for MEL and CYA analysis. Data acquisition, processing and system operation were employed by Analyst V1.5 software. Chromatographic separation was performed by Waters Atlantis HILIC (150 mm × 2.1 mm i.d., 5 μm particle size). The mobile phases consisted of 10% 100 mmol L^{-1} ammonium formate in acetonitrile with pH of 3.2 (A) and 0.1% formic acid in acetonitrile (B). The following isocratic elution program was used: 5% A for 2.5 min, increase to 100% at 4 min, held from 4 to 8 min, returned to 5% A at 8.5 min, and allowed to re-equilibrated for additional 4 min. The flow rate is 0.4 mL min^{-1}. Injection volume of sample was 10 μL and column temperature was set at 35 °C.

The electrospray interface was switched from negative ion mode to positive ion mode at 4.0 min. CYA was analyzed in negative ion mode (ESI−) at voltage of −4.5 kV while MEL was analyzed in positive mode (ESI+) at voltage of 5.5 kV. The optimized MS/MS parameters were set as follows: nebulizer gas pressure 70 psi, auxiliary heater gas pressure 65 psi, and curtain gas pressure 25 psi, collision gas pressure 5 psi. The MS method including negative and positive scan event with the selective reaction monitoring (SRM) transition was listed as Table 1.

2.3. Preparation of mixed-mode solid phase extraction column (MCT™ column)

About 100 mg of hydrophilic functional silica gel sorbent and 50 mg of cation exchange sorbent were homogeneously mixed together, then the mixer was added to a 3 mL SPE tube with a polypropylene frit and tamped down. The column has been available commercially and provided by Shanghai Anpel company with the product name of MCT now.

2.4. Preparation of standards

Stock solution of 100 μg mL^{-1} MEL and CYA were prepared using 10.0 mg of standards with 100 mL of acetonitrile/water (1:1, v/v) solution. $^{15}N_3$-MEL and $^{13}C_3$-CYA stock solution had a concentration of 10 μg mL^{-1} using 1.0 mg of standards with 100 mL of acetonitrile/water (1:1, v/v) solution. Working solution of different levels was prepared by diluting appropriate aliquots of stock solution. The calibration working solution at 0, 10, 20, 50, 100, 200, and 500 μg L^{-1} with $^{15}N_3$-MEL and $^{13}C_3$-CYA concentration of 100 and 200 μg L^{-1}, respectively, were prepared before use. All solutions were stored at −4 °C.

2.5. Samples

Egg, pork, liver, kidney of pig, shrimp, honey, soybean milk, soybean powder, protein powder, milk and other dairy products were purchased from local supermarket or collected by the Shanghai Entry-Exit Inspection and Quarantine Bureau of the People's Republic of China and stored at −18 °C.

Table 2
Method validation performance (ranges of recovery in spiked matrices, validation levels, limit of detection, limit of quantitation, regression of coefficient).

Matrices	Compounds	VL (μg kg^{-1})	Recovery (%)	RSD (%, n = 6)	LOD (μg kg^{-1})	LOQ (μg kg^{-1})	R^2
Egg	MEL	25,50,100	92.0–123.0	5.7–9.6	8.6	21.1	0.9708
	CYA	50,100,200	73.8–117.5	5.5–12.7	18.7	53.2	0.9837
Meat	MEL	25,50,100	75.2–120.0	10.4–14.4	10.3	26.7	0.9799
	CYA	50,100,200	73.5–106.9	2.7–9.1	20.5	46.9	0.9655
Liver	MEL	25,50,100	90.2–123.8	4.8–10.5	8.1	20.0	0.9769
	CYA	50,100,200	76.6–122.9	3.2–13.8	12.6	31.7	0.9237
Shrimp	MEL	25,50,100	93.0–129.2	6.4–13.1	8.0	20.5	0.9895
	CYA	50,100,200	74.3–127.0	8.0–15.7	19.5	48.3	0.9827
Kidney	MEL	25,50,100	82.4–124.4	6.9–16.6	8.5	20.8	0.9737
	CYA	50,100,200	90.9–128.9	7.0–10.5	28.3	55.6	0.9357
Sausage casing	MEL	25,50,100	79.8–129.6	5.6–15.1	7.7	21.6	0.9520
	CYA	50,100,200	71.4–119.6	10.6–14.8	20.8	49.3	0.9098
Honey	MEL	25,50,100	78.8–105.0	2.4–8.3	10.2	29.7	0.9962
	CYA	50,100,200	103.0–128.6	5.3–6.6	15.2	39.3	0.9722
Liquid milk	MEL	25,1000,2500	77.0–94.0	9.3–21.7	9.2	30.1	0.9771
	CYA	50,250,2500	70.8–91.0	4.3–18.7	18.2	44.0	0.9190
Soybean milk	MEL	50,1000,2500	73.6–128.4	1.4–23.3	12.1	53.0	0.9891
	CYA	100,250,2500	70.0–117.0	3.6–8.6	27.3	78.3	0.9325
Soybean powder	MEL	50,1000,2500	70.4.–128.4	1.6–21.6	15.8	56.2	0.9750
	CYA	100,250,2500	70.8–90.4	1.7–6.7	29.9	90.0	0.9577
Protein powder	MEL	50,1000,2500	70.6–123.4	4.5–21.1	15.6	55.0	0.9474
	CYA	100,250,2500	71.9–104.1	9.7–20.9	38.1	82.8	0.9172
Milk powder	MEL	50,1000,2500	72.6–124.8	1.6–8.0	14.7	51.8	0.9614
	CYA	100,250,2500	79.4–126.8	4.9–5.3	32.7	89.0	0.9527
Condensed milk	MEL	50,1000,2500	82.2–125.0	4.5–15.2	14.0	56.8	0.9511
	CYA	100,250,2500	75.8–127.0	3.7–21.7	35.3	82.1	0.9279
Cheese	MEL	50,1000,2500	75.3–127.6	6.8–13.8	17.9	51.8	0.9914
	CYA	100,250,2500	72.6–107.3	2.8–11.9	13.6	49.9	0.9626
Butter	MEL	50,1000,2500	84.8–102.2	1.9–3.4	15.6	52.9	0.9994
	CYA	100,250,2500	73.8–103.9	1.5–6.2	14.9	61.8	0.9508
Icecream	MEL	50,1000,2500	89.7–128.6	8.8–10.0	17.0	49.4	0.9925
	CYA	100,250,2500	77.7–108.0	4.9–10.0	28.9	76.4	0.9383
Milk sugar	MEL	50,1000,2500	76.4–114.5	2.3–3.7	20.6	48.8	0.9803
	CYA	100,250,2500	73.6–110.5	2.6–8.9	39.4	99.1	0.9204
Biscuit	MEL	50,1000,2500	72.8–123.1	3.4–6.5	16.6	61.7	0.9542
	CYA	100,250,2500	80.8–103.2	2.3–10.2	30.4	82.8	0.9404

R^2: Regression of coefficient.

2.6. Extraction

About 10 mL of acetonitrile/water (1:1, v/v) solution was added to each 50 mL plastic centrifuge tubes containing 2 g of sample with regard that 1 g of honey, butter, cheese, toffee, biscuit, powdered milk, and soybean flour samples was enough. After 100 μg $^{15}N_3$-MEL and 200 μg $^{13}C_3$-CYA internal standard solution was added, the sample were capped and shaken for 30 s, and adjust pH value to 2–3 with 1 mol L^{-1} hydrochloric acid followed by a vigorous agitation for 2 min. The tubes were put into an ultrasonic bath for 15 min, and then centrifuged at 8000 × g for 5 min at 4 °C. The supernatant was transferred to a clean tube, 5 mL of hexane was added and samples were mixed gently for 2 min, and then centrifuged at 8000 × g for 5 min at 4 °C. The hexane layer was removed while the aqueous layer was diluted with acetonitrile/water (1:1, v/v) to obtain a final volume of 8 mL for SPE.

MCT^{TM} (Anpelclean, Shanghai, China) cartridge containing 3 mL solid phase extraction column with 150 mg of mixed sorbent were conditioned with 3 mL methanol followed by 3 mL acetonitrile/water (1:1, v/v). The 8 mL of sample was loaded and wash with 2 mL of acetonitrile/water (1:1, v/v). The cartridge was dried under vacuum for 5 min and eluted by gravity with 2 mL methanol and 4 mL of 5% ammonia hydroxide in methanol (v/v). The elution was collected in a glass tube. The samples were evaporated to dryness under nitrogen flow at 40 °C and reconstituted with 1 ml of 100 mmol L^{-1} ammonium formate in water/acetonitrile (1:9, v/v). All samples were filtered through a 0.2 μm nylon filter for HILIC–MS/MS analysis.

3. Results and discussion

3.1. Optimization of MS condition

Preliminary experiments were carried out by flow injection of 0.5 μg mL^{-1} MEL and CYA standard solution in positive and negative mode, respectively, in order to obtain suitable MS parameters of the two compounds. Nebulizer gas (GS1), curtain gas (CUR), auxiliary heater gas (GS2), and flow rate of each gas and ion source temperature was optimized. Values of final collision energy (CE), declustering potential (DP), collision cell exit potential (CXP), collision cell entrance potential (EP) and electrospray capillary voltage (IS) were listed as Table 1. A protonated molecular ion $[M+H]^+$ (m/z 127) was selected as precursor ion for MEL to generate product ion

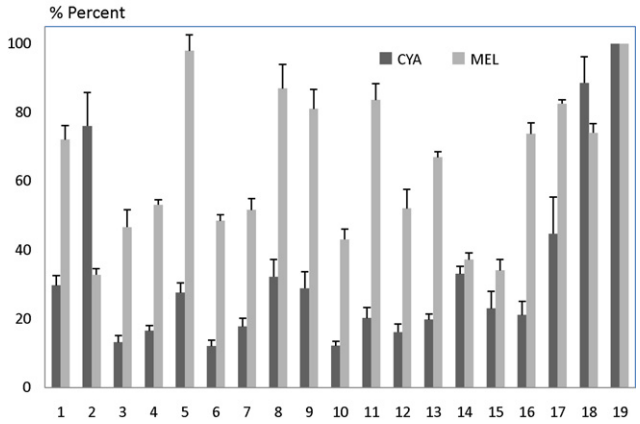

Fig. 1. Matrix effect on MEL and CYA response (1: shrimp, 2: honey, 3: pork, 4: egg, 5: liver, 6: kidney, 7: sausage casing, 8: powdered milk, 9: protein powder, 10: cheese, 11: biscuit, 12: soybean powder, 13: soybean milk, 14: liquid milk, 15: condensed milk, 16: icecream, 17: milk sugar, 18: butter, 19: standard).

fragment of *m*/*z* 85.1 and *m*/*z* 68.4 corresponding to loss of CH_2N_2 and CH_5N_3, respectively. The isotope internal standard of $^{15}N_3$-MEL was optimized in similar ways to generate a quantitative transition of 130.1 > 87.4. The response of CYA and $^{13}C_3$-CYA were both optimized by the predominant $[M-H]^-$ ion of *m*/*z* 127.9 and 130.9. Product ion of *m*/*z* 42 was proved to be the group of CNO from quasi-molecular ion corresponding to *m*/*z* 43.0 from $^{13}C_3$-labeled isotope CYA. The neutral loss of CHNO produced a qualitative transition of 127.9 > 85.0 where transition of 127.9 > 42.0 was operated as the quantification.

3.2. HILIC separation

A good separation between target compounds was necessary for a positive/negative switching mode of mass spectrometry detection method. HILIC can provide a good retention for polar compounds which made a great complement to conventional reversed phase chromatography. Furthermore, the combination of a polar stationary phase with high proportion organic solvent mobile phase offered an enhanced sensitivity and excellent compatibility between the HPLC and MS determination, especially for the detection of MEL and CYA. Mechanism of HILIC retention was generally accepted as a combination of hydrogen bonding, electrostatic and dipole–dipole interaction, similar with the chromatography integrated with advantages of reverse phased liquid chromatography (RPLC), normal phase liquid chromatography (NPLC) and ion chromatography (IC). So the retention behavior of HILIC was related with proportion of organic solvent mobile phase, flow rate, column temperature and pH value, concentration of mobile phase salt buffers. Optimization procedure was performed by optimizing the addition of mobile phase modifier. We investigated different concentration of formic acid and acetic acid from 0.05% to 0.2% (v/v) in mobile phase B and ammonium formate and ammonium acetate buffers from 1 to 10 mmol L^{-1} in mobile phase A. By comparing the sensitivity and retention time, the peak shape of CYA could be improved with the increasing of the concentration of ammonium formate. Finally the best results were obtained with 0.1% formic acid in mobile B and 100 mmol/L ammonium formate in mobile A.

3.3. Optimization of mixed-mode solid phase extraction

As the solubility of MEL and CYA in water is 3240 and 2000 mg L^{-1}, respectively [6], water was normally used as preferred extraction solvent with the addition of different modifiers like acetic acid, formic acid, trichloroacetic acid, hydrochloric acid and acetonitrile or methanol. The simultaneous extraction of MEL and CYA was preferred followed by SPE clean-up procedure. In this paper, a mix sorbents integrated SPE was used to retain both MEL and CYA. MEL was enriched on a cation exchange sorbent while a hydrophilic silica gel sorbent was used to concentrate the CYA. The retention mechanism was similar with that of HILIC chromatography. So, an aqueous solution including different percentage of acetonitrile (3:7, 4:6, 5:5, 6:4, 7:3, v/v) was employed to evaluate extraction efficiency of MEL and CYA together. The proportion of 5:5 provided the best result; furthermore, this ratio between water and acetonitrile was also suitable for subsequent loading on column of SPE for a high percentage of acetonitrile was contribute to retention of CYA on SPE column. It has been reported that a basic extraction condition was benefit for prevention of formation of MEL–CYA complex [22], however, MEL could be hydrolyzed to CYA at a basic condition [23]. In this paper, we found that an acid extraction solution at pH value between 2 and 3 can provide a better and more stable recovery. A stability test during consecutive 12 days was performed using MEL and CYA mix solution both with the concentration of 50, 100, 200 and 500 μg L^{-1}. The results were expressed as average of three injections with a relative standard deviation. There was no significant difference between the response of MEL and CYA at every collection time for all levels of concentrations (*t*-test, $P < 0.05$). Otherwise, MEL could not only bind proton to form cation for ion-exchange retention but also formation of neutral CYA was held on hydrophilic silica gel sorbent at acid condition. Furthermore, the SPE flow rate was a critical site. It was important to operate under gravity for CYA according to the retention mechanism of mixed-SPE column. A rapid decrease of recovery for CYA would be found if flow of loading and elution (above 1–2 d s^{-1}) was not well controlled.

Table 3
The results of the recovery experiments in several real samples.

Matrix	Compounds	VLs (μg/kg)	Recovery %	RSD (%, $n = 6$)	Matrix	Compounds	VLs (μg/kg)	Recovery %	RSD (%, $n = 6$)
Kidney	MEL	25	84.0–124.4	16.6	Soybean powder	MEL	50	70.4–128.4	21.6
		50	82.4–111.4	10.2			1000	83.1–87.0	1.6
		100	88.7–105.7	6.9			2500	76.0–84.8	2.8
	CYA	50	97.9–117.5	7.0		CYA	100	72.1–85.5	5.7
		100	108.9–128.9	7.2			250	74.8–90.4	6.7
		200	90.9–112.9	10.5			2500	70.8–75.6	1.7
Meat	MEL	25	83.2–120.0	14.4	Milk powder	MEL	50	99.6–124.8	8.0
		50	75.2–100.8	10.4			1000	75.5–84.4	1.6
		100	93.5–119.5	11.2			2500	72.6–83.4	5.3
	CYA	50	80.5–106.9	9.1		CYA	100	79.4–86.5	5.3
		100	73.5–85.5	4.1			250	82.4–93.2	4.9
		200	92.0–99.0	2.7			2500	96.7–126.8	5.3

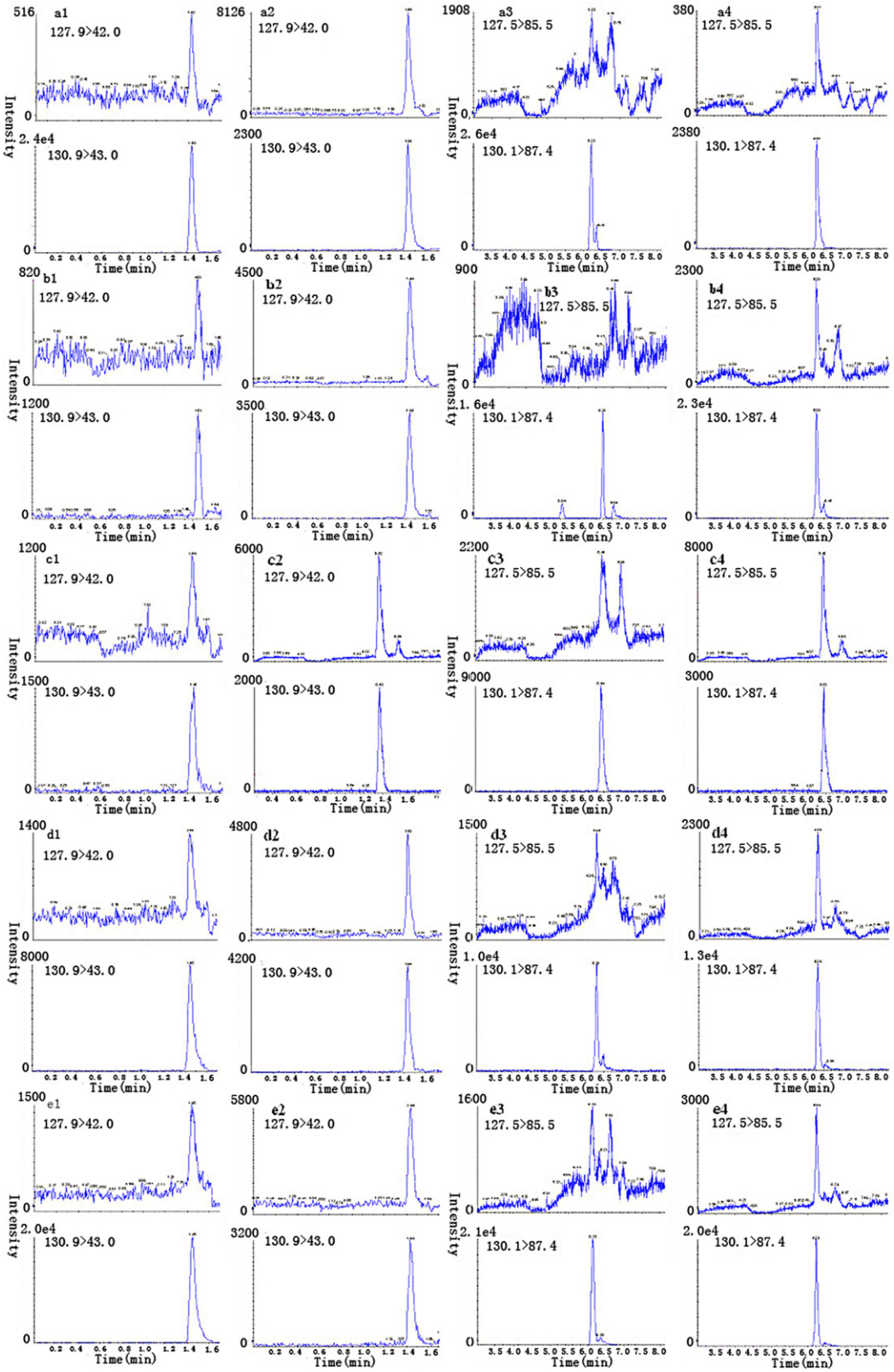

Fig. 2. SRM chromatogram of different types of food matrices (a1 and a3: pork blank, a2 and a4: pork fortified with CYA of 50.0 μg/kg and MEL of 25.0 μg/kg, b1 and b3: honey blank, b2 and b4: honey fortified with CYA of 50.0 μg/kg and MEL of 25.0 μg/kg, c1and c3: milk powder blank, c2 and c4: milk powder fortified with CYA of 100.0 μg/kg and MEL of 50.0 μg/kg, d1 and d3: soybean powder blank, d2 and d4: soybean powder fortified with CYA of 100.0 μg/kg and MEL of 50.0 μg/kg, e1 and e3: cheese blank, e2 and e4: cheese fortified with CYA of 100.0 μg/kg and MEL of 50.0 μg/kg).

3.4. Matrix effect

Matrix effects or ion suppression by the sample matrix are commonly observed during LC/MS/MS analysis. Total 18 kinds of food matrices tested with potential CYA and MEL contamination in this experiment mainly involved in tissue, dairy and soybean products such as egg, pork, soybean milk, milk, etc. The detailed information was list in Table 2. Matrix effect was evaluated by comparing the

response of two levels of concentration of MEL and CYA standards in mobile phase A (Rs) with same concentration spiked in blank matrix solution after the clean-up procedure (Rm). The ration between Rm and Rs was calculated with value of 100 × Rm/Rs representing the matrix effect of CYA and MEL in different kinds of matrices. The standard of MEL and CYA was spiked in mobile phase and matrices above at level of 0.2 μg mL^{-1}. Fig. 1 was shown the result. Matrix effect value of MEL ranged from 30.2% to 99.1% whereas stronger ionization suppression was found on CYA with a matrix effect value ranged from 11.5% to 88.3% (Fig. 1), especially for pork, kidney and cheese. The significant decrease of MS response could be explained by ion suppression of target compound due to the presence of unknown matrix interferes co-eluted with MEL and CYA. A matrix-matched calibration and isotope labeled internal standard ($^{15}N_3$-MEL and $^{13}C_3$-CYA) were both used to compensate the matrix effect. It was noted that a suitable dilution before injection using mobile phase A could decrease the matrix effect to some extent.

3.5. Method validation

A validation procedure based on EU Commission Decision 2002/657/EC [24] was conducted to evaluate the method performance including the precision, selectivity, specificity and applicability. Validation levels (VLs) regarding 18 kinds of matrices (listed as Table 2) was chosen according to MRL and MRPL values of MEL and CYA in corresponding laws and regulations. The matrix-matched calibration curves were prepared and subsequently analyzed to provide the linear range from 0 to 500 ng mL^{-1} and R^2 ranged from 0.9098 to 0.9994. When evaluating the 18 kinds of matrices at three VLs, the recovery of MEL and CYA were observed to be 60–97.2% with RSD of 8.9–23.7% (n = 6) and 10.2–37.3% with RSD of 33.4–48.7% (n = 6), respectively, based on a solvent standard calibration curve. A matrix-matched calibration curve provided the recovery of MEL and CYA to be 72.1–98.6% with RSD of 9.2–25.5% (n = 6) and 37.6–56.7% with RSD of 27.2–36.5% (n = 6), respectively. We also tried to use MCX and MAX SPE column separately for clean-up of MEL and CYA, a similar result could be obtained according to operation described in Ref. [8]. When isotope labeled internal standard ($^{15}N_3$-MEL and $^{13}C_3$-CYA) and matrix-matched calibration were both used, a corrected recovery could be observed to be 70.0–129.6% (1.4–23.3% RSD, n = 6) and 70.0–128.9% (RSD 1.5–21.7%, n = 6). A detailed data for every matrix was shown in Table 2. The limit of detection (LOD) of method was determined by multiplying the 99% confidence level Student's t-test with standard deviation of the 25, 50 or 100 μg/kg spike level of MEL and CYA. The limit of quantitation (LOQ) was established as 10 times the standard deviation at the 25, 50 or 100 μg/kg spike level of MEL or CYA. The detailed LOD and LOQ data for each species was listed in Table 2. A detailed recoveries of MEL and CYA in pork, kidney, soybean powder and milk powder at three validation levels were shown in Table 3. The representative SRM chromatograms of quantitative transition for pork, honey, milk powder, soybean powder and cheese spiked at VLs were shown in Fig. 2.

3.5.1. Application to real sample

The proposed method was applied to determine the levels of the MEL and CYA in 5 positive milk powder samples collected by the Shanghai Entry-Exit Inspection and Quarantine Bureau of The People's Republic of China during the year of 2009 and 36 unknown samples purchased from local supermarket including liquid milk, kidney, milk powder, cheese, etc. The detection results of positive milk powder were confirmed by proposed method with MEL values ranged from 234 to 2580 μg kg^{-1} in accordance with previous results. It was interesting that there was no CYA confirmed within these positive MEL samples. However, 2 samples of kidney of porcine were detected to provide CYA positive with the concentration of 352 and 587 μg kg^{-1}. It can be inferred that kidney was mainly considered as metabolic organ where MEL can be transformed to CYA.

3.6. Conclusion

A sensitive and rapid method based on the mixed-mode SPE technique coupled to HILIC–MS/MS for determining the residues of MEL and CYA was developed for the first time. MEL and CYA could be simultaneous extracted on a SPE column containing hydrophilic functional silica gel and cation exchange sorbents. MEL and CYA could be qualified and quantitated in 18 kinds of matrices by isotope labeled internal standard and matrix-matched calibration. The method provided a comprehensive solution for screening of MEL and CYA in food samples.

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