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A sensitive and validated method for determination of melamine residue in liquid milk by reversed phase high-performance liquid chromatography with solid-phase extraction

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ABSTRACT

A sensitive and validated method for the determination of melamine residue in liquid milk is developed using reversed phase high-performance liquid chromatography-diode array detection (RP-HPLC-DAD) with solid-phase extraction (SPE). The conditions of the extraction, SPE and HPLC were investigated and optimized. The linearity is satisfactory in the range of 0.1–50 μg/mL with a correlation coefficient of 0.9998. Under the optimal conditions, the method limit of detection (LOD) and method limit of quantification (LOQ) were 18 μg/kg and 60 μg/kg, respectively. The recovery of melamine for milk samples spiked with 0.10–3 mg/kg was in the range of 85.5–99.3% with the RSDs ($n = 3$) of 2.3–3.7%. The intra-day assay precision (RSD) was 5.6% for five replicates of quality control milk sample at 2 mg/kg level. Confirmation of the identities of melamine was achieved by monitoring the two transitions in multiple-reaction monitoring (MRM) mode, and has been applied successfully for the determination of melamine residue in liquid milk samples. The confirmatory method can permit the detection of melamine residues at levels as low as 60 μg/kg in different liquid milks.

1. Introduction

Melamine has been found in animal food because these animals were fed melamine-contaminated feeds. In 2008, melamine has caused the death of certain infants in China, who had drunk milk contained this compound. Certain liquid milk and milk powder were adulterated with melamine to increase their total nitrogen concentration. Melamine as the environmental contaminants has caused the attention in the world. It is important to monitor melamine in raw milk and milk products.

A series of analytical techniques are available to determine melamine in a variety of pet foods and animal feeds. Recently melamine in pet food was determined by Kim et al. (2008) using enzyme immunoassay, high-performance liquid chromatography (HPLC) and ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS). Analysis of melamine and related compounds in human foods is an important work for health safety. HPLC is more attractive than GC because no preliminary derivatization procedures are required. Muñiz-Valencia et al. (2008) developed a simple HPLC method for the determination of melamine and its degradation products in rice protein concentrate with the decision limit of 65 μg/g and detection capability of 75 μg/g for melamine. A method for the determination of melamine residue in plant origin protein powders was developed by Ding et al. (2008) using HPLC-diode array detection (HPLC-DAD) for preliminary screening of the samples for melamine with an LOQ of 10 mg/kg and HPLC–MS/MS was used in the confirmatory of melamine with LOQ of 0.5 mg/kg. An isotope dilution LC–MS method was reported for determine melamine and cyanuric acid in catfish, pork, chicken, and pet food with LOD of 10 μg/kg (Varelis & Jeskelis, 2008). HPLC–MS/MS method has been used for the quantification and confirmatory of melamine in catfish, trout, tilapia, salmon and shrimp with LOD of 3.2 μg/kg (Andersen et al., 2008), porcine muscle tissue with LOD of 1.7 μg/kg (Filigenzi, Tor, Poppenga, Aston, & Puschner, 2007), chard with LOD of 10 μg/kg (Sancho, Ibañez, Grimalt, Pozo, & Hernandez, 2005), and kidney tissue (Filigenzi, Puschner, Aston, & Poppenga, 2008). Recently, Feng et al. (2008) established an LC–MS/MS method for determination of melamine and cyromazine residues in milk and dairy products with the LOD of 10 μg/kg. Solid-phase extraction (SPE) with OASIS MCX column was used for clean-up of pet food prior to HPLC (Wang et al., 2008), and for clean-up of the foods prior to HPLC–MS/MS detection (Liu et al., 2008). Recently, Cleanert PCX SPE column is popular, and frequently used for separation and pre-concentration purposes to detect

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melamine in feet and milk powder by liquid chromatography–quadrupole mass spectrometry (Ming & Wang, 2008), and in raw milk and dairy products by HPLC (China National Standardizing Committee, 2008a, 2008b). Agela Technologies has developed an effective, improved and reliable method for determining melamine in raw milk, milk powder and dairy products by using the solution kit containing Cleanert PCX SPE column and Venusil ASB C8 chromatographic column (Beijing Agela Sci-Tech. Company Limited, 2008). An interim method for the determination of melamine residue in foods using LC–MS/MS was proposed by the Food and Drug Administration (FDA) (Smoker & Krynitsky, 2008), and the method limit of quantification (LOQ) for melamine was 25 μg/kg for tissue and liquid formula and 200 μg/kg for dry infant formula products. This method is the principal analytical method for detection and quantification of melamine in foods. To our knowledge, there are few reports for the determination of melamine in liquid milk and milk products. Recently, a national standard method (GB/T 22400–2008) for rapid determination of melamine in raw milk using LC has been issued in China with the limit of detection of 50 μg/kg (China National Standardizing Committee, 2008a, 2008b). A HPLC method presented by Yan et al. (2008) and a hydrophilic interaction chromatography–electrospray ionization–MS/MS method reported by He, Liu, Huang, Yang, and Liao (2008) were applied for the determination of melamine in milk powder and dairy milk with LOQ of 1 mg/kg and 50 μg/kg, respectively. It is of critical importance to develop simple and sensitive methods for melamine detection in food systems information on toxicity of melamine and on the levels of melamine compounds in edible tissues would be useful for future assessments.

To meet detection need for melamine contaminated, a reliable and simple SPE–HPLC method was developed in our work for the determination of melamine in liquid milk with method LOD of 18 μg/kg and LOQ of 60 μg/kg. The proposed method was validated by LC–MS/MS, and has been applied successfully for routine determination of melamine of different milk samples.

2. Materials and methods

2.1. Chemicals and reagents

Cleanert PCX-SPE cartridges (3 mL/60 mg) were obtained from Beijing Agela Technologies Company (Beijing, China). Sodium *n*-heptanesulfonate (chromatographic grade) was used as an ion pair reagent. Melamine was purchased from Kermel Chemical Reagents Development Center (Tianjin, China). An individual stock standard solution, 1000 μg/mL, was prepared by dissolving the melamine in a mixture of methanol and water (1:4, v/v), and was stable for al least 1 month if stored at 4 °C. A fresh working standard solution was prepared daily by diluting the stock solution with a mixture of methanol and water (1:4, v/v) for different studies. The solution was filtered through 0.45 μm microporous membrane of mixed cellulose ester. The acetonitrile was filtered through 0.22 μm microporous membrane of polyvinylidene fluoride before used. A 10 mM sodium *n*-heptanesulfate (pH adjusted with citric acid)–acetonitrile (83:17, v/v) were used as mobile phase for HPLC. All the reagents were of analytical grade except for additional illustration. Doubly distilled water obtained from quartz distillation apparatus.

2.2. Instrument

The HPLC equipment was a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) with a binary pump, a gradient controller (SCL-10Avp), an on-line-degasser (DGU-12A), a column thermostat (CTO-10Avp), and a diode array detector (SPD-M10Avp). CLASS-VP workstation was used as the data acquisition system. The analytical column was a ZY1104 C18 column (250 × 4.6 mm I.D. 5 μm). An ultrasonic cleaner (Ultrasonic Instrument Co., Ltd., Kunshan, China) and PHS-3C pH meter (Shanghai precision & scientific instrument Co., Ltd., Shanghai, China) were used in sample treatment. An TGL-16 M centrifuge (Xiangyi Centrifuge Co., Ltd., Hunan, China) was used in sample treatment.

Confirmation of melamine in milk sample was performed by LC–MS/MS (Thermo Electron Corp., San Jose, CA, USA) consisting of a Surveyor MS pump with an on-line-degasser, a Surveyor autosampler, and a TSQ Quantum triple stage quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source operated in positive ion mode. LC separations were performed on an SeQuant ZIC HILIC column, 2.1 × 150 mm PEEK, 5 μm (The Nest Group) a guard column (2.1 mm × 12.5 mm) that was at 30 °C. LC mobile phase was composed of 10 mM ammonium acetate and acetonitrile (90:10, v/v) at a flow rate of 0.2 mL/min. The injection volume was 10 μL and between injections, the needle was rinsed with methanol. TSQ Quantum mass spectrometer was calibrated with a solution of polytyrosine-1,3,6 according to the manufacturer. For method development, a standard solution containing 1 μg/mL of melamine was infused at 10 μL/min with 200 μL/min mobile phase into the ESI source. The optimized source parameters were as follows: sheath gas pressure, 20 (arbitrary units); auxiliary gas flow, 30 (arbitrary units); spray voltage, 4000 V; capillary temperature, 350 °C; tube lens offset, 78 V; and source collision-induced decomposition (CID), 10 V. For quantification, the mass spectrometer was set to the data acquisition mode of multiple-reaction monitoring (MRM). The acquisition parameters were: scan width (*m/z*) 0.01, scan time 0.1 s, peak width (FWHM) 0.7 for both Q1 and Q3, and collision gas pressure 1.5 mTorr, the primary transition: *m/z* 127/85, Collision Energy 25 eV, Secondary transition: *m/z* 127/68; Collision Energy 25 eV. Data acquisition and analysis were accomplished with LC quan software v.2.5 (Thermo Electron Corp., San Jose, CA, USA).

2.3. Sample extraction and clean-up

A 50 mL of 1% trichloroacetic acid solution and 2 mL 2.2% lead acetate solution were added to 5 g of liquid milk sample in order to eliminating protein and extracting analyte. A 30 mL of the mixture was placed in ultrasonic cleaner for 20 min to mix well, standing for 2 min. Then mixed solution was centrifuged for 10 min at 10,000 rpm. A 10 mL volume of the supernatant was applied to a PCX-SPE cartridge which had been previously conditioned with 3 mL of methanol and 3 mL of water. SPE cartridge was washed in turn with 5 mL of water and 5 mL of methanol and the eluate was discarded. Melamine was eluted with 6 mL of 25% ammonia solution–methanol (1:20, v/v). The eluate was evaporated to dryness at 50 °C under a stream of nitrogen and residue was re-dissolved in 0.5 mL of methanol–water (1:4, v/v). Then the solution was filtered through a 0.45 μm microporous membrane of mixed cellulose ester for HPLC analysis.

2.4. HPLC analysis

After the C18 column was conditioned with a mobile phase of 10 mM sodium *n*-heptanesulfonate–acetonitrile (83:17, v/v, pH 2.7) at 1 mL/min at 30 °C, a 20 μL volume of sample solution was injected in the column, and then eluted with the mobile phase. The linear equation describing the relationship between melamine concentration and its peak area was determined by least-squares weighted for UV detection at 235 nm. Quantification was carried out by using matrix-matched standards calibration.