Journal of Chromatography B, 957 (2014) 7–13

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Packed-fiber solid-phase extraction coupled with high performance liquid chromatography–tandem mass spectrometry for determination of diethylstilbestrol, hexestrol, and dienestrol residues in milk products

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ARTICLE INFO

Article history:
Received 16 November 2013
Accepted 23 February 2014
Available online 2 March 2014

Keywords:
Liquid dairy products
Packed-fiber solid-phase extraction and high performance liquid chromatography–tandem mass spectrometry
Stilbenes

ABSTRACT

A sensitive analytical method based on packed-fiber solid-phase extraction and high performance liquid chromatography–tandem mass spectrometry (PF SPE-HPLC–MS/MS) has been developed for determination of three synthetic stilbenes in milk. The stilbenes are extracted with acetonitrile, using sodium chloride, and purified with PF SPE using a cartridge containing electrospun polystyrene nanofibers. Parameters affecting the efficiency of PF SPE, such as pH and amount of salt, were optimized. Under optimal conditions, the limits of detection and quantification were 5–13 pg/g and 15–37 pg/g, respectively. Absolute recoveries varied between 60% and 85% at three different levels. The method was successfully applied for the determination of estrogenic stilbenes in a total of 69 milk samples. The method is sensitive and cost-effective in stilbene detection, and has potential in quality control of dairy products.

1. Introduction

Synthetic stilbenes, e.g., diethylstilbestrol, hexestrol, and dienestrol, are nonsteroidal hormones used to treat reproductive diseases and promote growth in animals [1]. Extensive research shows that such stilbenes, particularly diethylstilbestrol, in the human diet can produce endocrine disorders and even cancer in hormone-dependent organs; thus, they are banned worldwide for use in food-producing animals [2,3]. Liquid dairy products could be contaminated by stilbenes through the milk supply chain if these drugs were used illegally. Therefore, an efficient and sensitive method for the determination of stilbenes should be developed and validated to monitor stilbene residues in food and thereby ensure the safety of the food supply.

Stilbenes may be present in milk at very low concentration levels (micrograms or nanograms per liter) [4]; thus the analytical methods used for dairy samples need to be sensitive. During the past decade, analytical strategies including high-performance liquid chromatography (HPLC), liquid chromatography–mass spectrometry and its tandem development (LC–MS, LC–MS/MS), and gas chromatography–mass spectrometry (GC–MS) have been reported for the analysis of stilbenes and other estrogens in milk and dairy products [5–12]. Among them, LC–MS/MS has been widely adopted as the main tool for identification and quantification by virtue of its superior sensitivity, specificity, and efficiency [13].

In all these methods, sample preparation remains a bottleneck in the analytical process. In reported methods, samples were prepared both with and without deproteinization prior to purification [5–12]. The pretreatment technologies included solid-phase extraction (SPE) [5–7,10,14], dispersive solid-phase extraction (SPME) [15], dynamic liquid–liquid–solid microextraction (DLLSME) [8], automated solid-phase extraction (ASPE) [9], molecularly imprinted polymer-coated polypropylene hollow fiber tube extraction (MIP-HFT) [11] and magnetic solid-phase extraction (MSPE) [12]. Some of them can only be applied for LC analysis but not for LC–MS/MS, other pretreatment techniques combining

Abbreviations: PF SPE, packed-fiber solid-phase extraction; DES, diethylstilbestrol; HEX, hexestrol; DS, dienestrol; MRM, multiple reaction monitoring; PS, electrospun polystyrene.

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http://dx.doi.org/10.1016/j.jchromb.2014.02.036
1570-0232/

with LC–MS/MS did not validate their method by testing the matrix effect.

With the advent of nanotechnology research, some new materials have been used successfully as functional sorbents in SPE. Owing to their unique mechanical and chemical properties, both cheap and easy to obtain, electrospun nanofibers have the potential to serve as a good sorbent material for SPE-based techniques [16,17]. The main advantage of nanofiber materials is their large surface area to volume ratio, which allows the use of a smaller sorbent bed mass, reducing the amount of organic solvents and increasing extraction efficiencies for trace analyses [18]. In 2007, Kang and co-workers developed a packed-fiber solid-phase extraction (PF SPE) method using an electrospun nanofiber to extract trazodone from human plasma and obtained better extraction results [19].

In this study, PF SPE coupled with high performance liquid chromatography–tandem mass spectrometry (PF SPE-HPLC–MS/MS) has been used for the first time for the simultaneous determination of three stilbenes including diethylstilbestrol (DES), hexestrol (HEX) and dienestrol (DS) in liquid dairy products. The key PF SPE factors affecting the extraction efficiency of stilbenes, i.e., pH and amount of sodium chloride, were systematically optimized. A stable-isotope labeled standard was used to minimize matrix effects. This work offers a practical approach that applies nanotechnology in food safety testing and improves analytical quality and laboratory efficiency.

2. Experimental

2.1. Chemicals, standards, and stock solutions

HPLC grade acetonitrile (ACN) and methanol (MeOH) were purchased from Merck (Darmstadt, Germany). Ultrapure water (18.2 MΩ) was obtained using a Milli-Q Water System (Millipore, Bedford, MA, USA). Analytical grade sodium chloride (NaCl), potassium dihydrogen phosphate (KH_2PO_4), and phosphoric acid (H_3PO_4) were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China). Dienestrol (99.5%), hexestrol (98.5%), and *trans*-diethylstilbestrol (99.0%) were purchased from the laboratories of Dr. Ehrenstorfer (Augsburg, Germany), and diethylstilbestrol-d_6 (DES-d6) was purchased from RIVM (Bilthoven, Netherlands).

A mixed stock solution of the three stilbenes was prepared in methanol at a concentration of 10 μg/mL and stored at 4 °C in darkness. A preliminary stability study showed that the stock solutions were stable for almost 3 months. The stock solution was used to spike the sample solutions for the following experiments. An internal standard solution containing approximately 50 ng/mL of DES-d6 was prepared in methanol.

2.2. Design and preparation of PF SPE column

The electrospun polystyrene (PS) nanofibers were prepared by was loading PS solution into a glass syringe, which was fitted to a steel needle with a filed flat tip. A high-voltage generator was linked with the needle through a copper pin. A grounded iron drum mantled with a copper grid was served as the collection screen. A dense web of the fibers was collected on the copper grid. The detailed explanation with illustrated procedure was described in previous published literature [20]. A scanning electron microscope image of the selected PS nanofibers is given in Fig. 1(A). The diameter of the fibers was about 200–600 nm. The PF SPE columns were prepared manually by packing 5 mg of PS fibers into a 5-mL plastic cartridge (purchased from Suzhou Dongqi Biological Technology Co., Ltd., China) with a narrow tip to hold the sorbent. A schematic of the PF SPE device is shown in Fig. 1(B).

2.3. Sample collection

A total of 69 fluid dairy samples were collected through a spot-check of dairy samples collected in Jiangsu Province in China and stored at 4 °C. These samples consisted of 19 batches of plain milk, 18 batches of reconstituted milk, 15 batches of fortified milk, and 17 batches of flavored milk.

2.4. Sample preparation

A homogenized fluid dairy sample (10 g) was weighted into a 50-mL polypropylene centrifuge tube and mixed with 100 μL internal standard solution (DES-d6). After adding 0.5 g of sodium chloride and 10 mL of acetonitrile, the tube were capped and vortexed for 2 min at a high speed (Stuart, UK). Then, the tube was centrifuged for 5 min at 3000 rpm (Anke, China). Finally, the organic layer was transferred to an evaporating flask and evaporated to dryness on a water bath at 50 °C (Buchi, Switzerland). The residue was immediately reconstituted with 1.0 mL of phosphate buffer (0.05 M, pH 9.6) before loading onto the PF SPE cartridge.

2.5. PF SPE procedure

The extraction columns were conditioned by washing with 100 μL of methanol, followed by 100 μL of water. After that, all the sample extracts were drawn through the columns using a 24-port SPE Vacuum Manifold (Supelco, USA). The analytes were quantitatively eluted with 100 μL of MeOH by the air pressure forced by a gas-tight plastic syringe (10 mL), which was fitted to the top of the extraction cartridge. Finally, 1.0 μL of eluate was analyzed immediately by LC–MS/MS. All the operations were performed at room temperature.

2.6. Instrumental and analytical conditions

The separation of the stilbenes from the fluid dairy extracts was carried out using a high performance liquid chromatography system (Agilent 1200 Series HPLC system, Agilent Technologies, Santa Clara, USA) consisting of vacuum degasser, auto sampler and a binary pump, equipped with a reversed-phase XDB-C18 analytical column (3.0 mm × 50 mm; 2.1 μm particle size, Agilent Technologies, Santa Clara, USA). An isocratic mobile phase of 35% acetonitrile and 65% water was pump mixed from separate containers. The flow rate was 0.2 mL min^{-1}. The HPLC system was connected to a triple-quadrupole mass spectrometer (Agilent 6410B QQQ tandem mass spectrometer, Agilent Technologies, Santa Clara, USA) equipped with an electrospray interface operated in the negative ionization mode, using a capillary voltage of 4000 V, a nebulizer pressure of 40 psi, a drying gas flow rate of 9.0 L/min, and a gas temperature of 350 °C. For the LC–MS/MS analysis, the mass spectrometer was operated in multiple reaction monitoring (MRM) mode. The MRM data was collected and analyzed with Agilent MassHunter Data Acquisition software (Version B.02.02).

2.7. Method validation

Peak area ratios of stilbene/DES-d6 plotted against the corresponding concentrations were calculated to construct calibration curve. Limit of detection (LOD) is estimated by the concentration that gave a signal equal to the blank plus 3 times the standard deviation, while limit of quantification (LOQ) is defined as the amount of the analytes that gave a signal equal to the blank plus 10 times the standard deviation.

The precision of the method, expressed as percent relative standard deviation (%RSD), was estimated by the repeated analysis