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Multiresidue determination of estrogens in different dairy products by ultra-high-performance liquid chromatography triple quadrupole mass spectrometry☆

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ABSTRACT

In this work, a simple and fast methodology has been validated and applied for the analysis of a group of 22 estrogenic compounds including eight phytoestrogens (i.e. daidzein, enterodiol, glycitein, enterolactone, genistein, formononetin, prunetin, biochanin A), six mycotoxins (β-zearalanol, β-zearalenol, α-zearalanol, α-zearalenol, zearalanone, zearalenone) as well as four synthetic (i.e. ethynylestradiol, diethylstilbestrol, dienestrol, hexestrol) and four natural estrogens (i.e. estriol, 17β-estradiol, 17α-estradiol, estrone) in different dairy products. Extraction was carried out using the QuEChERS method while separation, determination and quantification of the target analytes were achieved by ultra-high-performance liquid chromatography coupled to triple quadrupole mass spectrometry with an electrospray ionization interface. The methodology was validated for four dairy product samples with relevant interest for the population including skimmed and whole cheese and goat and cow kefir, using 17β-estradiol-2,4,16,16,17-d_5 as internal standard for natural and synthetic estrogens and β-zeralanol-10,10,11,12,12-d_5 as internal standard for mycotoxins and phytoestrogens. Recovery ranged from 70 to 119% for the four types of matrices with RSD values lower than 14% and the limits of quantification of the method achieved were in the range 0.025–2.50 μg/kg for all samples. Finally, the analysis of commercially available products was carried out finding the presence of daidzein, glycitein enterolactone and genistein in some of the studied samples.

1. Introduction

Natural estrogens are sex steroid hormones that stimulate the development of female characteristics and regulate the menstrual cycle of humans and, in general, the oestrous cycle in mammals. The most important representatives of these natural organism-synthesized compounds, also named as *endoestrogens*, are estrone (E_1), estradiol (E_2) or estriol (E_3), which have been demonstrated to be present in milk and their derivatives [1].

In addition to the previous group of compounds, there can also be found a wide number of substances called *exoestrogens* that have an important estrogenic activity (either enhancing or suppressing it). This is the case of ethynylestradiol (EE_2), a synthetic derivative of E_2 which is a human contraceptive [2] or synthetic stilbenes such as dienestrol (DS), hexestrol (HEX) or diethylstilbestrol (DES) which are commonly used as growth rate cattle promoters [3]. Another

Abbreviations: ACN, acetonitrile; DES, diethylstilbestrol; DS, dienestrol; E_1, estrone; E_3, estriol; EE_2, ethynylestradiol; ESI, electrospray ionization; GC, gas chromatography; HEX, hexestrol; HF-LPME, hollow-fiber liquid-phase microextraction; IS, internal standard; IT, ion trap; LC, liquid chromatography; LLE, liquid-liquid extraction; MeOH, methanol; MRLs, maximum residues limits; MRM, multiple reaction monitoring; MS, mass spectrometry; PP, polypropylene; QqQ, triple quadrupole; SPE, solid-phase extraction; UHPLC, ultra-high-performance liquid chromatography; ZAN, zearalanone; ZEN, zearalenone; α-ZAL, α-zearalanol; α-ZEL, α-zearalenol; β-ZAL, β-zearalanol; β-ZEL, β-zearalenol; β-ZAL-D_5, β-zeralanol-10,10,11,12,12-d_5; 17α-E_2, 17α-estradiol; 17β-E_2, 17β-estradiol; 17β-E_2-D_5, 17β-estradiol-2,4,16,16,17-d_5.

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important group of exoestrogens is constituted by the so-called mycoestrogens, such as zearalenone (ZEN) and its derivatives zearalanone (ZAN), zearalanols (ZALs) and zearalenols (ZELs), which are, in fact, mycotoxins that can be present in cereal crops and that can be subsequently transferred to milk and, consequently, to their derivatives by animal feeding and also because some of them are used as veterinary drugs [4,5]. Obviously, other exoestrogens or endocrine disruptors such as pesticides, polychlorinated biphenyls, bisphenols, phthalates, etc. are also included in this group.

Apart from these substances, other naturally occurring compounds with estrogenic activity are the so-called phytoestrogens, which are, in fact, isoflavones (i.e. biochanin A, daidzein, formononetin, genistein, glycitein, etc.), lignans (i.e. enterolactone, enterodiol) or coumestans (i.e. coumestrol). Most of them are naturally found in plants whereas others like the lignans enterolactone or enterodiol are obtained as products of animal metabolism. In both cases, and as a result of the vegetable composition of animal feed, they can appear in milk and, consequently, in dairy products [6,7].

Nowadays, there exist several European regulations regarding the use of hormones as animal growth promoters because of their possible toxic effect on public health. In this sense, Directive 2003/74/EC [8], which amends Directive 96/22/EC [9], currently prohibits the specific use of some substances with hormonal activity for the fattening of farm animals to ensure human health protection within the European Union. Among them, 17β-E_2, its ester like derivatives, and stilbenes and their derivatives, as well as their salts and esters, are included. It should be noted that α-ZAL, which is also used in several countries for growth promotion, is also banned in Europe. Despite such legislation, there are no specific maximum residue limits (MRLs) established for dairy products for these compounds but the possibility of the widespread abuse of hormonal substances in some parts of Europe still exits.

As a result of the estrogenic activity of all these families of compounds, there is increasing interest in their determination in milk and/or its derivatives, which represents an important group of food commodities widely consumed nowadays by the world population. In fact, several studies have pointed them to be responsible for many disorders of the human reproductive system [10,11] or even cancer [12], though in this last case, and for some of them, there is still controversy in whether they do cause cancer or not [13,14]. However, and probably because of the complexity of dairy products, the analysis of these compounds in such products has not been so widely tackled as for other simpler matrices like, for example, water samples [15,16]. In fact, only some phyto-, natural and synthetic estrogens have been analyzed in a few occasions in cheese samples (cow cheese) [6,7,17–19], while the analysis of the presence of these groups of compounds in kefir samples has not been reported in the bibliography. In these studies, different extraction techniques were applied including liquid-liquid extraction (LLE) [7,19], LLE combined with solid-phase extraction (SPE) [16,18] and the application of miniaturized techniques as hollow-fiber liquid-phase microextraction (HF-LPME) [17].

As it is well known, one of the sample preparation methods most frequently used worldwide, though mainly applied for pesticide residue analysis, is the so-called QuEChERS method [20]. The methodology is highly used in official laboratories that require multiresidue methods in order to maximize sample throughput by minimizing sample preparation, to ensure rapid turnaround time and to carry out effective control. Such approach is very flexible and different versions have been independently developed and applied in monitoring laboratories, mainly in combination with gas chromatography (GC) and liquid chromatography (LC) coupled to mass spectrometry (MS). As a result, the excellent and inherent advantages as well as the results provided by the QuEChERS sample preparation approach combined with both techniques have leaded to its extremely high popularity. In fact, many companies currently sale QuEChERS kits, also adapted to consumers' requirements. Apart from the pesticide residue analysis field, the method has also been successfully applied to the extraction of other groups of compounds like PAHs [21], pharmaceuticals [22], PCBs [23], etc. also from matrices different than fruits or vegetables like mussels [24,25], sewage sludge [22] or fish [25], among others. However, up to now, and to the best of our knowledge, the QuEChERS method has only been used for the extraction of some estrogenic compounds from milk or dairy products such as yogurt in very few occasions [26–29]. However, none of them have determined such compounds in cheese or kefir samples using the QuEChERS method.

Due to the low levels at which these compounds can be found (μg/kg-ng/kg range), the analysis of estrogenic compounds is frequently performed by chromatographic techniques, being LC coupled to MS via an electrospray ionization (ESI) interface, working in either the positive or, the most common, negative mode [1,11]. The coupling of ultra-high-performance liquid chromatography–tandem MS (UHPLC–MS/MS) using triple quadrupole (QqQ) as analyzer offers relevant advantages in terms of sensitivity as well as specificity which are of great importance for the analysis of complex matrices such as those studied in the present work.

Therefore, this work aims at the evaluation and application of a simple and effective method based on the QuEChERS extraction followed by UHPLC-QqQ-MS/MS determination for the analysis of a wide group of estrogenic compounds constituted by four natural (E_1, 17α-E_2, 17β-E_2 and E_3), four synthetic (DS, DES, HEX and EE_2), eight phyto- (daidzein, enterodiol, glycitein, enterolactone, genistein, formononetin, prunetin and biochanin A) and six mycoestrogens (ZEN, α-ZEL, β-ZEL, ZAN, α-ZAL and β-ZAL) in cheese and kefir of different animal origin. Several real samples were also analyzed. To the best of our knowledge, this is the first time that these compounds are simultaneously extracted from this type of dairy products using the QuEChERS approach. Furthermore, and since certain phytoestrogens were also found in some of the analyzed samples, this manuscript reports the first data available in the literature regarding phytoestrogens content in kefir samples and also constitutes one of the few studies providing data of their occurrence in cheese samples.

2. Experimental

2.1. Chemicals and materials

Analytical standards of biochanin A (CAS 491-80-5), daidzein (CAS 486-66-8), DES (CAS 56-53-1), DS (CAS 84-17-3), E_1 (CAS 53-16-7), 17α-E_2 (CAS 57-91-0), 17β-E_2 (CAS 50-28-2), 17β-E_2-D_5 (CAS 221093-45-4), E_3 (CAS 50-27-1), EE_2 (CAS 57-63-6), enterolactone (CAS 78473-71-9), enterodiol (CAS 80226-00-2), formononetin (CAS 485-72-3), genistein (CAS 446-72-0), glycitein (CAS 40957-83-3), HEX (CAS 84-16-2), prunetin (CAS 552-59-0), ZAN (CAS 5975-78-0), α-ZAL (CAS 26538-44-3), β-ZAL (CAS 42422-68-4), ZEN (CAS 17924-92-4), α-ZEL (CAS 36455-72-8), β-ZEL (CAS 71030-11-0) from Sigma-Aldrich Chemie (Madrid, Spain) and β-ZAL-D_5 from Witega Laboratorien Berlin-Adlershof GmbH (Berlin, Germany) were used without further purification (purity ≥ 95%).

Stock solutions of each analyte of about 100 mg/L were precisely prepared in methanol (MeOH) and stored in the darkness at 4 °C. Working analyte mixtures 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) of HPLC grade was from VWR International (Geldenaaksebaan, Belgium), ACN and MeOH of HPLC–MS hypergrade, were from Merck (Darmstadt,