Food Chemistry 149 (2014) 319–325

Contents lists available at ScienceDirect

Food Chemistry

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

Analysis of oestrogenic compounds in dairy products by hollow-fibre liquid-phase microextraction coupled to liquid chromatography

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ARTICLE INFO

Article history:
Received 16 July 2013
Received in revised form 30 September 2013
Accepted 14 October 2013
Available online 25 October 2013

Keywords:
High-performance liquid chromatography
Hollow-fibre liquid-phase microextraction
Oestrogens
Milk derivatives

ABSTRACT

In this work, the potential of a hollow-fibre liquid-phase microextraction (LPME)-based method has been studied and validated for the extraction of a group of nine oestrogenic compounds four of them being natural (oestriol, 17β-oestradiol, 17α-oestradiol and oestrone), four being synthetic (17α-ethynyloestradiol, diethylstilbestrol, dienestrol and hexestrol) and one metabolite (2-hydroxyoestradiol) in different dairy products (whole and skimmed natural yogurt, a probiotic yogurt-type drink and cheese). The methodology includes a prior protein precipitation with acidified acetonitrile for all samples and an additional defatting step with *n*-hexane for cheese, the matrix with the highest fat content. Later separation, determination and quantification were done by high-performance liquid chromatography coupled to a diode array detector and a fluorescence detector set in series. Calibration, sensitivity, precision and accuracy of the method were carried out in the selected matrices, providing good linearity, LODs in the low μg/kg or μg/L range, good precision and appropriate accuracy.

1. Introduction

The association between the consumption of dairy products and the possibility of increasing or even lowering the risk of the development of certain diseases is varied, as data is still inconclusive or lacking (Davoodi, Esmaeili, & Mortazavian, 2013; Yerlikaya, Acu, & Kinik, 2013). The need to know the risk or benefit-producing constituents and their concentrations in milk, yogurt, butter and cheese matrices has encouraged the development of analytical approaches for their determination.

One of the groups of substances that milk and dairy products contain and which are known to produce problems in the human organism is the lipophilic oestrogenic hormones. They basically induce endocrine disorders such as sexual maturation and reproductive functions problems in women after prepubertal exposure (Rasier, Toppari, Parent, & Bourguignon, 2006), and are also suspected procarcinogenic agents (Bradlow & Sepkovic, 2004; Rasier et al., 2006; Soloway, 2007; Storgaard, Bonde, & Olsen, 2006). For this reason, efforts have been made for their quantification, especially in milk, both by immunoassays or chromatographic methods (Socas-Rodríguez, Asensio-Ramos, Herrera-Herrera, Hernández-Borges, & Rodríguez-Delgado, 2013a). However, the number of works related with the study of milk derivatives is still very low (Socas-Rodríguez et al., 2013a). In fact, and to the best of our knowledge, this issue has only been addressed using chromatographic techniques on four occasions (Hartmann, Lacorn, & Steinhart, 1998; Shi et al., 2011; Su et al., 2011; Yuan et al., 2012) and using radioimmunoassay (RIA) in one (Pape-Zambito, Roberts, & Kensinger, 2010). However, in these works the analysis of natural oestrogens has been, in general, the aim of the study, without determining synthetic oestrogenic molecules or metabolites. In this respect, oestrone (E_1), 17α-oestradiol (17α-E_2), 17β-E_2, and oestriol (E_3) have been analysed in yogurt (Hartmann et al., 1998; Shi et al., 2011), cream (Hartmann et al., 1998; Pape-Zambito et al., 2010), butter (Hartmann et al., 1998; Pape-Zambito et al., 2010; Su et al., 2011) and cheese (Hartmann et al., 1998), while 17β-E_2 and E_3 have been also determined in milk tablets (Yuan et al., 2012) and 17α-ethynyloestradiol (EE_2) in butter (Su et al., 2011). Regarding sample preparation, these compounds have been extracted basically by liquid–liquid extraction (LLE) followed by column chromatography (Hartmann et al., 1998), solid-phase extraction (SPE) (Shi et al., 2011; Yuan et al., 2012) or matrix solid-phase dispersion (MSPD) (Su et al., 2011), which are procedures known to consume high or relatively high amounts of organic solvents.

Over the last years, trends in sample pretreatment have focused on the use of easy, quick and miniaturised techniques with low solvent consumption. This is the case of liquid-phase microextraction (LPME), which has been successfully applied for the extraction of a wide variety of analytes from a large range of matrices, including

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http://dx.doi.org/10.1016/j.foodchem.2013.10.066

foods (Asensio-Ramos, Ravelo-Pérez, González-Curbelo, & Hernández-Borges, 2011). One of its variations is hollow-fibre LPME (HF-LPME), in which the extraction of the analytes from an aqueous donor phase occurs into a liquid membrane supported on a hydrophobic fibre, and then inside the solvent contained in the lumen. This solvent can be the same as in the lumen (two-phase) or different (three-phase) and after the extraction, it can be directly injected into the instrument, or submitted to a desorption step in an appropriate solvent.

In a recently work by our group (Socas-Rodríguez, Asensio-Ramos, Hernández-Borges, & Rodríguez-Delgado, 2013b), a method based on two-phase HF-LPME was optimised and validated for the extraction and preconcentration of a group of four natural oestrogens (E_3, 17β-E_2, 17α-E_2 and E_1), four synthetic (EE_2; diethylstilbestrol, DES; dienestrol, DS; and hexestrol, HEX) and one metabolite (2-hydroxyestradiol, 2-OHE_2) from milk with different fat content after a simple deproteinisation, demonstrating a good performance of the whole method with LODs in the low μg/L range. The proposed procedure was simple, selective, effective and with low consumption of organic solvents.

The aim of this paper is to extend our previously developed methodology for the extraction of oestrogenic residues from milk derivatives, which is a scarcely studied field. To fulfil this objective, some minor but necessary modifications in the sample preparation step were necessary due to the different characteristics of the matrices. The complete method was validated for the analysis of whole and skimmed yogurt, a dairy *Lactobacillus* probiotic product and white cheese. To the best of our knowledge, this is the first time that the analysis of synthetic stilbenes and metabolites of oestrogens is addressed in dairy products different from milk. Furthermore, it is the first work which reports the analysis of oestrogens in a probiotic product, as well as the first application in general of LPME for the analysis of these compounds in milk derivatives.

2. Materials and methods

2.1. Chemicals and materials

Analytical standards of E_1 (1 1,3,5(10)-oestratrien-3-ol-17-one), 17α-E_2 (1,3,5(10)-oestratriene-3,17α-diol), 17β-E_2 (1,3,5-oestratriene-3,17β-diol), E_3 (1,3,5(10)-oestratriene-3,16α,17β-triol), EE_2 (17α-ethynyl-1,3,5(10)-oestratriene-3,17β-diol), DES ((E)-3,4-bis (4-hydroxyphenyl)-3-hexene), DS (3,4-bis(4-hydroxyphenyl)-2,4-hexadiene), HEX (4,4′-(1,2-diethylethylene)diphenol) and 2-OHE_2 (1,3,5(10)-oestratriene-2,3-17β-triol) from Sigma–Aldrich Chemie (Madrid, Spain) were used without further purification (purity ⩾ 95%).

Stock solutions of the analytes were prepared in methanol (MeOH, 100 mg/L for 2-OHE_2 and 1000 mg/L for the rest) and stored in the dark at 4 °C. Working mixes of the analytes were daily prepared by dilution with the appropriate volume of mobile phase.

All chemicals were of analytical reagent grade (unless otherwise indicated) and used as received. Acetonitrile (ACN) and MeOH of HPLC grade, hydrochloric acid (25%, w/w) and glacial acetic acid were from Merck (Darmstadt, Germany), sodium hydroxide and formic acid (98%, w/w) were from Panreac Química (Barcelona, Spain), while sodium chloride (purity > 99.5%) was from Sigma–Aldrich Chemie, 1-octanol from Fluka (Sigma–Aldrich Chemie) and *n*-hexane from VWR (Barcelona, Spain). Distiled water was deionised using a Milli-Q gradient system A10 from Millipore (Bedford, MA).

The Accurel Q3/2 polypropylene HF membrane (600 μm ID, 200 μm wall thickness and 0.2 μm pore size) was acquired from Membrana GmbH (Obernburg, Germany) and used as received.

2.2. Apparatus and software

HPLC analyses were performed in a Waters HPLC system (Milford, MA,) equipped with a binary pump (Model 1525), a column heater (Model 5CH 1500 series), an autosampler (Model 717 Plus), a fluorescence detector (FD, Model 2475 Multi λ) and a DAD (Model 2998) connected in series, working with Waters Empower 2 software. Separation was carried out at 40 °C in a Nova-Pak C_{18} column (150 mm × 3.9 mm, 4 μm) using a Guard-Pak C_{18} pre-column (4 μm), both from Waters.

Considering 1 mM formic acid in ACN as mobile phase A and 1 mM formic acid at pH 3.50 as mobile phase B, the initial composition of the mobile phase was 15/85 (v/v) A/B which was changed to 28/72 (v/v) A/B in 1 min (linear step) and then to 37/63 in 5 min (linear step). This composition was maintained for 17 min and then changed to 100% A in 1 min (linear step), which was also kept for 5 min and returned to the initial composition. The flow rate and the injection volume were 1 mL/min and 20 μL, respectively. The DAD was used to detect E_1 at $\lambda = 215$ nm and DES and DS at $\lambda = 230$ nm, while FD was used to detect 17α-E_2, 17β-E_2, E_3, EE_2 and HEX at $\lambda_{ex} = 280$ and $\lambda_{em} = 310$ nm and 2-OHE_2 at $\lambda_{ex} = 280$ and $\lambda_{em} = 320$ nm. Both detectors worked in multichannel mode to produce multiple chromatogram traces.

2.3. Daily product selection

Four types of dairy products (a whole and skimmed yogurt, a probiotic liquid product and white cheese) were selected to extend the method previously developed by our group for the analysis of oestrogens in cow milk (Socas-Rodríguez et al., 2013b). The content of proteins (3.3–11.6 g), carbohydrates (2.8–10 g) and fats (0.1–14 g) per 100 g of sample indicated on the commercial packaging of each product are shown in Table 1. The selected matrices were chosen according to their wide range of nutritional content, with the aim of validating the robustness of the methodology. All samples were purchased in local supermarkets of Tenerife, Canary Islands, Spain.

2.4. Yogurt extraction

Three grams of spiked or non-spiked whole or skimmed yogurt were introduced into a 50-mL centrifuge tube. Then, 9 mL of ACN and 150 μL of acetic acid were added and the sample was vortex-shaken for 3 min to produce protein precipitation. The mixture was maintained in the dark for 15 min and centrifuged at 4400 rpm (3000 × *g*) for 15 min in a 5702 centrifuge from Eppendorf (Hamburg, Germany). The extracts were evaporated to dryness at 40 °C and 180 mbar using a Rotavapor R-200 equiped with a V-800 vacuum controller, and a V-500 vacuum pump, all of them from Büchi Labortechnik. The residue (3 mL) was then redissolved in 7 mL Milli-Q water (total volume of 10 mL), 1 g NaCl was added and the mixture was filtered through a 0.45 μm Minisart SRP 15 PTFE filter from Sartorius (Goettingen, Germany) into a 20-mL vial.

Table 1
Nutritional information of the selected matrices.

	Whole yogurt (WY)	Skimmed yogurt (SY)	Probiotic product (PP)	White cheese (WC)
Carbohydrates (g)	3.9	4.5	11.6	3.3
Proteins (g)	3.2	4.3	2.8	10
Fats (g)	2.1	0.1	2.6	14