Highly sensitive determination of cyromazine, melamine, and their metabolites in milk by molecularly imprinted solid-phase extraction combined with ultra-performance liquid chromatography

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ABSTRACT

A novel molecularly imprinted solid-phase extraction–ultra-performance liquid chromatography (MISPE–UPLC) method for effective separation and simultaneous determination of cyromazine, melamine, and their metabolites (ammelide and ammeline) in milk samples was developed. Molecularly imprinted polymers (MIP) were synthesized in an ethanol–water system, with melamine as the template and methacrylic acid as the organic functional monomer. The MIP were applied as a specific sorbent for the selective solid phase extraction of cyromazine, ammelide, melamine and ammeline. The molecular recognition mechanism was investigated by molecular simulation and the experiment was validated by using Fourier transform infrared spectroscopy and ¹H nuclear magnetic resonance spectroscopy. A new mechanism based on the formation of both an amide group and hydrogen bonds was developed. A binding study demonstrated that the MIP showed excellent affinity to and high selectivity for melamine and related compounds. Under optimized conditions, we achieved good linearity of the calibration curves with correlation coefficients >0.999. Low limits of quantification (LOQ) for the method were determined to be 1.25, 1.25, 2.59, and 6.42 µg/kg for cyromazine, ammelide, melamine, and ammeline, respectively, which were 3 orders of magnitude smaller than the maximum residue limit (MRL). The high sensitivity of this method allows detection at the microgram per kilogram level. The proposed MISPE–UPLC method is a highly selective and sensitive method for determination of cyromazine, melamine, and their metabolites (ammelide and ammeline) for use in the control and quality assurance of milk.

Key words: ultra-performance liquid chromatography, molecularly imprinted solid-phase extraction, cyromazine, melamine, milk

INTRODUCTION

Bovine milk is one of the most important components of the human diet. The pesticide cyromazine can be metabolized to melamine during dealkylation reactions in both plants and animals. Melamine can be hydrolyzed to the metabolites ammelide and ammeline by microbes in the body (Cook and Hutter, 1981; Jutzi et al., 1982; Shelton et al., 1997). Battaglia et al. (2010) studied the transfer of melamine from feed to milk and from milk to cheese and whey. In recent years, use of cyromazine has caused actual environmental and human health problems (Wang et al., 2008). To ensure human food safety, China has set maximum residue limits (MRL) for cyromazine residue in the range of 0.5 to 1 mg/kg, depending on the particular vegetable (Chinese National Standard, 2012; no MRL for crops and products derived from crops), and the World Health Organization has set the MRL for melamine in powdered infant formula and in other foods and animal feed at 1 and 2.5 mg/kg, respectively (World Health Organization, 2010). Therefore, it is necessary to develop an effective and reliable method for quantifying cyromazine, melamine, and their residues in milk and dairy products.

Several papers have reviewed advances in analytical methodology of melamine in foods (Chu et al., 2010; Sun et al., 2010a; Wu and Zhang, 2013). Chromatographic methods are widely used for food analysis, and liquid chromatography (LC) is more attractive than GC because no preliminary derivatization procedures are required. A series of LC methods has been used for the determination of melamine alone in food (Filigenzi et al., 2007; Tittlemier et al., 2009; Sun et al., 2010b; Venkatasami and Sowa, 2010; Gosciniy et al., 2011; Filazi et al., 2012). For determination of cyromazine

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and melamine residues in food, 3 LC methods have been reported, with a limit of quantification (**LOQ**) of 0.02 to 0.06 mg/kg (Hwang et al., 2003; Sancho et al., 2005; Wei et al., 2009). A reversed phase–LC method was described for simultaneous analysis of melamine and its metabolite cyanuric acid in liquid milk and egg samples, with a limit of detection (**LOD**) of 6.2 to 11.5 µg/kg (Sun et al., 2009). Simultaneous detection of cyromazine, melamine, and their metabolites in animal origins samples was achieved by LC–UV detection, and an LOQ of 40 µg/kg was obtained (Yu et al., 2010). The determination of melamine, ammeline, ammelide, and cyanuric acid in rice and cereal flours by LC (Ehling et al., 2007; Muñoz-Valencia et al., 2008) and in food of animal origin by LC–tandem mass spectrometry (MS/MS; Filigenzi et al., 2008) has been reported, but the sensitivity of the methods needs to be improved.

Choice of analytical technique depends on the sample preparation method. Extraction and clean-up are 2 crucial steps that affect the LOD of the overall method. After solvent extraction, solid-phase extraction (**SPE**) has been used to clean extracts of melamine in food and biological samples using the Oasis MCX cartridge (Hercules, Beijing, China; Filigenzi et al., 2007; Tittlemier et al., 2009), PCX-SPE cartridges (Sun et al., 2010a), and Strata X-C cartridge (Lv et al., 2010), but recovery and precision need to be improved further. The classical SPE method has low selectivity and is time consuming. New sorbents such as molecularly imprinted polymers (**MIP**) have been developed to improve selectivity. Coupling MIP with SPE combines the advantages of molecular recognition with traditional separation methods. The application of molecularly imprinted SPE (**MISPE**) allows an analyte to be preconcentrated while removing interfering compounds from the matrix (Qiao et al., 2006). A MIP with 2,4-diamino-6-undecyl-1,3,5-triazine (DAUTA) as a mimic template was used for purification and concentration of cyromazine and melamine from milk samples with LOD of 0.05 and 0.12 µg/mL, respectively (Wang et al., 2011). Cyromazine-MIP were applied to SPE and matrix solid-phase dispersion for determination of melamine in milk and feed with LOD of 50 to 160 µg/kg (He et al., 2009; Yan et al., 2012). Melamine-MIP were applied for SPE of melamine from milk and dairy products, followed by LC–time-of-flight (TOF)–MS analysis, with LOQ of 60 µg/kg (Yang et al., 2009). Magnetic molecularly imprinted polymers have been synthesized by using melamine as the template molecule and Fe₃O₄ magnetite as the magnetic component for the determination of melamine in milk (Wang et al., 2013; He et al., 2014). The molecular recognition mechanisms based on the formation of hydrogen bonds were suggested for these MISPE and magnetic molecular imprinted SPE

(MMISPE; He et al., 2009, 2014; Yang et al., 2009; Yan et al., 2012; Wang et al., 2013). However, the target analyte of these MISPE and MMISPE methods is melamine, not cyromazine, ammeline, or ammelide. Thus, molecular recognition mechanisms need to be studied further.

The objective of this study was to prepare a melamine-imprinted polymer using melamine (**MEL**) as template molecule and methacrylic acid (**MAA**) as functional monomer, to validate the formation of the amido group and hydrogen bonds by Fourier transform infrared spectroscopy and ¹H nuclear magnetic resonance (NMR) spectroscopy, and to develop a new imprinted SPE-ultra-performance-liquid chromatography (UPLC) method for the determination of cyromazine, melamine, and their metabolites in milk.

MATERIALS AND METHODS

Instrumentation

The UPLC equipment was an Acquity UPLC H-Class system (Waters, Milford, MA), which consisted of an Acquity Quaternary Binary Solvent Manager, an Acquity Sample Manager-FTN, an Acquity diode array detector, and a high-temperature column heater. Empower III workstation was used as the data acquisition system. An Acquity UPLC BEH (bridged ethyl hybrid) HILIC (hydrophilic interaction chromatography) column (100 × 2.1 mm, 1.7 µm i.d.) was used as the analytical column and was connected to an inline precolumn. An Avance III 500 MHz NMR spectrometer (Bruker, Fällanden, Switzerland), WQF-510A Fourier-transform infrared spectrometer (Beifen-Ruili, Beijing, China), and JSM-7500 scanning electron microscope (Jeol, Tokyo, Japan) were used to observe the images of MIP. A TGL-16M centrifuge (Xiangyi Centrifuge Co., Hunan, China) and RE-2000A rotary evaporator (Yarong Biochemistry Instrument Co., Shanghai, China) were used in sample preparation.

Chemicals and Reagents

Cyromazine, melamine, ammeline, and ammelide (>99.5% purity for each) were obtained from Hebei Institute of Food Quality Supervision Inspection and Research (Shijiazhuang, China). Methacrylic acid (purified by distillation to remove inhibitor) and azodiisobutyronitrile (**AIBN**) of analytical grade were purchased from Tianjin Kermel Chemical Reagents Centre (Tianjin, China). Methanol (HPLC grade), acetonitrile (HPLC grade), ethanediamine (analytical grade), and acetic acid (analytical grade, 36%) were purchased from Dikma Technologies Inc. (Tianjin, China). Ethyl-

ene glycol dimethacrylate (**EGDMA**) was from Sigma-Aldrich (St. Louis, MO). Solid-phase extraction empty tube and sieve plate were purchased from Bonna-Agela Technologies Inc. (Tianjin, China).

Mobile phase A was prepared by dissolving 0.77 g of ammonium acetate in 1,000 mL of water, and adjusting the pH to 4.2 using acetic acid. Mobile phase B was acetonitrile. Cyromazine and melamine stock standard solutions, 1 mg/L, were prepared by dissolving cyromazine and melamine, respectively, in acetonitrile, and were stored at 4°C in amber glass bottles. Ammeline and ammelide stock standard solutions, 1 mg/L, were prepared by dissolving them in a mixture of water:acetonitrile (30:70, vol/vol), and were stored at 4°C in amber glass bottles. Fresh working standard solutions were prepared daily by diluting the stock solution with the mobile phase for different studies. The solution and methanol were filtered through 0.22- μ m microporous, polyvinylidene fluoride membranes before use. Distilled water was further purified by a Molelement 1820a ultrapure water apparatus (Molecular Devices Limited, Shanghai, China), and then filtered through a 0.4- μ m microporous membrane of mixed cellulose ester.

Preparation of MIP

The MEL-imprinted polymers were prepared by thermal-initiated polymerization within a 50-mL thick-walled glass conical flask, which was equipped with a ground glass stopper. The polymerization mixture consisted of 1.0 mmol of melamine, 9.0 mmol of MAA, 20 mmol of EGDMA, and 40 mg of AIBN dissolved in 12 mL of solvent (ethanol:water, 4:1, vol/vol). The solution was sonicated for 10 min and purged with nitrogen gas for 15 min before being sealed under a nitrogen stream. The sealed flask was placed in a thermostatically controlled water bath at 60°C for 24 h. After polymerization, the macroporous monolith was ground and sieved through 38–74 μ m (200–400 mesh) sieves, and then the imprinted particles were suspended in acetone to remove the small particles. The product was dried under vacuum at room temperature. The obtained particles were extracted with ethanol containing 10% acetic acid to remove the templates for 48 h, and then dried in a vacuum drying oven at 50°C and stored in a desiccator at ambient temperature before use. A nonimprinted blank polymer (**NIP**, in the absence of template) was prepared and treated in an identical manner.

Determination of Binding Capacity of the MIP

To investigate the binding property of the synthesized MIP, static absorption and Scatchard analysis were

used in this study. Twenty milligrams of the polymer (MIP or NIP) and 1 mL of different concentrations of melamine solution (0.2–3.0 mM) were placed in a 10-mL glass flask, and dissolved in methanol:water (8:2, vol/vol). The flasks were sealed and placed in the dark at room temperature for 48 h. Then, the solutions were centrifuged and filtered, and the free concentrations were quantified by UPLC at 235.7 nm. The equilibrium adsorption capacity (Q) was calculated according to the following equation: $Q = V(C_o - C_e)/m$, where V is the volume of solution (mL); m is the mass of the MIP; and C_o and C_e are the initial and the equilibrium concentrations of melamine in methanol:water (80:20, vol/vol) solution, respectively.

To evaluate the specific rebinding of melamine, the MIP cartridge was reused following regeneration with 10 mL of methanol:acetic acid (90:10, vol/vol). We tested 5 recyclings of the MIP cartridge.

Sample Preparation

Ten milliliters of milk purchased from a local market was transferred to a 50-mL centrifuge tube. After centrifugation at $10,733 \times g$ for 10 min at 4°C, the fat on the upper layer was removed. Then, 10.0 mL of acetonitrile was added, and the tube was thoroughly shaken for 2 min on a vortex to precipitate the proteins. After a second centrifugation at $10,733 \times g$ for 10 min at 4°C, the obtained supernatant was concentrated to 3 mL by using a rotary evaporator at 45°C for 10 min. Both blank samples and spiked samples were pretreated by this method.

MISPE Procedure

A MISPE column was prepared by packing MIP (100 mg) into an empty SPE cartridge (60 mm $\times$ 8.0 mm, i.d.). The cartridge was capped with 2 fritted polyethylene sieve plates at the bottom and top ends. The MISPE column was preactivated with 3 mL of methanol:acetic acid (90:10, vol/vol) and equilibrated with 3 mL of water. After the 3 mL of concentrated extract was passed through the cartridge at a flow rate of 0.3 mL/min, the cartridge was equilibrated for 30 min to complete interaction and adsorption with the analytes. The loaded cartridges were washed in turn with 3 mL of water and 3 mL of methanol, and the eluate was discarded. The analytes in the cartridge were eluted with 3 mL of methanol:acetic acid (70:30, vol/vol). The eluate was evaporated to dryness under a stream of nitrogen at 45°C. The obtained residue was redissolved in 1.0 mL of acetonitrile:water (94:6, vol/vol) for UPLC analysis. The cartridges were regener-

ated with 10 mL of methanol:acetic acid (90:10, vol/vol) before subsequent studies.

UPLC Analysis

After the UPLC column was conditioned with a mobile phase of 1 mM ammonium acetate-acetic acid solution (pH = 4.2):acetonitrile (6: 94, vol/vol), a 10- μ L volume of sample solution was injected into the column and eluted with the mobile phase at 0.4 mL/min and 35°C. Melamine, cyromazine, ammeline, and ammelide were detected at 235.7, 239.2, 234.0, and 221.0 nm, respectively. Matrix-matched calibration curves for the relationship between concentrations of analytes and their peak areas were calculated by using weighted least-squares regression.

RESULTS AND DISCUSSION

Polymerization of MIP

To obtain MIP with higher specific recognition ability for target molecule in water environment, different monomers such as MAA and acrylamide were investigated. Polymers prepared with MAA as a functional monomer showed better molecular recognition ability under polar conditions than did acrylamide. Due to the functional monomer having 2 or more dual-hydrogen bonds, 4 molar ratios between template and monomer of 1:4, 1:6, 1:8, and 1:10 were tested in this experiment.

After optimization, a molar ratio of 1:9:20 (template: MAA: EGDMA) was used to prepare the water-compatible MIP. Additionally, thermal-initiated polymerization was chosen in this work due to the degradation of melamine caused by UV irradiation. Because of solubility limitations of melamine, melamine-imprinted polymer could not be synthesized successfully in a non-polar solution. Coupled with restrictions on the mechanical strength of the MIP, only the melamine MIP prepared in the ethanol-water system with a 1:9 molar ratio between template and functional monomer was successful.

Recognition Mechanism of the MIP: Molecular Simulation

To predict the interaction sites between melamine and MAA, the HyperChem V7.0.1 program (Science Plus, Groningen, the Netherlands), a sophisticated molecular modeling program for exploring quantum chemical calculations, was used in this work.

Theory of PM3 semiempirical level was used as the computation model with chemically useful accuracy for molecules with hydrogen bonds and amido bonds. Based on this, the structures of the associated complexes of melamine with MAA were optimized (Figure 1).

The changes (ΔE) in bond energy (E) were calculated according to equation $\Delta E = E_{\text{MIP}} - E_{\text{template}} - \Sigma E_{\text{monomer}}$ for binding by hydrogen bond, and equation $\Delta E = E_{\text{MIP}} + \Sigma E_{\text{water}} - E_{\text{melamine}} - \Sigma E_{\text{MAA}}$ for binding

Figure 1. Structures of molecularly imprinted polymers (MIP) formed with 1:3 melamine:methacrylic acid. (a) H...O bond; (b) N...H bond; (c) amido group. Color version available online.

Table 1. Configuration of hydrogen bond and amido group, molar ratio of monomer and template, and bond energy (E ; kcal/mol) of monomer, template, and molecularly imprinted monomer (MIP)

Configuration		Molar ratio	E_{MIP}	E_{water}	E_{MEL}	E_{MAA}	$\Delta E (E_{\text{MIP}} - E_{\text{water}} - E_{\text{MEL}} - E_{\text{MAA}})$
Hydrogen bond state	N...H-O	1:1	-2658.6006		-1474.9246		-11.206
		1:2	-3838.8077			-1172.4701	-18.943
		1:3	-5018.4757				-26.141
	H...O	1:1	-2651.5536		-1474.9246		-4.159
		1:2	-3823.9776			-1172.4701	-4.113
		1:3	-4996.3578				-4.023
Amido group	$\begin{array}{c} \text{H} \quad \text{O} \\ \quad \\ -\text{N}-\text{C}- \end{array}$	1:1	-2502.2820	-217.2217	-1474.9246		-72.109
		1:2	-3451.3004			-1172.4701	-65.879
		1:3	-4399.9010				-59.231

¹ E_{MIP} , E_{water} , E_{MEL} , and E_{MAA} = bond energies of molecularly imprinted monomer, water, melamine, and methacrylic acid, respectively; ΔE = change in bond energy.

by amido bond. It was used to judge whether the simulated molecule was in the most stable situation. The bond energy E of each potential compound was computed, and the obtained ΔE values are given in Table 1. Moreover, length and angle of hydrogen bond and the angle defined by the 2 heavy atoms and hydrogen (donor atom-H-acceptor atom) were computed.

Absolute ΔE values showed that the possibility of formation of the MIP via hydrogen bond of H...O (Figure 2a) was small due to very small $|\Delta E|$, and the MIP formed via amido group (Figure 2c) was the most stable formation. When the distance between the heavy atoms fell within the sum of their van der Waals radii, and the angle defined by the 2 heavy atoms and hydrogen (donor atom-H-acceptor atom) was $>90^\circ$ (Körtvélyesi and Pálíncó, 2000), the hydrogen bond can be confirmed. The computed hydrogen bond length for N...H-O was 1.78, 1.78, and 1.78 Å for 1:1, 1:2, and 1:3 (melamine:MAA), respectively, which is within the sum (2.90 Å) of their van der Waals radii, and the computed angle for N...H-O was 172° ($>90^\circ$). Therefore, the MIP can be formed via hydrogen bond of N...H at the site of N on triazine rings (Figure 2b). Moreover, the hydrogen atom in the amino group of MEL and the hydroxide group in carboxyl group of MAA could be combined to be water, forming an amido group (Figure 2c) and having high $|\Delta E|$ values. Based on the above analysis, it is predicted that MIP with hydrogen bond of N...H and MIP with amido group structure could be formed, and the MIP with amido group structure was most stable.

Recognition Mechanism of the MIP: Experimental Validation

Fourier transform infrared (FTIR) spectroscopy is a suitable method to determine the functional groups and types of bonds present in MIP. Melamine and MAA were used as reference to confirm that polymerization and interaction with template had occurred. The FTIR

spectra for melamine, pure monomer, and MIP are shown in Figure 2.

From Figure 2, the C=N stretch vibrations showed a medium band for melamine compound at $1,632 \text{ cm}^{-1}$, whereas the intensity of this vibration band for MIP disappeared due to overlapping with C=O absorptions. The C=C stretch vibrations showed a very strong band for MAA at $1,636 \text{ cm}^{-1}$, whereas intensity of this vibration band for MIP was weak due to overlapping with C=O absorptions (Yusof et al., 2013). The presence of the broad transmittance peak of -COOH- in the MAA sample was clear, and antisymmetric stretching vibration peaks of N-H of melamine at 3,471 and $3,419 \text{ cm}^{-1}$ were also observed. However, in case of MIP, the vibration peak of N-H disappeared and a broad absorption band at $3,507 \text{ cm}^{-1}$ appeared, which suggested hydrogen bonding of O on the carbonyl group of MAA with H on amino group of melamine. The presence of 2 significant peaks around $1,727 \text{ cm}^{-1}$ (C=O stretching) and $1,145 \text{ cm}^{-1}$ (C-O stretching) supports the existence of poly(EDGMA) as a cross-linker in the obtained MIP (Pan et al., 2009). We found that the flex liberation peak of N-H in melamine at $1,652 \text{ cm}^{-1}$ completely disappeared. At the same time, the stretching vibration peak of R-CONH-R at $1,596 \text{ cm}^{-1}$ appeared. This finding reveals that the amino of melamine had been combined with the carboxyl group of MAA to form an amido group.

In this work, the amido group formed between melamine and MAA was investigated further by ^1H NMR spectra. The ^1H NMR spectra of melamine, MAA, and their complex are shown in Figure 3. The chemical shift (δ) of H on amino of melamine was $\delta 4.839 \text{ ppm}$, and the chemical shift of H on hydroxyl of MAA was $\delta 11.827 \text{ ppm}$. The H of the amino group of melamine with the hydroxyl in carboxyl group of MAA formed the amido group, resulting in a chemical shift of the H of melamine from $\delta 4.839$ to $\delta 6.015 \text{ ppm}$, and the H on hydroxyl of MAA at $\delta 11.827 \text{ ppm}$ disappeared. This

Figure 2. Infrared and ^1H nuclear magnetic resonance (NMR) spectra of melamine (MEL), methacrylic acid (MAA), and molecularly imprinted polymer (MIP) Fourier-transform infrared scans in the region between 500 cm^{-1} and $4,500\text{ cm}^{-1}$. Color version available online.

finding shows that the amido group was formed due to the hydroxyl of MAA could combine with a hydrogen atom of the amino group of melamine to form water.

Dual-hydrogen bonds and amido group are expected to be formed between melamine and MAA as a key interaction necessary for binding site construction, whereby a carboxylic group of MAA works as both a hydrogen bond acceptor and a donor interacting with a hydrogen atom of the amino group and a nitrogen atom of the triazine body, respectively (Welhouse and Bleam, 1993). In our work, we showed that, in addition to hydrogen bonding, amido bonding played a significant role in the recognition of melamine–MIP systems.

Binding Capacity of the MIP

To estimate the binding properties of the MIP, static absorption experiments at room temperature were used and the data were further processed by Scatchard analysis based on the equation $Q/C_{\text{free}} = (Q_{\text{max}} - Q)/K_D$, where Q is the amount of melamine bound to the MIP at equilibrium, Q_{max} is the maximum binding capacity, C_{free} is the free concentration of analyte at equilibrium, and K_D is the dissociation constant.

Figure 3 shows experimental binding isotherms of MIP and NIP versus melamine concentrations. Within the melamine concentration range, MIP exhibited a

Figure 3. Binding isotherm for molecularly imprinted polymer (MIP) and nonimprinted blank polymer (NIP). Q = the amount of melamine bound to the MIP at equilibrium; C_{initial} = the initial concentration of analyte at equilibrium. Color version available online.

Figure 4. Scatchard plot of molecularly imprinted polymers (MIP) over a concentration range from 0.2 to 3.0 mmol/L. Q = the amount of melamine bound to the MIP at the equilibrium; C_{free} = the free concentration of analyte at equilibrium. Color version available online.

higher capacity for melamine than did NIP. The weak adsorption of template on NIP is due to nonspecific interaction with the polymer matrices. As shown in Figure 4, 2 distinct sections within the plot can be seen as straight lines, indicating that the binding sites can be classified into 2 distinct groups with different specific binding properties: one is amido bond and the other is intermolecular forces. The respective K_D and Q_{max} values can be calculated according to the slopes and intercepts of the 2 linear portions of the Scatchard analysis. The results are shown in Table 2.

Optimization of MISPE Conditions

To completely eliminate matrix interferences and concentrate the analytes, we investigated the effect of washing and eluting conditions of MISPE. The purpose of the wash step is to remove all interfering compounds from the complex milk matrix without eluting out the target analytes. Different volumes of water, methanol, and acetonitrile in the range of 1.0 to 6.0 mL were in-

vestigated. The interfering components were removed, and cyromazine, melamine, ammeline, and ammelide were fully retained when using 3 mL of water and 3 mL of methanol, so this combination was chosen as the washing solvent.

The nature of the elution solvent is also important because the target analytes should be efficiently desorbed while other matrix components are retained in the cartridge. To achieve the best recovery, we evaluated a series of acidic elution solutions, including methanol:acetic acid (70:30, vol/vol), ethyl acetate:acetic acid (70:30, vol/vol), and acetonitrile:acetic acid (70:30, vol/vol), and the obtained recoveries are listed in Table 3. The best recoveries (74.6–100%) were obtained when using 3.0 mL methanol:acetic acid (70:30, vol/vol) as the eluting solution.

The synthesized MIP showed high selectivity, and we observed no interference from the matrix. Blank samples were treated under the same conditions and no melamine peak was observed in the chromatogram at the same retention time, which indicated that all tem-

Table 2. Result of Scatchard analysis of molecularly imprinted polymers (MIP; $n = 3$)¹

Binding site	Linearity	r	K_D (mmol/L)	Q_{max} (mmol/g)	RSD (%)
Higher affinity site	$Q/C_{\text{free}} = 546.02 - 18.60Q$	0.986	0.054	0.0295	≤3.1
Lower affinity site	$Q/C_{\text{free}} = 78.18 - 1.55Q$	0.997	0.645	0.0504	≤2.7

¹ Q is the amount of melamine bound to the MIP at equilibrium; C_{free} is the free concentration of analyte at equilibrium; K_D = dissociation constant; Q_{max} = maximum binding capacity; RSD = relative standard deviation.

Table 3. Recoveries of the 4 analytes with different elution solvents (n = 3)

Analyte	Ethyl acetate:acetic acid (70:30, vol/vol)		Acetonitrile:acetic acid (70:30, vol/vol)		Methanol:acetic acid (70:30, vol/vol)	
	Recovery (%)	RSD ¹ (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Cyromazine	91.2	2.3	97.0	2.6	98.9	1.9
Melamine	92.7	2.9	98.8	2.1	100.1	2.3
Ammeline	78.1	3.7	84.7	4.3	83.5	3.0
Ammelide	63.4	4.1	73.6	3.1	74.6	3.7

¹Relative standard deviation.

plates were already removed from the polymer matrix and no template leakage occurred during MISPE.

The recycling experiments showed that MIP had very good performance. The recoveries of MEL were >90% even though each MIP cartridge was used up to 5 times. Thus, the selectivity of MIP to MEL is specific and stable.

Optimization of UPLC Separation Conditions

Because melamine, cyromazine, ammeline, and ammelide are small and highly polar molecules, it is difficult to achieve sufficient retention using traditional C18 columns. The separation condition of these analytes was investigated by using an Acquity UPLC BEH HILIC column and ammonium acetate-acetic acid solution:acetonitrile as mobile phases.

The pH of ammonium acetate solution influences the stability and ionization of analytes. Cyromazine could be hydrolyzed at pH ≤2 to melamine, and the dissociation constants pK_a are 5.0 for melamine, 4.5 and 9.4 for ammeline, and 1.8 and 6.9 for ammelide (Sun et al., 2009b). Therefore, the pH of ammonium acetate solution was adjusted to 4.0 to 5.3 with acetic acid. The effect of pH on the retention time and peak area of the analytes was investigated.

Figure 5 shows no obvious change in retention time in a pH range of 4.0 to 5.3, but the peak areas of cyromazine and melamine increased with decreasing pH due to the increase in protonation. The best response for the analytes was achieved at pH 4.2, which was used in further work.

We also investigated the effect of the concentration of ammonium acetate solution on retention time and peak area of the 4 analytes. We observed no obvious effect of ammonium acetate concentration in the range of 0.5 to 2.0 mM on retention times and peak areas of the 4 analytes. Therefore, 1.0 mmol/L ammonium acetate solution was used in subsequent work. We then investigated effects of different proportions of ammonium acetate solution in the mobile phase on peak area and retention time of the 4 analytes in and isocratic elution

program. The isocratic elution program with 1 mM ammonium acetate-acetic acid solution (pH 4.2):acetonitrile (6:94, vol/vol) as a mobile phase was favorable for the analysis.

Performance of the Method: Specificity

After MISPE for several milk samples, cyromazine, melamine, ammeline, and ammelide were selectively separated, and no interference from the milk matrices was observed at the retention time of target analyte.

Figure 5. Effect of pH on retention time and signal response (absorbance, arbitrary units). For each pH value in the lower panel, peak (bar) 1 = ammelide, 50 µg/kg; peak 2 = cyromazine, 50 µg/kg; peak 3 = melamine, 65 µg/kg; peak 4 = ammeline, 250 µg/kg. Signal response is the average value of 3 measurements and contains less than 3% relative standard deviation. Color version available online.

Figure 6. Chromatograms of (A) blank milk sample, (B) blank milk sample treated by molecularly imprinted solid-phase extraction (MISPE), and (C) spiked milk sample treated by MISPE, generated using an Acquity ultra-performance liquid chromatography BEH hydrophilic interaction chromatography (HILIC) column (Waters, Milford, MA); mobile phase = 2 mmol/L ammonium acetate-acetic acid solution (pH = 4.2):acetonitrile (94:6, vol/vol). Spiked level: 1 = ammelide, 2.0 $\mu\text{g/kg}$; 2 = cyromazine, 2.0 $\mu\text{g/kg}$; 3 = melamine, 5.0 $\mu\text{g/kg}$; 4 = ammeline, 10.0 $\mu\text{g/kg}$.

Without MISPE, cyromazine and milk matrices could not be separated. Figure 6 shows a representative chromatogram for blank milk and spiked milk after MISPE. The method demonstrated acceptable selectivity of the synthesized MIP to cyromazine, melamine, ammeline, and ammelide. The study of matrix effects for the 4 analytes in a concentration range of 13 to 200 $\mu\text{g/L}$ in

milk showed that the retention times of the 4 analytes in solvent and matrix were in agreement.

Performance of the Method: Linearity, Detection Limit, and Precision

Under optimized conditions, we evaluated the linearity of matrix-matched calibration curves for the analysis of spiked real milk samples with the measured peak areas of the spiked standards against their concentrations (number of points, $n = 7$). The linear regression equations are listed in Table 4, which show good linearity for each analyte, with correlation coefficients (r) > 0.999 .

The LOD was determined as the sample concentration that produced a peak of height 3 times the level of the baseline noise, and the LOQ was calculated as the sample concentration that produced a peak of height 10 times the ratio of signal to noise. The LOD and LOQ values for instrument and method are given in Table 4.

The instrument (solution) LOD of melamine, cyromazine, ammeline, and ammelide ranged from 4 to 20 $\mu\text{g/L}$, and their LOQ ranged 13 and 66.7 $\mu\text{g/L}$. The relative standard deviation was 2.2 to 4.6% for 11 determinations of the 4 analytes at 10 $\mu\text{g/L}$ for each.

For 10-mL milk samples and 1-mL final solution, the method LOD was in the range of 0.4 to 2 $\mu\text{g/L}$ and the method LOQ was in the range of 1.3 to 6.7 $\mu\text{g/L}$. Considering the sample weight, the method LOD was in the range of 0.38 to 1.9 $\mu\text{g/kg}$, and the method LOQ were 1.25, 1.25, 2.59, and 6.42 $\mu\text{g/kg}$ for cyromazine, ammelide, melamine, and ammeline, respectively. The LOQ values are 3 orders of magnitude smaller than the MRL (World Health Organization, 2010; Chinese National Standard, 2012). Moreover, the sensitivity of the method was higher than those of the LC method (Hwang et al., 2003; Sancho et al., 2005; Ehling et al., 2007; Muñiz-Valencia et al., 2008; Sun et al., 2009; Yang et al., 2009; Venkatasami and Sowa, 2010; Yu et al., 2010; Wang et al., 2011; Filazi et al., 2012; Yan et al., 2012), LC-MS/MS method (Filigenzi et al., 2008; Sun et al., 2009; Yu et al., 2010; Gosciniy et al., 2011),

Table 4. Linear regression equation, correlation coefficient (r), linearity range, relative standard deviation (RSD), limit of detection (LOD), and limit of quantification (LOQ) for the 4 analytes

Analyte	Regression equation ¹	r	Linearity range ($\mu\text{g/mL}$)	RSD (%) ($n = 5$)	Solution		Method	
					LOD ($\mu\text{g/L}$)	LOQ ($\mu\text{g/L}$)	LOD ($\mu\text{g/L}$)	LOQ ($\mu\text{g/L}$)
Melamine	$A = 3,212.95C + 1,744.36$	0.9998	0.027–2.7	3.7	8.0	27	0.8	2.7
Cyromazine	$A = 2,496.82C + 4,706.68$	0.9991	0.013–1.3	2.5	4.0	13	0.4	1.3
Ammeline	$A = 1,579.13C - 4,270.42$	0.9994	0.067–6.7	2.2	20	67	2.0	6.7
Ammelide	$A = 2,821.71C - 958.05$	0.9999	0.013–1.3	4.6	4.0	13	0.4	1.3

¹A = peak areas; C = analyte concentration.

Table 5. Determination of 4 analytes in spiked milk samples

Analyte	Spiked level ($\mu\text{g/kg}$)	Measured ($\mu\text{g/kg}$)	Recovery (%)	RSD ¹ (%) (n = 3)
Melamine	5	4.87	97.4	3.1
	30	30.1	100	5.9
	100	98.5	98.5	1.7
Cyromazine	2	1.98	99.0	2.2
	10	9.83	98.3	4.7
	50	48.9	97.8	3.6
Ammeline	10	7.76	77.6	4.5
	50	41.9	83.8	3.7
	100	80.9	80.9	4.9
Ammelide	2	1.43	71.5	3.3
	10	68.1	70.1	6.9
	50	35.1	70.2	4.1

¹Relative standard deviation.

and LC–time-of-flight/MS (Yang et al., 2009) as well as standard methods (Chinese National Standard, 2008).

Analysis of Spiked Milk Samples

The MISPE–UPLC method was used to analyze cyromazine, ammelide, melamine, and ammeline in milk samples. To investigate the effect of sample matrix on the accuracy, a recovery experiment was carried out by spiking 3 levels of the analytes into milk samples. The results are given in Table 5. Recoveries of analytes in milk samples varied from 70.1 to 100% with a relative standard deviation of 1.7 to 6.9%, indicating that the proposed MISPE–UPLC method has feasibility for rapid determination of these analytes in complex samples, even in the presence of relatively high concentrations of potential interferents.

CONCLUSIONS

Melamine molecularly imprinted polymers prepared in a water-methanol system showed high affinity and selectivity to melamine, cyromazine, ammeline, and ammelide. In addition to hydrogen bonding, an amido bond was a dominant feature in the specific recognition of melamine-MIP. Under optimal conditions, melamine, cyromazine, ammeline, and ammelide were selectively retained and all matrices interferences were eliminated simultaneously. The proposed MISPE–UPLC method could be applied for selective extraction and highly sensitive determination of cyromazine, melamine, and their metabolites (ammelne and ammelide) at the microgram per kilogram level in complex milk samples.

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