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A novel dispersive micro solid phase extraction using PCX as the sorbent for the determination of melamine and cyromazine in milk and milk powder by UHPLC-HRMS/MS

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ABSTRACT

A novel dispersive micro solid phase extraction (DMSPE) cleanup method based on the PCX sorbent (a kind of cation exchange polymer material) was applied to the analysis of melamine and cyromazine residues in milk and milk powder, and ultra high performance liquid chromatography-high resolution mass spectrometry (UHPLC–HRMS) was used as instrument detection. Milk powder samples were first extracted with 1% formic acid in acetonitrile/water (1:1 v/v), and milk samples were cleaned up directly without any pre-extraction. Then, melamine and cyromazine in the extracts or milk were adsorbed to the PCX powder. Subsequently, the analytes in PCX sorbent were eluted with ammonium hydroxide/acetonitrile (2.5:97.5 v/v) through a simple unit device equipped with 1 mL syringe and 0.22 μm nylon syringe filter. All the samples were analyzed by UHPLC–HRMS/MS on a Waters Acquity BEH HILIC column with 0.1% formic acid and 4 mM ammonium formate in water/acetonitrile as the mobile phase with gradient elution. The matrix effect, recovery, and repeatability, within laboratory reproducibility, CCα and CCβ of the DMSPE cleanup method were investigated. The proposed method provided a significant improvement for the determination of melamine and cyromazine in milk and milk powder in terms of efficient, rapid, economical, and miniaturized sample preparation methods, which yielded fewer matrix effects compared with SPE method. The established cleanup method is expected to be widely applied for the sample preparation of alkaline contaminants at trace levels in the future.

1. Introduction

The occurrence of matrix effects in pollutant residue analysis of food is well known and the removal of interference and minimization of matrix effect is the key to accurate, robust, and sensitive quantitative assay [1,2]. The cleanup is considered to be the most laborious but effective process to overcome matrix effects. Currently, the cleanup methods including solid phase extraction (SPE), gel permeation chromatography (GPC), liquid-liquid extraction (LLE), and dispersive solid phase extraction (DSPE), etcetera are widely used to overcome the matrix interferences [3–6]. SPE cleanup can reduce organic solvent usage and human labor for sample preparation with high pre-concentration factors and simplicity of phase separation. However, the SPE cleanup procedure is still time-consuming and costly. DSPE is a relatively new technique for cleanup operations, and its pre-concentration is based on the solid phase extraction concept introduced in 2003 [7]. Although DSPE is an efficient approach used to acquire high selectivity of analysis, the purification effect is worse than that of SPE [8,9]. Recent research activities are being focused on the development of efficient, rapid, economical, and miniaturized sample preparation methods [10–12]. More recently, dispersive micro solid phase extraction (DMSPE) has been reported as a miniaturization model of DSPE or SPE based on use of micro amounts of the sorbent phase. The DMSPE exhibits some advantages over traditional DSPE (fewer matrix effects) and SPE in the aspects of less complex equipment, short time requirement and less solvent consumption [13–15]. For DMSPE cleanup technique, the solid sorbent is added directly to the extracts, and the cleanup procedure relies only on shaking and centrifugation. And graphene and carbon nanotubes (CNTs) are the commonly used solid sorbent materials. Based on the mixed-mode cation exchange (MCX) SPE method, the cation exchange polymer material would probably become a new adsorbent to carry out DMSPE method. PCX, as a high molecular polymer, is a cation exchange sorbent material which can adsorb the alkaline chemical substances directly and provide an effective separation. In this regard, PCX was tried to serve as a sorbent for DMSPE cleanup method in residue analysis of alkaline substance in the present study.

Cyromazine (N-cyclopropyl-1,3,5-triazine-2,4,6-triamine), as a triazine pesticide and insect growth regulator, is widely used for

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fly control in animal manure and is effective in protecting flowers, fruits and vegetables in agriculture [16]. After exposure to cyromazine, the residue of cyromazine remains in animal tissues and related food products. Therefore, the allowable concentration of cyromazine is 0.1 mg/kg and 0.01 mg/kg for edible poultry tissue and milk products respectively by the Codex Alimentarius Commission (CAC) [17]. Melamine (1,3,5-triazine-2,4,6-triamine), as a degradation product of cyromazine, might also present in animal products and milk products [18]. In addition, melamine is one of the substances in the Negative List for Non-edible Food Ingredients as Intentional Adulteration in food that might be adulterated in protein-rich diets to increase the apparent protein content. However, the combination of melamine and cyanuric acid (a metabolite of melamine) might result in the formation of insoluble melamine-cyanurate crystal deposits in kidneys and cause renal failure in those who consume the adulterated foods [19]. So, melamine is not approved for use in human foods and animal feed. Therefore, rapid, sensitive and reliable methods for the simultaneous determination of melamine and cyromazine in food samples are highly demanded.

In this study, a novel DMSPE cleanup method based on a PCX sorbent is established for the simultaneous determination of melamine and cyromazine residues in milk and milk powder. In addition, the matrix effect, recovery, and repeatability, within laboratory reproducibility, CCα and CCβ of the DMSPE cleanup method were investigated. The new cleanup method is expected to be widely applied for the analysis of alkaline contaminants at trace levels in the future for sample cleanup.

2. Material and methods

2.1. Chemicals and reagents

Acetonitrile and methanol (HPLC grade) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid (HPLC grade) was purchased from Tedia (Weston, America). Ultra-pure water was prepared from a Milli-Q Plus system at 18.2 MΩ (Millipore, Bedford, MA, USA). Mixed-mode cation exchange (MCX) solid-phase extract cartridge (60 mg, 3 mL) was obtained from Waters Co., Ltd (Shanghai, China). Cleanert® PCX powder was obtained from Agela Technologies (Tianjing, China).

Melamine and cyromazine were purchased from Sigma Aldrich (Shanghai, China). Isotope labeled melamine-$^{13}C3,^{15}N3$ was obtained from Cambridge Isotope Laboratories (Tewksbury, MA, USA), and cyromazine-d_4 was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). The purity of all these standards was no less than 98.0%.

2.2. Samples preparation and quality control (QC) samples

Milk and milk powder sample were obtained from the local markets from Beijing and kept under 4 °C. Quality control (QC) samples at four concentration levels (0.2, 0.5, 2 and 10 μg/kg for milk; 2, 5, 20 and 100 μg/kg for milk powder) were prepared by adding the standard solutions to blank milk and milk powder.

2.2.1. Milk

1.0 g of milk was accurately weighed into 2 mL eppendorf tube with 25 mg PCX, which had been preconditioned with 1 mL acetonitrile. 10 μL of formic acid was added to the tube. The mixture was vortexed for 30 s, and then was poured off into a simple unit device equipped with 1 mL syringe and 0.22 μm nylon syringe filter. The extracting solution was passed through the unit device manually, and then was washed with 1 mL of acetonitrile. Then, the unit device was eluted with 2 mL of ammonium hydroxide/acetonitrile (2.5:97.5 v/v). The collected elution was prepared for UHPLC-MS analysis.

2.2.2. Milk powder

A portion (1.0 g) of sample was accurately weighed into a 15 mL centrifuge tube. The sample was extracted with 10 mL of 1% formic acid in acetonitrile/water (1:1 v/v) and ultrasonicated for 5 min at room temperature, and then centrifuged at 5000 rpm for 3 min. 1 mL of the extract solution was transferred into a 2 mL eppendorf tube with 25 mg preconditioned PCX. And then the subsequent procedures were identical to those described in Section 2.2.1.

2.3. Chromatographic conditions

UHPLC analysis was performed on a UHPLC Ultimate 3000 system (Dionex) with the column oven temperature maintained at 40 °C, using an Acquity BEH HILIC (2.1 mm × 100 mm, 1.7 μm particle size) analytical column (Waters, USA). The aqueous solvent (A) consisted of a mixture of 0.1% of formic acid and 4 mM ammonium formate in water, and the organic phase (B) was acetonitrile with 0.1% formic acid. The gradient started at 98% B was reduced to 90% B in the next 4 min and then linearly ramped to 40% B in the following 2 min. This was followed by re-equilibration at 98% B for 3 min prior to the next injection. The flow rate was set to 300 μL/min with a resulting overall runtime of 9 min. The injection volume was 5 μL.

2.4. Mass spectrometry conditions

Q-Exactive Mass Spectrometer (Thermo Fisher Scientific, Bremen, Germany) with the heated electrospray ionization (HESI) was operated in the positive (ESI^+) electrospray ionization mode. The system was controlled by Xcalibur 2.2 (Thermo Fisher Scientific). The spray voltage was 3.5 kV for the positive mode. The temperature of ion transfer capillary, sheath gas, auxiliary gas, sweep gas, and S-lens RF level were set to 325 °C, 30, 10, 0 (arbitrary units), and 55 V, respectively. The instrument was calibrated in the positive mode every three days using the calibration solutions, including caffeine, MRFA, and a mixture of fluorinated phosphazines ultramark 1621, provided by the instrument manufacturer.

The Q-Exactive detector was operated in targeted single ion monitoring (tSIM)/dd-MS^2 (Top N) mode. By tSIM /dd-MS^2 (Top N) mode, tSIM spectra were acquired at mass resolving power of 70000 full width at half-maximum (FWHM) in an isolation window of 4 Da without use of any locked mass. Data-dependent acquisition of tandem mass spectra was triggered automatically using an inclusion list that comprised information of *m/z* values and retention times (RT). Fragmentation mass spectra were recorded at a mass resolving power of 17500 FWHM with the use of a normalized collision energy (NCE) of 35% and a quadrupole isolation window of 4 Da. Using this scan mode, the parent ions were selected in the quadrupole 127.0728 *m/z* for melamine and 167.1039 *m/z* for cyromazine) for quantitative analysis by tSIM. The qualitative analysis was performed by dd-MS^2 (Top N) with all fragmented ions (85.0514, 110.0464 *m/z* for melamine and 85.0517, 125.0822 *m/z* for cyromazine) originating from the parent ion (Fig. 1).

2.5. Method validation

Validation of the method was based on the European Commission Decision 2002/657/EC in terms of selectivity, linearity, accuracy, recovery, matrix effects, decision limit (CCα), and detection capability (CCβ) [20].

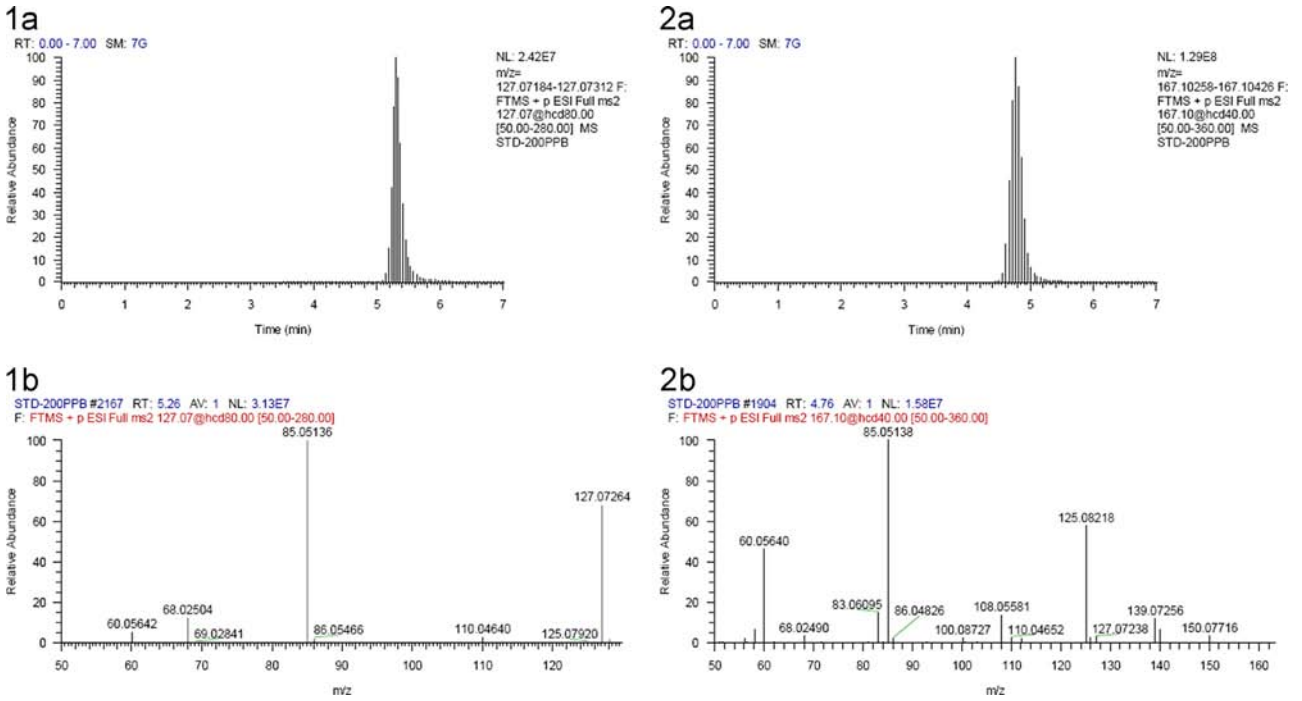

Fig. 1. Q Orbitrap MS chromatograms and spectra: (1a and 2a) extracted ion chromatogram of melamine $[M+H]^+$ *m/z* 127.0728 and cyromazine $[M+H]^+$ *m/z* 167.1039 with a mass tolerance of 5 ppm; (1b and 2b) mass spectrum from chromatograms 1a and 2a for melamine and cyromazine respectively.

Specificity of the method was performed by analyzing the blank samples and matrix interferences were checked compared with the elution time of the analytes.

The linearity of the method was generated by analysis of five calibration curves at concentrations of 0.05, 0.1, 0.2, 0.5, 1, 2 and 5 μg/L. Calibration curve was constructed using working standard solutions and by plotting the peak area ratio of the quantitative ion of standard to respective internal standard for melamine and cyromazine versus the analyte concentration. The concentration of the internal standards was 0.5 μg/L for melamine-$^{13}C3,^{15}N3$ and cyromazine-d_4.

Values of CCα and CCβ were estimated from the matrix calibration curve prepared by spiking blank milk and milk powder matrices at four concentration levels in the low concentration range. CCα was determined as the corresponding concentration at the intercept plus 2.33 times its the standard deviation. The value of CCα plus 1.64 times the corresponding standard deviation equals CCβ. In addition, limit of detection (LOD), and limit of quantification (LOQ) were estimated for a signal-to-noise (*S/N*) ratio of more than three and ten, respectively from the chromatograms of samples spiked at the lowest concentration validated.

Accuracy is determined by determining precision and trueness. Precision (intra-day repeatability and inter-day reproducibility, in terms of % RSD_r and RSD_R) and trueness (percentage biases) were estimated by analyzing six replicates of QC samples at four concentration levels (0.2, 0.5, 2 and 10 μg/kg for milk; 2, 5, 20 and 100 μg/kg for milk powder). Intra-day repeatability of the method was evaluated and analyzed in the same run of the day on the UHPLC-HRMS/MS. For inter-day reproducibility, the four concentrations were analyzed in three different days. In order to assess the bias, the percentage deviation of the target value was calculated from the difference between the experimentally determined mean content and nominal concentration.

Extraction recoveries of melamine and cyromazine were measured in blank milk and milk powder which were fortified at four concentration levels (0.2, 0.5, 2, and 10 μg/kg for milk; 2, 5, 20 and 100 μg/kg for milk powder) with six replicates at each level. The fortified milk and milk powder samples were prepared and analyzed as described in Sections 2.2.1 and 2.2.2, respectively. The recoveries were calculated from the measured compared to the expected concentrations.

Matrix effect (ME) was determined by constructing calibration curves in blank extract and in the pure solvent ($n=3$). The effects were expressed in terms of signal suppression/enhancement (SSE) and calculated as follows: SSE=slope of spiked extract/slope of pure solvent standard.

3. Results and discussion

3.1. Optimization of chromatographic conditions

The separation and retention effects of different liquid chromatographic columns were initially studied. Three different types of analytical columns including Waters BEH C_{18} (2.1 mm × 100 mm, 1.7 μm), Waters HSS T_3 (2.1 mm × 100 mm, 1.8 μm), and Waters BEH HILIC (2.1 mm × 100 mm, 1.7 μm) were tested in their optimal elution conditions respectively, which were shown in Supplementary Data. The results showed that melamine and cyromazine had inferior retention and chromatographic behavior in C_{18} and T_3 columns owing to their high polarity. However, the HILIC column had a better retention effect and less interference from other impurities. As for the mobile phase, acetonitrile and water with a variety of modifiers were compared. From the analysis of the results shown in Fig. 2, ammonium formate mixed with formic acid showed a better performance for the ionization of melamine than the ammonium acetate mixed with formic acid. Additionally, the concentration of formic acid greater than 0.1% s did not produce a significant enhancement in performance for the ionization of melamine and cyromazine. Therefore, a mixture of 4 mM ammonium formate and 0.1% formic acid solution was selected as the mobile phase, by which a sufficiently good performance for ionization of melamine and cyromazine was achieved with good peak symmetry under the optimized linear gradient mode.

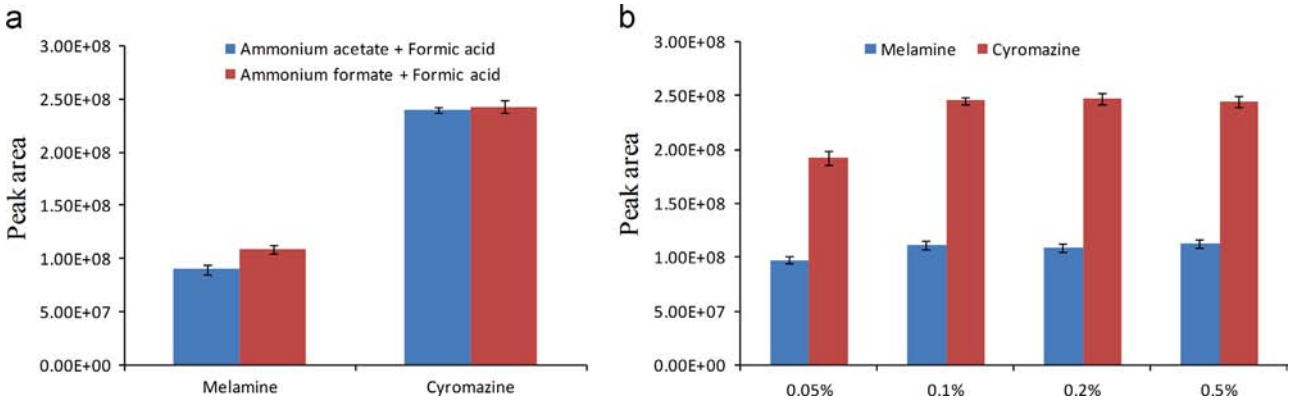

Fig. 2. Effects of the mobile phase on the ionization of melamine and cyromazine. (a) ammonium formate (4 mM) and formic acid (0.1%, v/v), ammonium acetate (4 mM) and formic acid (0.1%, v/v) and (b) the concentrations of formic acid (0.05%, 0.1%, 0.2% and 0.5%, v/v).

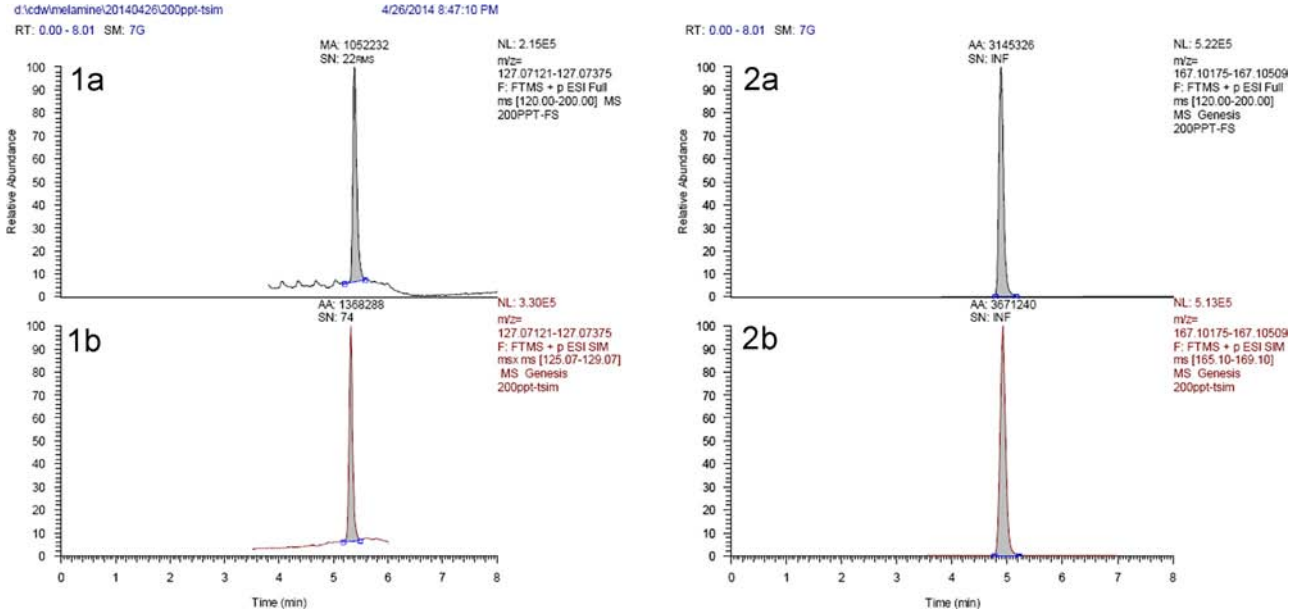

Fig. 3. Chromatographs for melamine (1) and cyromazine (2) at the concentration of 0.2 μg/kg in blank milk extract acquired with different modes (a: full mass; b: targeted single ion monitoring).

3.2. Optimization of MS acquisition modes

Full scan and tSIM modes are two kinds of commonly used quantitative models for Q Exactive. For melamine and cyromazine, Full scan mode and tSIM mode were evaluated in terms of the signal to noise (*S/N*) ratios. The signal to noise (*S/N*) ratios of melamine and cyromazine (0.2 μg/kg in blank milk extract) in different acquired modes were shown in Fig. 3. This figure highlights the fact that a narrower mass range in tSIM modes using the quadrupole increases the *S/N* ratio considerably, which leads to better method detection limits. Therefore, the tSIM mode was selected as the quantitative model for melamine and cyromazine in milk and milk powder.

3.3. Sample preparation

3.3.1. Optimization of extraction

Milk can be directly cleaned up without extraction. 1% formic acid in acetonitrile/water (1:1 v/v) was used to extract melamine and cyromazine from milk powder. The addition of water is conducive to the dissolution of milk powder and to enhance homogenization and permeability of the analytes into the extraction solution. Considering the physic-chemical properties of melamine and cyromazine, 1% formic acid would help to achieve more satisfactory extraction efficiency.

3.3.2. Optimization of DMSPE procedure

MCX SPE cleanup procedure had previously been widely used for triazines [21]. In this study, the same operation procedure was used for purification of melamine and cyromazine. However for a DMSPE using PCX sorbent, the parameters that affected the extraction efficiency, such as pH, the amount of PCX, the adsorption time, and the type of eluent were carefully studied.

3.3.2.1. Effect of pH. The effects on recovery caused by different pH values (2–8) and the lack of pH adjustment (pH=6.5) for the milk were investigated with 25 mg of PCX. There were no obvious differences in the recoveries of the melamine and cyromazine when the pH of the milk varied from two to five (Fig. 4**a**). However, there were significant reduction in the recoveries of the melamine and cyromazine at pH values greater than 5.0. It is well known that the melamine and cyromazine will be ionized at pH values lower than their pKa values (for example, the pKa value for melamine is

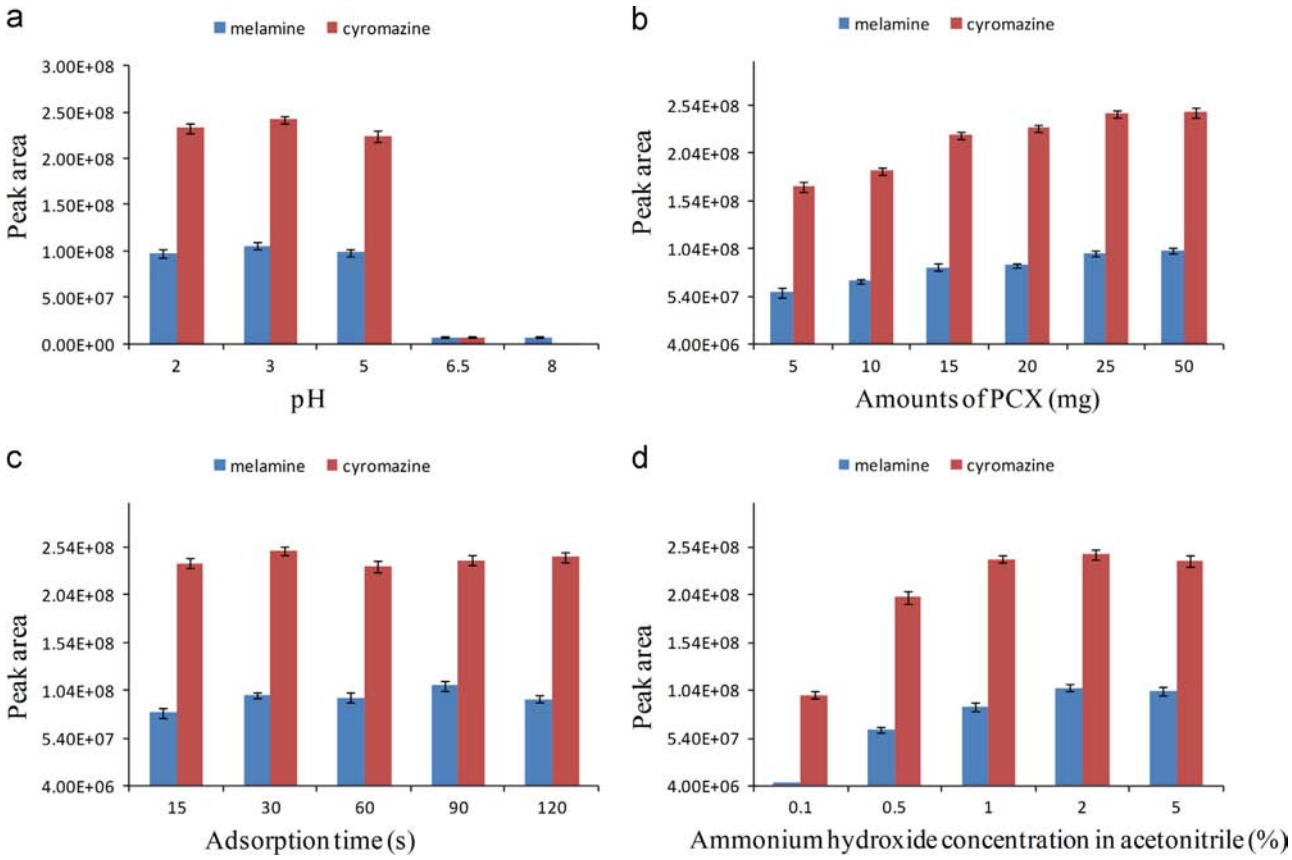

Fig. 4. Effects of the DMSPE cleanup conditions for melamine and cyromazine ($n=3$).

8.95) and are easily adsorbed in the PCX sorbent. Therefore, 1% formic acid for milk powder extract and 10 μL formic acid added to 1.0 g of milk were sufficient to lower the pH value less than 5.

3.3.2.2. The optimization of the amount of PCX adsorbent. The effects of the amount of PCX (5–50 mg) on recovery were carefully investigated at 100 μg/kg by the blank fortified milk sample using a 30 s adsorption time. The results are shown in Fig. 4**b**. It was found that the adsorption rates were higher as the amount of PCX increased from 5 to 25 mg both for melamine and cyromazine. Moreover, with the amount of PCX greater than 25 mg, there was no obvious increase in the adsorption rate. To obtain the best results, 25 mg of PCX was chosen as the optimal amount.

3.3.2.3. The optimization of the adsorption time. The effects of the adsorption time for melamine and cyromazine were investigated for the milk at the level of 100 μg/kg at different shaking times (15, 30, 60, 90, and 120 s). The results are shown in Fig. 4**c**. As expected, adsorption times greater than 30 s did not produce a significant enhancement of adsorption efficiency, demonstrating the quick adsorption process of melamine and cyromazine into PCX. As a result, an adsorption time of 30 s was chosen for all the subsequent experiments.

3.3.2.4. The selection of the elute solution. As an analytical practice for SPE, it is well known that alkaline substances are easily eluted from the strong cation exchange column in alkaline condition. In addition, it is desirable to use a mobile phase in the sample preparation process for the convenience of injecting directly into a LC system. As a result, the acetonitrile solutions containing different amount of ammonium hydroxide was used to elute melamine and cyromazine from the PCX adsorbent. The results showed that the recoveries increased significantly when the concentration of ammonium hydroxide increased from 0.1 to 2% (Fig. 4**d**). Moreover, ammonium hydroxide at concentrations above 2% did not show an obvious increase in recovery.

3.3.3. Design of experiments

In order to obtain the optimum DMSPE operating procedure and take into account interactions between factors, a Box-Behnken design (BBD) approach was carried out based on the previous experimental results obtained by the univariate method. The software Design-Expert (version 8.0.5b, Stat-Ease, Inc., Minneapolis, MN, USA) was used for experimental design, data analysis and model building for response surface methodology (RSM). BBD requires an experiment number according to $N=2k(k-1)+c_p$, where k is the factor number and c_p is the replicate number of the central point [22]. The effects of the amount of PCX (10–30 mg, A), the adsorption time (15–60 s, B) and the concentration of ammonium hydroxide in acetonitrile (0.5–2.5%, C) on the efficiency of extraction for analytes were considered and optimized in BBD (Table 1). In total, 17 experiments were performed in triplicate, with five repetitions of the center point. After performing the 17 run experiments, the responses Y (R1 for melamine and R2 for cyromazine) were calculated based on the efficiency of extraction, and all statistical analysis were performed by Design-Expert software. The ANOVA analysis for response surface quadratic model of melamine showed that the model was significant with *F*-value of 43.79 and *p*-value of less than 0.0001. The lack of fit of the model relative to its pure error showed an *F*-value of 3.11 and a *p*-value of 0.1510 and so was not significant, which indicated that the fitted model was considered adequate to predict the efficiency of extraction under any sets of the variables combination. Model coefficients for the

Table 1
Variables and levels evaluated in the Box-Behnken design.

Independent variables	Unit	Symbol	Coded level		
			−1 (Low)	0 (Center)	+1 (High)
The amount of PCX	mg	A	10	20	30
The adsorption time	s	B	15	37.5	60
The concentration of ammonium hydroxide	%	C	0.5	1.5	2.5

Table 2
ANOVA for response surface quadratic model of melamine.

Source	Sum of squares	df	Mean square	*F*-value	*P*-value
Model	3327.63	9	369.74	43.79	< 0.0001
A	1526.20	1	1526.20	180.74	< 0.0001
B	4.34	1	4.34	0.51	0.4966
C	620.38	1	620.38	73.47	< 0.0001
AB	45.56	1	45.56	5.40	0.0532
AC	34.81	1	34.81	4.12	0.0819
BC	1.32	1	1.32	0.16	0.7041
A^2	654.22	1	654.22	77.47	< 0.0001
B^2	9.35	1	9.35	1.11	0.3277
C^2	213.15	1	213.15	25.24	0.0015
Lack of fit	41.36	3	13.79	3.11	0.1510
Pure error	17.75	4	4.44		
Corrected total	3386.74	16			
SD	2.91	R^2	0.9825		
CV	3.63	adj-R^2	0.9601		

response of melamine and cyromazine were shown in Table 2 and Table S1 (Supplementary Data). The final equation in terms of coded factors of melamine was: Response = 87.75 + 14.65 A + 0.78 B + 8.02C + 3.38 AB + 2.21 AC + 0.43 BC - 12.46 A^2 - 1.49B^2 - 4.00C^2. The three-dimensional (3D) surface response plots and their related counters, obtained using the fitted model, were shown in Fig. 5. Fig. 5 showed that the concentration of ammonium hydroxide and the amount of PCX were two important factors which had a significant positive effect on the extraction efficiency. However, the adsorption time did not produce a significant effects. The optimum conditions were selected by the analysis data obtained from the response surface plots and the regression coefficient plots. The factor settings that maximize the efficiency of extraction of analytes were chosen in response optimization. Among these settings, the most desirable factor levels ranged as follows: 22–30 mg for the amount of PCX, 23–60 s for the adsorption time, and 1.8–2.5% for the concentration of ammonium hydroxide. In order to obtain the simultaneous extraction of the melamine and cyromazine with ideal maximum response, the best combination was found to be 25 mg PCX, 30 s adsorption time and 2.5% ammonium hydroxide in acetonitrile.

3.4. Method validation

3.4.1. Specificity

As described in Section 2.5, the results showed that there were no interfering peaks at the retention times of melamine and cyromazine (Fig. 6).

3.4.2. Linearity, CCα and CCβ

To obtain the internal calibration curves for the analytes, the peak area ratio of the quantitative ion of each standard to internal standard was plotted at the concentrations of 0.05 to 5 μg/L with the internal standard of melamine-$^{13}C3,^{15}N3$ (0.5 μg/L) and cyromazine cyromazine-d_4 (0.5 μg/L). As shown in Table 3, the coefficients of determination (R^2) of the calibration curves for melamine and cyromazine were above 0.999 indicating the good linearity in the analytical range. The homoscedasticity of calibration data was also confirmed by the *F*-test statistical method and residual plots (plots of residuals versus concentration) [23]. In the F-test, the data are assumed to be uniformly distributed when the tabulated *F*-value (F_{tab}) is greater than the experimental *F*-value (F_{exp}) ($F_{tab} > F_{exp}$). The F_{tab} is obtained from the *F*-table distribution critical values with confidence levels of 95% for $f1 = f2 = (n\text{-}1)$ degrees of freedom, and the F_{exp} is expressed as the ratio between the variances obtained at the lowest SD^2 and highest SD^2 of the working concentration range. The results (Table 3) indicated that the calibration data were homoscedastic for melamine and cyromazine as the F_{exp} were lower than the $F_{tab.}$ Additionally, the residual plots showed a random distribution around the axis of concentrations and the residuals were within a band parallel to the axis of concentration, further confirming the homoscedasticity of the calibration data [23]. Therefore, the linear regressions were appropriate for calibration data of melamine and cyromazine.

The CCα and CCβ were estimated according to the European Commission Decision 2002/657/EC. The results of CCα and CCβ were shown in Table 3, the CCα values for melamine and cyromazine were in the range of 0.06–0.84 μg/kg in milk and milk powder, and the CCβ values fall within a range of 0.21–2.7 μg/kg. Additionally, the LOD values for melamine and cyromazine were in the range of 0.05–0.06 μg/kg (milk) and 0.60 μg/kg (milk powder), and the LOQ values were both 0.20 in milk and 2.0 μg/kg in milk powder.

3.4.3. Accuracy and recovery

The results of accuracy experiment (Table 4) exhibited acceptable intra- and inter-day precision and trueness; the average repeatability (RSD_r), and reproducibility (RSD_R) were 1.4–7.9% (mean bias from −6.2 to 3.2%), and 2.7–6.2% (mean bias from -5.6 to 2.3%), respectively for melamine, and 0.9–5.5% (mean bias from −4.6 to 3.4%) and 1.7–8.7% (mean bias from −5.7 to 4.2%) for cyromazine, respectively, which indicated that the established method was accurate enough for the determination of melamine and cyromazine in milk and milk powder. To assess extraction efficiency of the method, recoveries at four concentration levels were performed, as provided in Table 4. 78.1–107.1% of recoveries were obtained for melamine and cyromazine in milk and milk powder, indicating that proposed method was suitable for the simultaneous determination of melamine and cyromazine.

3.4.4. Comparison on matrix effects

As described in Section 2.5, slope ratios from MCX SPE method for milk and milk powder samples were 1.32 ± 0.04 and 1.44 ± 0.02 for melamine, 1.16 ± 0.02 and 1.13 ± 0.05 for cyromazine. However, slope ratios from DMSPE method for milk and milk powder samples were 1.21 ± 0.03 and 1.16 ± 0.05 for melamine, 1.03 ± 0.02 and 1.04 ± 0.03 for cyromazine. The results inferred that DMSPE method yielded fewer matrix effects than the SPE method under the same enrichment factor (1.0). According to Frenich et al. [24], signal suppression or enhancement effect was considered tolerable if the value was between 0.8 and 1.2. The values outside this range indicate a strong matrix effect. It can be concluded that there was a slight matrix effect for melamine and cyromazine in milk and milk powder by using DMSPE method. Therefore, the pure solvent standard calibration was used for melamine and cyromazine and there was no need to use the matrix matched calibration for DMSPE method. However, in order to compensate the matrix effects and quantify accurately the concentrations of melamine and cyromazine

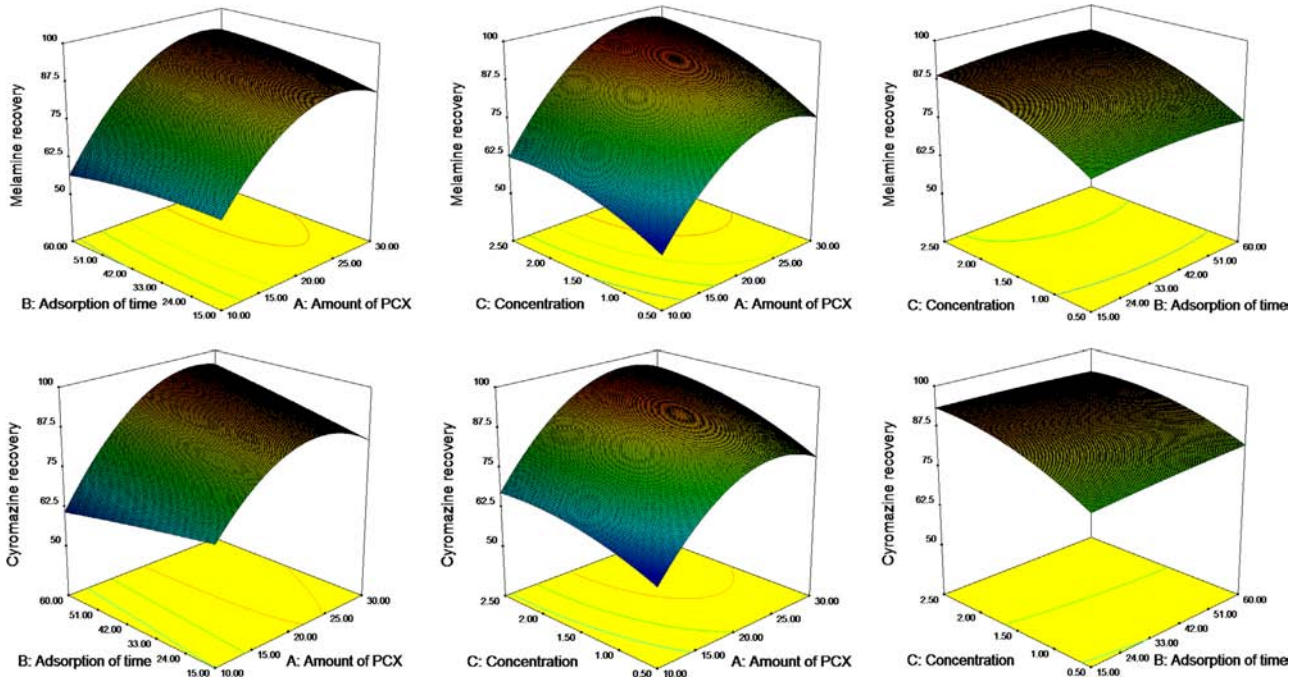

Fig. 5. Three-dimensional graphs of the effects of the amount of PCX (A), the adsorption of time (B) and the concentration of ammonium hydroxide (C) on efficiency of extraction: (a) fixed $C=1.5\%$; (b) fixed $B=37.5$ s; (c) fixed $A=20$ mg.

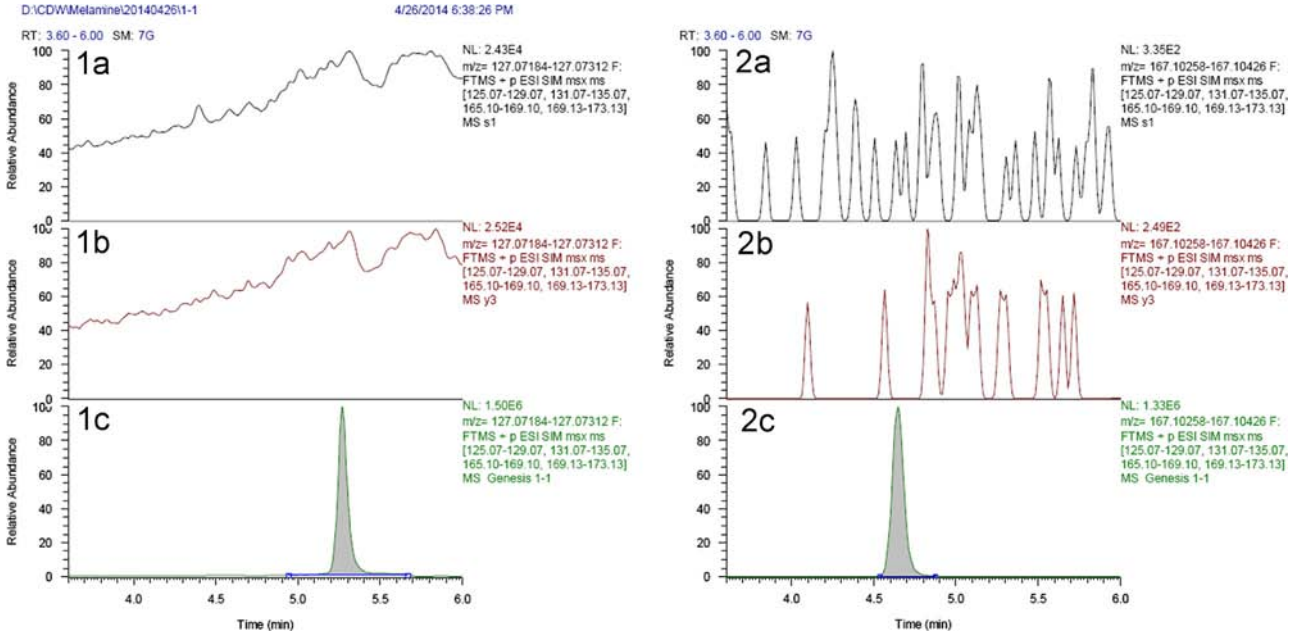

Fig. 6. Extracted ion chromatographs for melamine (1) and cyromazine (2) in the blank milk (a), blank milk powder (b) and spiked standard at 1 μg/kg in milk (c).

Table 3
Calibration curve equations, correlation coefficients (R^2), CCα, and CCβ for melamine and cyromazine.

Analytes	Precursor ion (m/z)	Fragment ion (m/z)	Linear range (μg/L)	Linearity equation	R^2	F_{exp}[a]	CCα/(μg/kg)		CCβ/(μg/kg)	
							milk	milk powder	milk	milk powder
Melamine	127.0728	85.0514; 110.0464	0.05–5	$Y=0.5351X-0.0316$	0.9995	0.721	0.10	0.84	0.21	2.7
Cyromazine	167.1039	125.0822; 85.0514	0.05–5	$Y=0.2719X+0.0005$	0.9999	0.438	0.06	0.72	0.26	2.3
Melamine-$^{13}C3,^{15}N3$	133.0739	89.0521	/	/	/		/		/	
Cyromazine-d_4	171.1289	86.0577	/	/	/		/		/	

[a] The theoretical value (F_{tab}) of $F_{4,4}$ ($p=0.05$) is 6.388

Table 4
Accuracy and recovery of melamine and cyromazine.

	Sample	Fortified concentration (μg/kg)	Extraction recovery[a] (%)	Intra-day		Inter-day		SSE
				RSD_r (%)	Biases (%)	RSD_R (%)	Biases (%)	
Melamine	Milk	0.2	84.9 ± 2.1	7.9	−6.2	6.2	−5.6	1.21 ± 0.03
		0.5	88.8 ± 3.3	3.8	−4.3	5.7	−3.3	
		2	104.8 ± 2.7	1.4	−0.6	2.7	−1.4	
		10	101.7 ± 2.3	2.5	1.6	3.4	2.3	
	Milk powder	2	84.8 ± 4.2	5.3	−4.1	5.9	−5.4	1.16 ± 0.05
		5	92.4 ± 3.9	4.8	−3.6	3.2	−3.1	
		20	99.1 ± 2.5	1.7	1.4	3.8	−2.8	
		100	106.2 ± 1.2	1.9	3.2	2.8	−1.3	
Cyromazine	Milk	0.2	79.3 ± 3.3	4.5	−4.6	8.7	−3.9	1.03 ± 0.02
		0.5	92.6 ± 3.7	3.1	−2.5	3.7	−2.6	
		2	103.7 ± 3.6	0.9	3.4	2.5	1.8	
		10	97.1 ± 1.6	2.3	2.6	4.6	4.2	
	Milk powder	2	78.1 ± 5.4	5.5	−4.2	6.1	−5.7	1.04 ± 0.03
		5	96.4 ± 3.6	2.7	−2.9	4.7	−3.9	
		20	95.2 ± 2.2	2.4	−1.8	1.7	2.2	
		100	107.1 ± 3.8	1.7	2.7	3.2	3.8	

[a] Mean ± SD (n=6)

Table 5
Comparison of various analytical methods developed for analysis of melamine and cyromazine in several matrixes.

Sample preparation	Analyte	Matrix	Analysis	LOD (μg/kg)	Ref.
MCX-SPE	Cyromazine	Animal edible tissues	LC-MS/MS	10	[25]
SCX-SPE	Melamine and cyromazine	Milk and milk-based infant formula	LC-MS/MS	50	[26]
MCX-SPE	Melamine	Milk and milk powder	GC–MS	10	[27]
MCX-SPE	Melamine	Raw milk and dairy products	GC–MS/MS	10	[28]
MCX-SPE	Melamine	Raw milk and dairy products	LC-MS/MS	5	[28]
QuEChERS	Melamine and cyromazine	Egg	LC-MS/MS	1.6–8	[19]
LLE	Melamine	Milk and milk-based infant formula	LC-MS/MS	25	[29]
MCX-SPE	Melamine and cyromazine	Animal muscle, milk and egg	GC–MS	5–10	[30]
PCX-DMSPE	Melamine and cyromazine	Milk and milk powder	LC-HRMS	0.05–0.6	This work

in the samples, the internal calibration was applied in the pure solvent standard calibration.

3.5. Comparison of reported analytical methods

The proposed dispersive micro solid phase extraction method can be compared with other reported methods for the analysis of melamine and cyromazine in several matrixes. Table 5 compiles the comparison of the analytical features of the selected references, including also sample preparation step. Compared to the reported methods, lower detection levels for the analysis of melamine and cyromazine were obtained in this study. SPE was widely used as a cleanup step in the reported method [25–28,30]. For the extraction and cleanup of melamine and cyromazine in milk and milk powder samples, the matrix effects of DMSPE cleanup method in this study were fewer than that of SPE method. Furthermore, the cost of the proposed cleanup method by PCX adsorbent for one sample is low enough (approximately 0.30 US dollars) compared to that of a solid phase extraction column (at least 3.0 US dollars for each). In addition, the total time required for the cleanup of one sample was only approximately 3 min and far less than that of the SPE method (at least 30 min). Therefore, the proposed method provided a relatively inexpensive and rapid analysis of melamine and cyromazine in milk and milk powder samples.

3.6. Application to real samples

Eleven milk and six milk powder samples available from local markets were analyzed using the established method. Six milk and four milk powder samples were found with melamine residue at concentrations of 0.1–12.9 μg/kg.

4. Conclusions

In the present study, a novel DMSPE cleanup method using PCX as the sorbent by UHPLC-HRMS/MS detection was established for the rapid analysis of melamine and cyromazine in milk and milk powder. The established method has the advantages of rapidness (3 min), economy (0.30 US dollars), convenience, high sensitivity, and good repeatability. The newly developed DMSPE cleanup method based on PCX sorbent material is expected to be widely applied for the analysis of alkaline contaminants at trace levels in the future for sample cleanup.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.10.036.

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