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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 (ammeline and ammelide) 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 validate by using Fourier transform infrared spectroscopy and ^{1}H nuclear magnetic resonance spectroscopy. A new mechanism based on the formation of both an amido 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 (ammeline and ammelide) 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 ammeline and ammelide 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; Goscinny 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; Muñiz-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 Fe_3O_4 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 ^{1}H 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-