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Determination of melamine and cyromazine in milk by high performance liquid chromatography coupled with online solid-phase extraction using a novel cation-exchange restricted access material synthesized by surface initiated atom transfer radical polymerization

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ABSTRACT

A novel strong-cation-exchange restricted access material has been synthesized by atom transfer radical polymerization (ATRP). In the synthesis, poly(3-sulfopropyl methacrylate-co-ethylene dimethacrylate), [p(SPM/EDMA)] was grafted on the silica by surface-initiated ATRP first. The poly(glycerol mono-methacrylate) [pGMMA] was then immobilized on the external surface, which created a chemical diffusion barrier for protein exclusion. The resulting Sil-g-p(SPM/EDMA)-g-pGMMA has both functions of protein exclusion and cation exchange, exhibiting the property of cation-exchange restricted access material. The application of Sil-g-p(SPM/EDMA)-g-pGMMA has been studied by the determination of melamine and cyromazine in bovine milk using the online solid-phase extraction/HPLC method. In the process, the Sil-g-p(SPM/EDMA)-g-pGMMA was used for the sample pre-treatment and a HILIC column was employed as the analytical column. The method has shown good accuracy, precision and low limits of detections. The result demonstrated that the Sil-g-p(SPM/EDMA)-g-pGMMA can be used for the cation extraction from biological samples by direct HPLC injection.

1. Introduction

There is an increased demand of high throughput methodologies with good sensitivity and reliability in the HPLC analysis of biological samples in recent years [1]. However, the traditional protein removal process is time consuming and is still the bottle neck in pursuing the high efficiency. In order to solve this problem, restricted access materials (RAMs) have been invented [2–4]. The RAMs can exclude proteins by the chemical or physical barriers [5] and retain small molecules at the same time, which facilitates the direct HPLC injection of proteinous samples. Different RAMs including chemically modified silica, total polymers, mesoporous, magnetic and molecularly imprinted materials have been fabricated and their applications have been explored [4,6–10]. The cation-exchange RAMs have been synthesized to selectively extract basic compounds in the pharmaceutical analysis [11]. They are also used in the proteomics analysis to extract low molecular weight peptides [12].

In recent years, atom transfer radical polymerization (ATRP) has been used to fabricate polymer grafted silica for the chromatographic analysis [13–18]. ATRP has been utilized to create an evenly distributed polymer on the support by the surface-initiated "grafting from" technique [13] and to synthesize multiple-functional materials by sequential polymerization using different monomers [19,20]. In the present study, a new strong cation-exchange restricted access material (SCE-RAM) was developed by grafting different polymers sequentially on the silica using surface-initiated ATRP technique. In the synthesis, poly(3-sulfopropyl methacrylate-co-ethylene dimethacrylate) [p(SPM/EDMA)] was grafted onto the silica surface first by the "grafting from" ATRP. Then a hydrophilic chemical barrier, poly(glycerol mono-methacrylate) [pGMMA], was established by using ATRP to graft glycidyl methacrylate (GMA) on the p(SPM/EDMA)-grafted silica followed by the hydrolysis of the epoxy groups. The resulting Sil-g-p(SPM/EDMA)-g-pGMMA has the property of protein exclusion in the HPLC analysis. Meanwhile the strong cation exchange property of the material is still remained.

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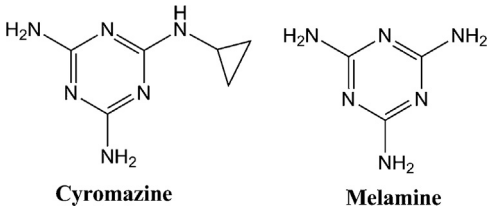

Fig. 1. The structures of cyromazine and melamine.

To explore the application ability of the Sil-g-p(SPM/EDMA)-g-pGMMA, an online solid-phase extraction (SPE)/HPLC for the cyromazine (CYR) and melamine (MEL) determination in bovine milk was developed. Cyromazine (*N*-cyclopropyl-1, 3, 5-triazine-2, 4, 6-triamine) is an insecticide used to control flies on animals or leaf miners [21,22], a portion of which can be degraded to MEL via dealkylation reactions. CYR was proved to cause mammary tumors in mouse [23]. Melamine (2, 4, 6-triamine-1, 3, 5-triazin) is a synthetic compound used in the manufacture of durable plastics and foam products. It is toxic at high-dose exposure and may form insoluble melamine cyanurate in kidneys, causing renal failure. Melamine was deliberately added to the food commodities by unethical manufactures to make fake protein result. Responding to these melamine scandals, the international health authorities enacted new regulations against the melamine contamination in animal feed and dairy products. Melamine may also be detected at low levels in food as a metabolites and degradation product of cyromazine [24,25]. The structures of melamine and cyromazine are shown in Fig. 1.

The melamine incidents promoted the development of fit-for-purpose analytical methods worldwide [26–29]. Because proteins are abundant in milk product, most analytical methods for the melamine determination in milk require protein removal and extraction in the sample preparations. Using RAM in the online SPE extraction can enhance the analytical efficiency and automation. Since MEL and CYR are weak alkaline compounds and positively charged in an acidic condition, SCE-RAM is well suited for their solid-phase extractions. We employed the Sil-g-p(SPM/EDMA)-g-pGMMA for SPE online coupled with HPLC column to facilitate the MEL and CYR determination process. To establish the online SPE/HPLC method, the SPE and separation conditions were studied and optimized.

2. Experimental

2.1. Materials and reagents

Silica gel (average particle size: 10 μm, average pore size: 10 nm, surface area: 380 $m^2\,g^{-1}$) was purchased from the Second Chemical Factory (Tianjin, China). Melamine (MEL) was kindly provided by the College of Life Sciences of Nankai University (Tianjin, China). Cyromazine (CYR) was from J&K Scientific Ltd. (Beijing, China). 3-Sulfopropyl methacrylate potassium (SPM potassium) was from Sigma-Aldrich Trading Co. Ltd. (Beijing, China). Ethylene dimethacrylate (EDMA) and 2, 2'-bipyridine (Bipy) were from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). Glycidyl methacrylate (GMA) was obtained from Yuanji Chemical Co. Ltd. (Shanghai, China). Bovine serum albumin (BSA) was from Tianjin Heowns Biochem Co. Ltd. (Tianjin, China). The purification processes for EDMA and GMA are in the Supplementary Material.

2.2. Grafting of p(SPM/EDMA) on the surface of silica by ATRP

The 3-(2-bromoisobutyryl) propyl triethoxysilane (BIBPTES) was synthesized according to the literature [30] and reacted with silica to form macroinitiator (denoted as BIBP-silica, Fig. S-1). The p(SPM/EDMA) was grafted on the surface of silica by the ATRP reaction (Fig. S-2). In the synthesis, BIBP-silica was used as the macroinitiator. The SPM and EDMA were the monomer and cross-linker respectively. The Bipy/$CuBr_2$/CuBr was employed as the catalytic system. The detailed procedure is in the Sections 1.2 and 1.3 of the Supplementary Material. The p(SPM/EDMA) grafted silica was denoted as Sil-g-p(SPM/EDMA).

2.3. Grafting of poly(GMMA) on the surface of Sil-g-p(SPM/EDMA) by ATRP

The poly(GMMA) was grafted on the surface of the Sil-g-p(SPM/EDMA) by the surface initiated ATRP of GMA, followed by the hydrolysis of the epoxy groups. The synthetic procedure is in the Supplementary Material. The resulting material, denoted as Sil-g-p(SPM/EDMA)-g-pGMMA, with p(SPM/EDMA) as the inner layer and pGMMA as the outer layer was produced (Section 1.4 in the Supplementary Material).

2.4. Chromatographic evaluation of Sil-g-p(SPM/EDMA) and Sil-g-p(SPM/EDMA)-g-pGMMA

A Shimadzu HPLC instrument (Shimadzu, Japan) equipped with two LC-20AD pumps and a SPD-20A UV detector was employed for the chromatographic evaluation. The particles of Sil-g-p(SPM/EDMA) or Sil-g-p(SPM/EDMA)-g-pGMMA were dry packed in the stainless steel columns. In the Sil-g-p(SPM/EDMA) evaluation, inorganic cations were detected by indirect UV method at 250 nm wavelength. $CuSO_4$ aqueous solution was used as the mobile phase. The retention factor (k) was calculated by $k = (t_R - t_0)/t_0$, where t_R is the retention time of the analytes and t_0 is the void time measured by H_2O. In the Sil-g-p(SPM/EDMA)-g-pGMMA evaluation, the ability of protein exclusion was analyzed by the recovery of BSA calculated by the ratio of peak area of BSA eluted from the column to that obtained without column.

2.5. Preparation of the standard solutions and spiked milk samples

The stock solution containing MEL and CYR (1.0 mg mL^{-1} for each compound) was prepared with deionized water. The standard solutions were prepared by dilution of the stock solution with mobile phase A [(5 mM ammonium acetate, pH 3.0)/ACN (98/2, v/v)].

Pasteurized bovine milk was purchased from the local supermarket. Bovine milk (1.0 mL) was mixed with 1.0 mL standard solution by vortexing, followed by centrifuge at 15,000 × *g* for 8 min. The central layer (1.0 mL) was withdrawn for the analysis. The spiked samples containing both MEL and CYR at concentration levels: 0.0050, 0.010, 0.10, 1.0, 10, 100 μg mL^{-1} were prepared. The blank milk sample was prepared by the same procedure except the standard solution was replaced by mobile phase A. All solutions were stored at +4 °C after preparation.

2.6. Online SPE/HPLC for the determination of MEL and CYR in bovine milk

A column-switching SPE/HPLC system (Fig. 2) was used for the determination of MEL and CYR in bovine milk. The system consisted of two LC-20 AD pumps, a SPD-20A PDA detector and a 6-port switching valve (FCV-20AH_6, Shimadzu). The position of the 6-port switching valve was controlled by the LabSolutions software. A stainless steel column (30 × 4.6 mm) packed with Sil-g-p(SPM/EDMA)-g-pGMMA (SCE-RAM in Fig. 2) was used as the SPE column. A 150 × 4.6 mm HILIC column (Luna 5 μm, 200 Å,