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Short communication

Hydrophilic interaction chromatography/tandem mass spectrometry for the determination of melamine in royal jelly and royal jelly lyophilized powder

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ABSTRACT

Melamine has become the focus of attention for the possible occurrence of nephrolithiasis and associated deaths, because it was added to foods to increase the apparent protein content by unethical manufacturers. An analytical method based on hydrophilic interaction chromatography/tandem mass spectrometry (HILIC-MS/MS) was developed and validated for the determination of melamine in the royal jelly (RJ) and royal jelly lyophilized powder (RJLP). Trace of melamine was extracted from the RJ and RJLP by ultrasonic-assisted extraction followed by clean-up procedure using mixed-mode cation exchange (MCX) solid phase extraction and separated on a hydrophilic interaction chromatography (HILIC) analytical column with acetonitrile/5 mM ammonium acetate buffer (88:12, v/v) as mobile phase. Detection was carried out by positive electrospray ionization (ESI+) in multiple reaction monitoring (MRM) mode. The chromatographic separation was obtained within 5 min and was linear in the concentration range of 0.01–8 μg/mL in RJ and 0.05–10 μg/mL in RJLP for melamine. The mean extraction recoveries for melamine were ranged from 89.6 to 100.4%. Method validation parameters were evaluated such as linearity, selectivity, precision, carryover and recovery, giving results within the acceptable range. The proposed method was successfully applied to the quantitation of melamine in RJ and RJLP. This approach will be of particular utility for the evaluation of melamine residue level and routine monitor of melamine in RJ and RJLP samples.

1. Introduction

Melamine is a triazine ring with three amino groups, which has been widely used to make plastics, fertilizer, glues and other products due to characteristics of fire resistance and heat tolerance [1–4]. Melamine has become a focus of discussion in March of 2007, because it has been found in samples of pet food and wheat gluten and resulted in the pet death [5,6]. In September of 2008, different brands of powdered infant formula, yogurt and biscuit and dessert contaminated with melamine were reported one after another in many countries including America, New Zealand, Netherlands, Australia and China [7]. All these adulterated milk products were most manufactured using ingredients made from melamine-tainted milk. In China, the unethical intermediates between dairy companies and dairy farmers were the arch-criminal in this scandal. Melamine can lead to nephrolithiasis and renal failure and even associated deaths, because ammonia crystals, generated from the digestion of the melamine, could obstruct and damage renal tubules leading to renal disease [8,9].

RJ is the only food of queen bees and accounts for their incredible size, fertility, and longevity. As a kind of high nutritional bee product, RJ and RJLP are highly appreciated as a health-beneficial food and a pharmaceutical product especially for the postoperative patients and the aged due to the abundant amounts of 10-Hydroxy-2-Decenoic Acid (HDA), amino acids, water, protein, carbohydrates, lipids and minerals [10]. It is doubtful that melamine can be added into RJ and RJLP to increase the protein content due to melamine's high nitrogen content (66% by mass versus approx) according to its molecular structure. Melamine can cause the protein content of RJ and RJLP to appear higher than the true value when the Kjeldahl or Dumas method is used for protein content analysis [11].

Melamine has been generally analyzed in different matrices by liquid chromatography with ultraviolet detection or diode array detection (DAD) [12–17], liquid chromatography/tandem mass spectrometry [18–22], gas chromatography [23], enzyme-linked immunosorbent assay [22,24], capillary electrophoresis [25], surface desorption atmospheric pressure chemical ionization

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mass spectrometry [26], capillary zone electrophoresis with quadrupole/time of flight mass spectrometry (CZE-Q/TOF) [27], CZE-DAD [28] and infrared spectroscopy [29]. A maximum of 1 mg/kg of infant formula was the limit in China [30] but any level of contamination level for melamine was still not established by FDA of America in infant formula.

Most of the above methods have been employed in various matrices, such as raw milk and dairy products, urine, soil, grains and animal tissues. However, no report on the application of melamine analysis in RJ and RJLP can be found in the literatures. In general, traditional extraction methods such as liquid–liquid partition or vortex were difficult to extract residual toxic substances from RJ and RJLP due to insufficient extraction yield. Ultrasound-assisted extraction has a lot of advantages including reduced extraction time, reduced solvent consumption and temperature independence alternative to conventional extraction processes [31]. Therefore, ultrasound treatment can be used for the extraction of toxin residue in RJ and RJLP.

HILIC, as a complementary method to conventional reversed phase liquid chromatography (RPLC), was used mainly for retention and separation of polar compounds due to their poor retention in RPLC column. The combination of a polar stationary phase with an aqueous/high proportion organic solvent mobile phase brought remarkable advantages including appropriate retention of polar analyte and compatibility between HPLC and MS spectrometry [32–34].

The aim of this study was to develop a simple and rapid method for the determination of melamine in RJ and RJLP on the basis of ultrasonic-assisted extraction, followed by solid phase extraction with MCX and HILIC-MS/MS. In addition, this study tried to present the evaluation result of detection method for melamine residue and to also verify whether determination of trace of melamine might be useful or appropriate in RJ and RJLP samples as in other matrices previously studied.

2. Experimental

2.1. Chemicals and reagents

Melamine (>99%, CAS 108-78-01) was purchased from Sigma–Aldrich. Acetonitrile, methanol and ammonium acetate (HPLC grade) were obtained from Fisher Scientific. Trichloroacetic acid was purchased from Beijing Chemical Reagent (Beijing, China). Pure water was prepared from a Milli-Q system (MilliPore, Bedford, MA, USA).

2.2. Preparation of calibration standards

Stock solution, containing 100 μg/mL of melamine was prepared using 1.0 mg of standard with 10 mL of 1:1 acetonitrile water and kept in a freezer at 4 °C for 3 months. Working solutions of different concentrations were prepared from above-mentioned stock solution afresh before use. Matrix standards were prepared by using blank RJ and RJLP sample extract which were added melamine working solutions in different concentrations. Matrix standards were prepared at 0.01–8 μg/mL in RJ extract and 0.05–10 μg/mL in RJLP extract and stored at 4 °C.

2.3. Sample preparation

Thirty of RJ and twenty of RJLP samples were obtained from apiaries and supermarkets and stored at −18 °C. Cold samples were equilibrated at room temperature for 1 h and then homogenized before analysis. Spiked samples were prepared by adding working standard solution having the desired concentration of melamine into 1.0 g RJ and 0.5 g RJLP which was left to stand at room temperature for 10 min and to allow sample equilibration. The approach of sample preparation was slightly modified according to the previous method developed by our group. A scheme of the sample preparation is shown in Fig. 1.

2.4. HILIC–MS/MS

An agilent 1200 liquid chromatography equipped with an autosampler, a binary pump and a thermostated column compartment was used for all analyses. The chromatographic separation was carried out by a Zorbax Rx-Sil (2.1 mm × 150 mm i.d., 5 μm particle size) analytical column (Agilent, Waldbronn, Germany) at 30 °C using isocratic elution with acetonitrile/5 mM ammonium acetate buffer (88:12, v/v) at a flow rate of 0.3 mL/min. The injection volume was 5 μL for analysis. In order to avoid carryover, the auto sampler needle was rinsed among a series of calibration standards, blank samples and spiked samples with mobile phase.

An Agilent 6460 triple quadrupole tandem mass spectrometer coupled to an electrospray ionization and Agilent Jet Stream interface was used. The system operation, data acquisition and data processing were controlled by the MassHunter software. The optimal MS/MS conditions were as follows: drying gas temperature 350 °C; drying gas flow rate 9 L/min; nebulizer gas pressure 40 psi; Sheath Gas Temp 400 °C; Sheath Gas Flow 8 L/min and capillary voltage 3.5 kV. Melamine was analyzed in ESI in positive ion (ESI+) mode. The collision energy was varied and optimized for each multiple reaction monitoring (MRM) transition with the characteristic fragmentation transitions m/z 127 > 85 at 20 V for quantitative analysis and 127 > 68 at 35 V for confirmatory analysis. The dwell time for melamine was 50 ms and fragmentor voltage was set at 100 V.

3. Results and discussion

3.1. Optimization of the liquid chromatographic condition

The optimal chromatographic separation is very crucial for appropriate retention and high sensitivity of analyte and low signal suppression of MS/MS detection. All parameters were optimized by a univariate approach for melamine and a serial of preliminary studies were carried out to optimize chromatographic condition using a matrix-matched standard solution of 0.2 μg/mL. Melamine is highly polar compound that is difficult to gain adequate retention in generic reverse phase chromatographic column that easy to co-elute with a host of relatively polar compounds within void volume. HILIC possessed the advantages of reversed phase liquid chromatography (RPLC), normal phase liquid chromatography (NPLC) and ion chromatography (IC) including mobile phases consisting of aqueous buffer solution and organic solvent (like RPLC), stationary phase with an embedded polar group (like NPLC) and electrostatic interaction between highly polar compounds and ions (like IC). HILIC retention is usually attributed to the distribution of the targeted analytes between mobile phase and a water layer in the hydrophilic stationary phase. Under HILIC conditions, the elution with organic mobile phase against hydrophilic stationary phase was improved by increasing the aqueous phase proportion being the strongest eluting solvent in the mobile phase [35,36]. In this study, a kind of chromatographic column bonded to highly pure porous silica micro-spheres, named ZORBAX Rx-SIL, was used for retention and separation of melamine and other impurities and available for polar hydrophilic compounds with high organic mobile phases in reversed phase mode.

In order to achieve the optimum response of melamine with sharp peak shape, different mobile phase compositions consisting of methanol or acetonitrile with formic acid, acetic acid, ammonium

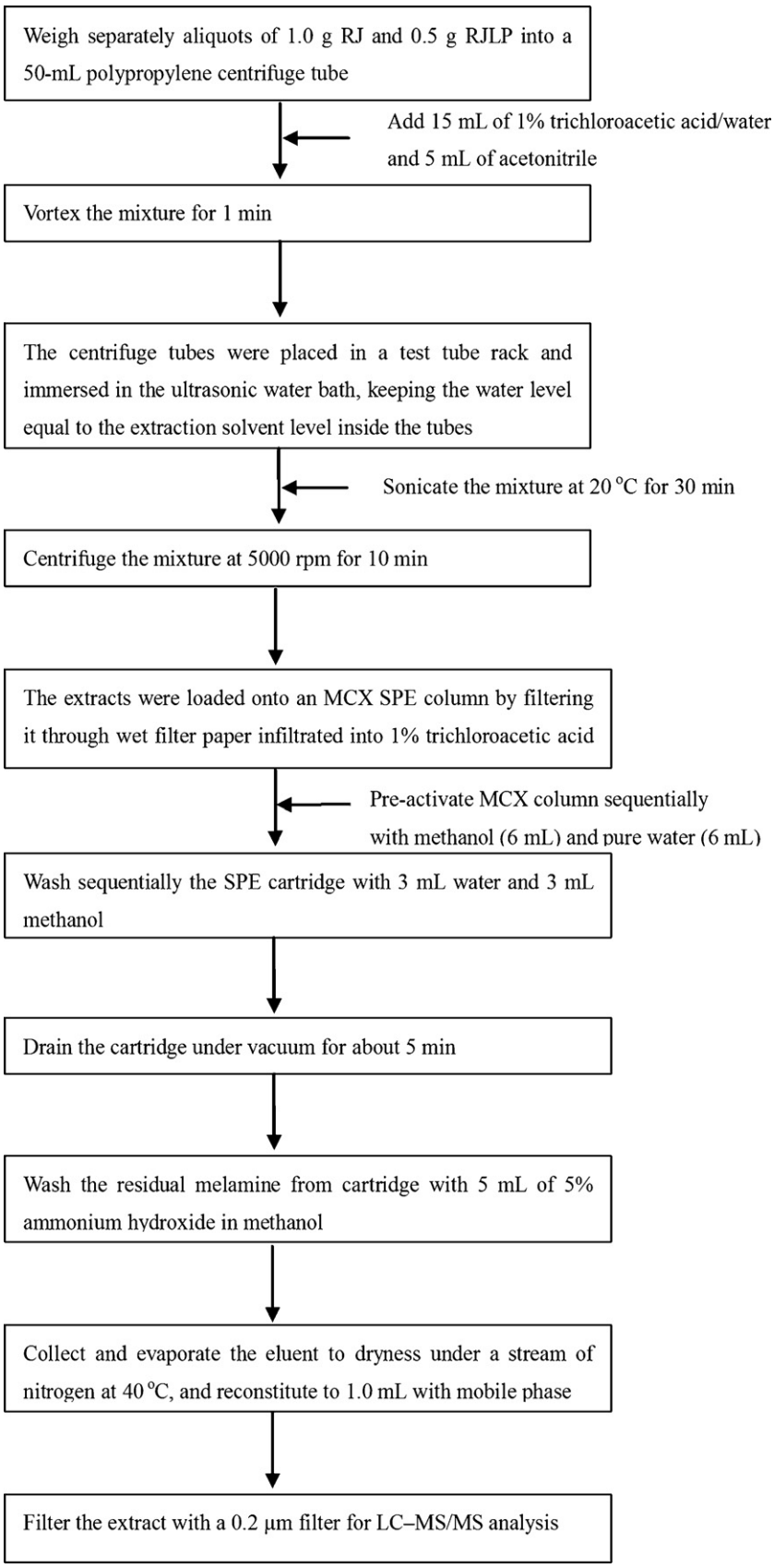

Fig. 1. The scheme of sample preparation.

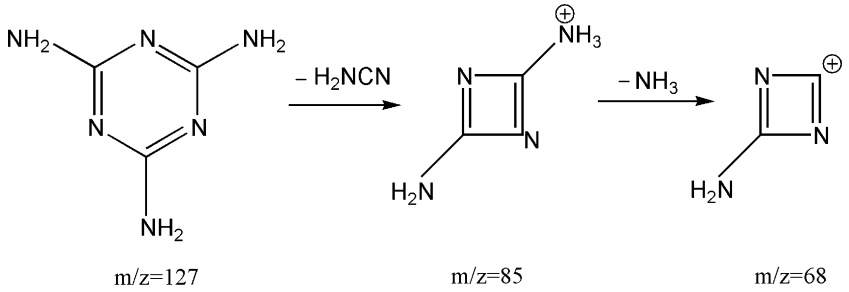

Fig. 2. Proposed fragmentation pathway of melamine.

formate or ammonium acetate buffers as modifiers in different volume ratios were tested in the HILIC–MS/MS analysis. Methanol or acetonitrile was evaluated as an option of mobile phase B and mobile phase A was fixed to be pure water contained 0.1% formic acid as mobile phase additive. The signal response of melamine in positive mode was better when the mixture of acetonitrile and buffer was used as a mobile phase, because the use of acetonitrile in place of methanol led to an obvious increase in response with a sharper peak shape and afforded to satisfactory sensitivity.

Moreover, it is imperative to optimize the modifiers in aqueous mobile phase A. The procedure was performed by comparing response and retention time of melamine under the addition of two acids (formic acid and acetic acid) at 3 concentrations (0.05, 0.1 and 0.2%, v/v) or two buffers (ammonium formate and ammonium acetate) at 3 concentrations (5, 10 and 20 mM) in aqueous phase. The peak shape and resolution are improved and the formation of $[M+H]^+$ ions is supported, making formic acid or acetic acid one of the most popular acidic additives in HILIC-MS/MS. But, in this study, the addition of 3 different concentrations of formic acid and acetic acid in aqueous phase have drastically shorten the retention time of melamine and easily led to the coelution of analyte and other impurities, and then ion suppression occurred.

When volatile acids are not suitable, volatile salts, like ammonium formate or ammonium acetate, may be the additives of choice in the mobile phase. Ammonium formate and ammonium acetate were analyzed as modifiers by adding them into aqueous phase of mobile phase, and ammonium acetate was selected because it led to the overall best results regarding sensitivity and appropriate retention time. Melamine with basic character was easily neutralized by the acid arising from dissociation of volatile modifiers. The decrease of ionization degree is more likely to occur when ammonium formate was added to the mobile phase, relative to ammonium acetate. Therefore, pure water–ammonium acetate/acetonitrile was chosen as mobile phase when ESI in positive mode was employed for the analysis of melamine. Moreover, one issue is the limited solubility of ammonium acetate in acetonitrile. The concentration of 5 mM ammonium acetate possessed sufficient solubility in acetonitrile when the proportion of acetonitrile was exceeded to 80% in the mobile phase [37]. So, taking into all these factors, acetonitrile/5 mM ammonium acetate buffer (88:12, v/v) was selected as the optimum mobile phase in this study.

3.2. Optimization of MS conditions

The MS condition optimization was performed, using melamine standard solution (0.2 μg/mL), by flow-injection analysis (FIA) in positive ion mode. In order to get the best response, two parameters including fragmentor and collision energy need to be optimized for melamine on Agilent 6460 MS Spectrometry. The suitable fragmentor voltage allows the highest transmission of the precursor ion into the mass analyzer. The appropriate collision energy provides the highest intensity of quantitation of the qualifier product ion, because collision energy is an important factor in the collision-induced dissociation (CID) process. The nebulizer spray was surrounded by super-heated nitrogen sheath gas so that more ions and fewer solvent droplets easily entered the sampling capillary which resulted in the increase of desolvation efficiency and improvement of signal intensity. ESI of melamine gave the protonated molecular ion of m/z 127, which corresponds to the molecular ion $[M+H]^+$ of melamine. The fragments of m/z 127 were m/z 127 > 85 with the loss of a molecule of $[M+H–CH_2N_2]^+$ and m/z 127 > 68 by loss of $[M+H–CH_5N_3]^+$ where m/z 85 is recommended as the quantification ion due to its high abundance. Proposed fragmentation pathway of melamine was shown in Fig. 2.

The optimizations of extraction and clean-up condition have been explicated in the previous method by our group, so it is omitted in this study.

3.3. Validation of the analytical method

3.3.1. Matrix effect

Matrix effect was evaluated by comparing the MS/MS responses of known amounts of melamine standard solution in acetonitrile/water (A) and matrix-matched standard solution with the same concentration in matrix extract after post-extraction and clean-up (B). Matrix effect percentage is calculated as 100 × B/A. The ratio is less than 100% which means ionisation suppression whereas the ratio is higher than 100% which indicates ionisation enhancement. Thus, matrix effect was studied in RJ and RJLP by adding melamine standard solution to the extractant at 0.05 and 0.2 μg/mL and processing every sample with the developed sample preparation method. Moreover, the effect of concentration of the matrix extracting solution, containing 2, 1 g/mL of RJ and 1, 0.5 g/mL of RJLP, using the same calibration curve was also tested and compared in this study.

The obtained results are shown in Table 1. Matrix effect value ranged from 53.1 to 73.5% which indicates ionization suppression in RJ and RJLP matrix. The significant decrease in the MS/MS response could be explained by ion suppression of the analyte due to the presence of a variety of endogenous matrix components co-eluting with melamine in RJ and RJLP products. The use of matrix-matched calibration can effectively compensate matrix effects for their co-elute analytes. The concentration of matrix had an impact on the

Table 1
Matrix effect on the melamine response.

The concentration of melamine (μg/mL)	Relative response (%)			
	RJ concentration		RJLP concentration	
	2	1	1	0.5
0.05	56.8	64.4	53.1	61.9
0.2	69.1	73.5	65.7	68.3

Relative responses were obtained by comparing the peak area of standard in neat solvent and in matrix extract spiked with the melamine at 0.05 and 0.2 μg/mL.

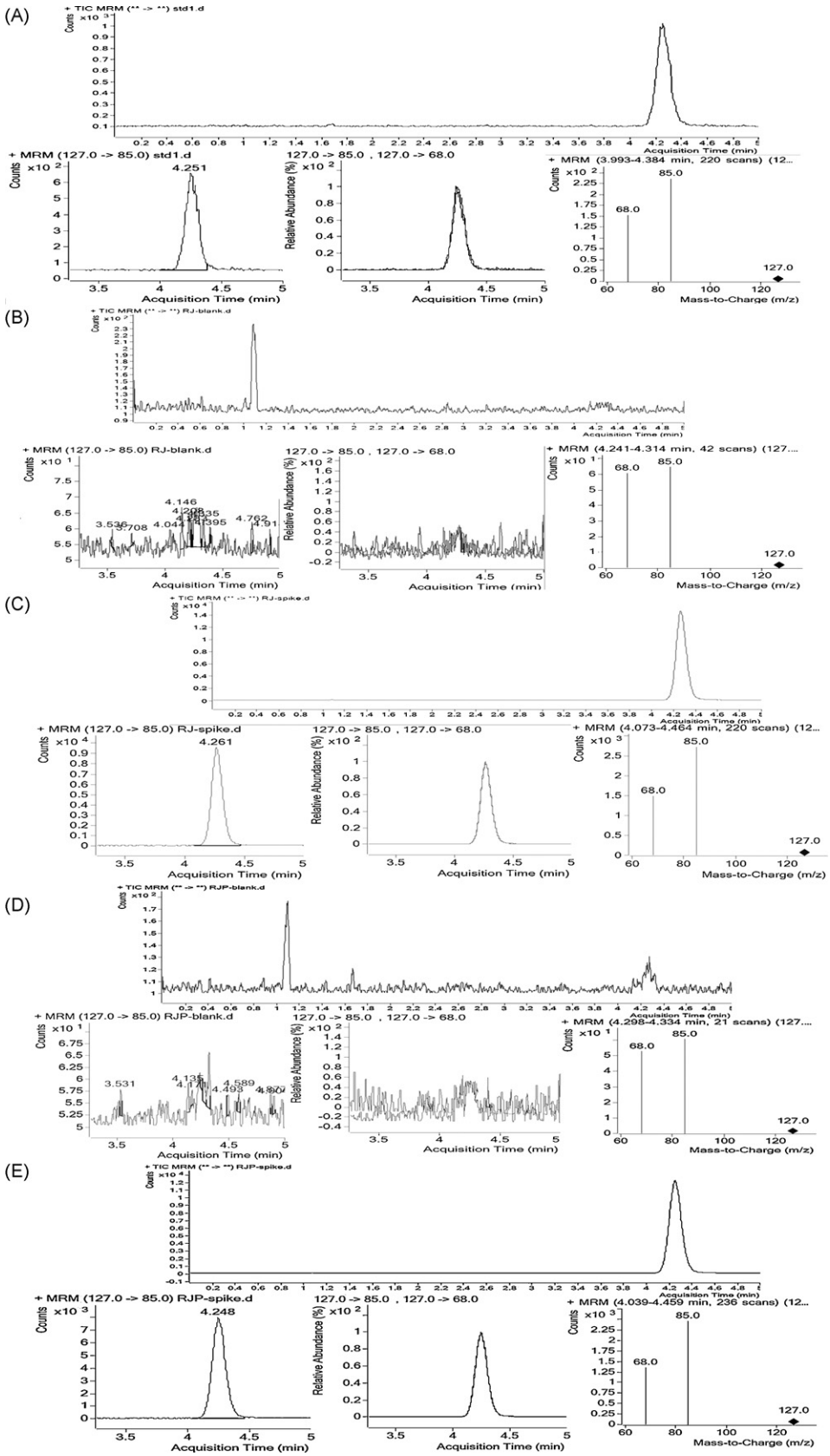

Fig. 3. (A) MRM ion chromatogram of standard solution at 0.01 μg/mL in RJ matrix; (B) MRM ion chromatogram of RJ blank sample; (C) MRM ion chromatogram of RJ spiked samples at 0.2 μg/g; (D) MRM ion chromatogram of RJLP blank samples; (E) MRM ion chromatogram of RJLP spiked samples at 0.4 μg/g.

Table 2
Method validation data (linearity, intra-day precision, inter-day precision, limit of detection, limit of quantitation, recovery).

	RJ		RJLP
Linearity (n = 5)			
Linear range	0.01–8 μg/mL		0.05–10 μg/mL
Regression of coefficient (R^2)	0.9988 (0.001)[a]		
Slope		389.88 (9.42)	
Intercept		0.1778 (0.002)	
Intra-day precision (n = 6; RSD, %)			
0.01/0.05[b] μg/g	8.41		12.7
0.1 μg/g	6.80		9.56
5 μg/g	3.75		5.13
Inter-day precision (n = 5; RSD, %)			
0.01/0.05[b] μg/g	10.7		13.9
0.1 μg/g	7.51		8.56
5 μg/g	5.22		7.17
Limit of detection (μg/g)		0.01	
Limit of quantitation (μg/g)		0.03	
Recovery (n = 5, %)			
0.01/0.05[b] μg/g	93.2(2.97)		89.6(4.53)
0.05/0.1[c] μg/g	97.9(2.58)		93.1(4.20)
0.2/0.4[d] μg/g	100.4(1.92)		99.2(3.08)

[a] Standard error values are shown in parentheses.
[b] The spiked concentration of melamine is 0.01 μg/g for RJ, 0.05 μg/g for RJLP.
[c] The spiked concentration of melamine is 0.05 μg/g for RJ, 0.1 μg/g for RJLP.
[d] The spiked concentration of melamine is 0.2 μg/g for RJ, 0.4 μg/g for RJLP.

matrix effect to some extent. Matrix extract with low concentration (1 g/mL of RJ and 0.5 g/mL of RJLP) can decreased the matrix effect to some extent.

3.3.2. Linearity

Six matrix-matched calibration standards were prepared and subsequently analyzed in quintuplicate. Linear range was tested following the developed procedure in the MRM mode and the range studied extracts was 0.01–8 μg/mL in RJ and 0.05–10 μg/mL in RJLP for melamine. Good linearity was found for melamine, with coefficients of determination higher than 0.99 when RJ and RJLP extract was used as the matrix. The standard error (SE) of slope and intercept was 9.42 and 0.002, respectively. In fact, RJLP is processed directly from RJ by freeze-drying, so the same matrix-matched calibration was used except different linear range in this study.

3.3.3. Selectivity

Six different blank RJ and RJLP samples respectively spiked at the 0.01 and 0.05 μg/mL were prepared with and without melamine in order to determine whether endogenous and extraneous substance interfere at the mass transitions for melamine according to the developed method. Under optimized LC and mass spectrometry conditions, no trace of interference substance was found at the same retention time as melamine by analyzing the matrix blank and spiked samples extracts.

Representative chromatograms of matrix-matched standard solution, blank RJ and RJLP sample, and spiked RJ and RJLP sample with melamine standard solution extracted by ultrasonic-assisted extraction are shown in Fig. 3A–E.

3.3.4. Precision

Precision of the method is checked by determining the repeatability (intra-day) and intermediary precision (inter-day) of within-laboratory. The repeatability of the chromatographic determination was evaluated by injecting 0.01, 0.05, 0.1 and 5 μg/mL of matrix-matched melamine standard solution for six times within 1 day. Good relative standard deviations (RSD) were obtained, which ranged from 3.75 to 12.7%. The intermediary precision was also evaluated by injecting the same standard solution on five successive days and RSD values lower than 13% were obtained. For the intra-day and inter-day precision, the results were suitable for three (low, medium and high) concentration levels and considered adequate for other concentrations within linear range, which showed satisfactory results during the method validation.

3.3.5. Carryover

The carryover was measured by injecting extracted blank RJ and RJLP samples following the injection of the highest concentration level and checking for the presence of compounds in the blank samples. No carryover was observed during multiple injections of RJ and RJLP samples with different concentrations when the instrument was run in batch sequence mode.

3.3.6. Limits of detection and quantification (LOD and LOQ)

The limit of detection (LOD) was set as the lowest concentration of melamine that could be detected with a signal to noise ratio of 3:1. The limit of quantification (LOQ) was defined as the lowest concentration of the calibration samples that could be quantified with an acceptable level. LOD and LOQ samples were analyzed in duplicate and evaluated as unknown samples on five successive days using the quantitation transitions under the MRM mode. The LOD obtained was 0.01 μg/g and the LOQ was set at 0.03 μg/g for RJ and RJLP.

3.3.7. Recovery

Recoveries through the method were performed by spiking samples at three levels (0.01, 0.05, 0.2 μg/g for RJ and 0.05, 0.1, 0.4 μg/g for RJLP, respectively), then the extraction and clean-up procedure were carried out for spiked samples according to the developed method. The results are summarized in Table 2. Good recoveries ranging from 89.6 to 100.4% with low ion suppression with SE values <4.53 were obtained for melamine studied.

3.3.8. Application to real samples

The proposed method was applied to determine the levels of melamine in RJ and RJLP samples from supermarket and apiary. In thirty-five royal jelly and twenty lyophilized powder samples, trace of melamine residue was not found. The most important reason is likely to be the lack of more typical samples because it is difficult to obtain a lot of samples from China in a short time. So the further

study will focus on the collection of a large quantity of samples for us.

4. Conclusion

A method to quantify melamine in RJ and RJLP was developed and validated by means of ultrasonic-assisted extraction and SPE technique followed by HILIC–MS/MS. This method was found to be more sensitive and selective with advantages of rapidity and easiness than previously reported methods based on LC–UV. The LC-MS/MS matrix effects were also evaluated in RJ and RJLP samples after SPE and signal suppression was noticed. As a result, it is evident that the matrix-matched calibration plays an important role in compensating the matrix effect and the satisfactory recoveries were obtained. On the basis of the satisfactory validation data, the developed method was suitable for the routine monitor and screen of melamine residue in RJ and RJLP samples.

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