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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,

Germany), magnesium sulphate monohydrate (97%) ($MgSO_4$), sodium chloride (NaCl) and ammonium hydroxide solution (≥25%) for trace analysis were from Sigma-Aldrich Chemie (Madrid, Spain) and octadecylsilane (C_{18}) sorbent was from Macherey-Nagel (Dürem, Germany). Water was deionized by a Milli-Q gradient system A10 from Millipore (Bedford, MA, USA).

2.2. Apparatus and software

UHPLC analyses were performed using a Waters ACQUITY UPLC H-Class (Milford, MA, USA) equipped with a quaternary solvent manager and a sample manager with flow-through needle (FTN) and working with the Masslynx™ software from Waters Chromatography. The UHPLC system was coupled to a MS Xevo QqQ detector (Waters Chromatography). Control of MS parameters and the collection and process of spectra data were developed using the same software from Waters Chromatography. Separations were carried out at 40 °C in an Acquity UPLC BEH C_{18} column (50 mm × 2.1 mm, 1.7 μm) using an Acquity UPLC BEH C_{18} precolumn (1.7 μm), both from Waters Chromatography.

The mobile phase used for the analysis of phyto- and mycoestrogens consisted of MeOH (A) and water (B), while for natural and synthetic estrogens MeOH/ACN (50/50, v/v) (A) and 2 mM ammonium hydroxide (B) were used, based on a previous study [29]. The gradient applied was the same for all groups of analytes: 10% A with a flow rate of 0.3 mL/min (initial), 10–40% A (0.5 min), 99.9% A with an increase of the flow until 0.4 mL/min (7.5 min), 99.9% A (2 min), 10% A with a decrease of the flow to 0.3 mL/min (2 min), 10% A (2 min). The injection volume was 5 μL at 10 °C.

The MS system was operated in multiple reaction monitoring (MRM) mode using 1 precursor and 2 product ions [30]. The typical source conditions for maximum intensity of precursor ions were as follows: capillary voltage 2.6 kV, source temperature 150 °C, desolvation temperature 150 °C, cone gas (N_2) flow rate 150 L/h, desolvation gas (N_2) flow 550 L/h, collision gas (Ar) pressure 0.5 bar. MS/MS experiments were performed by fragmentation of the deprotonated molecule $[M-H]^-$ or protonated molecule $[M-H]^+$, depending on each compound, which was selected as the precursor ion. MRM transitions as well as the values of cone voltage and collision energy of the target analytes were automatically optimized by the direct infusion of individual standards of estrogenic compounds at 2 mg/L in a mixture of A/B (50:50, v/v) for each group of compounds. Optimized MRM transitions as well as the values of cone voltage and collision energy of the target analytes are listed in Table S1 of the Supplementary material.

2.3. Samples selection

Four different dairy products, including two types of white cow cheese samples (skimmed and whole) and two kinds of kefir with goat and cow origin, were selected in order to validate the methodology. All samples were bought in local supermarkets of Tenerife (Canary Islands, Spain). The content of proteins, carbohydrates and fats were in the ranges 3.1–12 g, 3.5–4.4 g and 0.2–10 g per 100 g of sample, respectively, which is indicated on the commercial packaging of each product.

2.4. Dairy products extraction

The QuEChERS method [31] was applied for the extraction of the 22 estrogenic compounds from the selected dairy product samples. All products were previously homogenized using a T10 basic Ultra-Turrax from IKA (Staufen, Germany) for 3 min at a speed of approximately 11500 r.p.m. Then, 10 g of each sample were weighed into a 50 mL polypropylene (PP) centrifuge tube and 5 mL of Milli-Q water were added and mixed by shaking for 1 min. After that, 15 mL of ACN were added and the mixture was again shaken for 1 min, followed by the addition of 6 g of $MgSO_4$ and 1.5 g of NaCl, shaking during 1 min, ultrasounds for 5 min and centrifugation at 4400 r.p.m. for 15 min in a 5702 centrifuge from Eppendorf (Hamburg, Germany). The supernatant was transferred to a new PP centrifuge tube of 50 mL containing 180 mg of C_{18} sorbent and 1.8 g of $MgSO_4$, shaken for 1 min and centrifuged in the same conditions as in the first part of the procedure. Afterwards, 8 mL of the supernatant were collected and evaporated at 40 °C and 180 mbar using a Rotavapor R-200 equipped with a V-800 vacuum controller, and a V-500 vacuum pump, all of them from Büchi Labortechnik. Finally, the residue was reconstituted in 500 μL of 50/50 v/v MeOH/water and filtered using a Chromafil® Xtra PET-20/15 filter from Macherey-Nagel. In the case of whole cheese, a higher amount of C_{18} (500 mg) was necessary to remove the fat of the matrix during the clean-up step.

3. Results and discussion

3.1. UHPLC–MS/MS method

In order to proceed with the LC separation of the analytes, UHPLC–MS/MS conditions described in Section 2.2 were used. Results were found satisfactory with a very good separation of all the analytes and an analysis time of 5 min, using two different mobile phase compositions, one for the group of natural and synthetic estrogens and another for the combination of myco-and phytoestrogens. A repeatability study consisting on six consecutive injections (n = 6) of a mixture of the analytes at three levels of concentration (5, 250 and 500 μg/L) in three different days (n = 18) was carried out. Good repeatability for the retention times and peak areas were observed for all concentration levels in the same day, with RSDs lower than 0.4% and 14%, and between days, with RSD below 0.5% and 16%, respectively. Afterwards, instrumental calibration curves based on the relation of each analyte and the internal standard (IS) peak areas were obtained for each estrogenic compound by injecting seven concentration levels in quadruplicate (n = 7), obtaining determination coefficients (R^2) higher than 0.9968, as can be seen in Table 1. The ISs used were β-ZAL-D_5 for myco- and phytoestrogens and 17β-E_2-D_5 for natural and synthetic estrogens, which have a similar structure and behaviour to the group of the target analytes for which were applied. They were selected in order to correct the possible errors during sample preparation and to improve its reproducibility [32,33].

3.2. QuEChERS-UHPLC–MS/MS method validation

Dairy products are very complex samples with particular characteristics that can modify considerably the results obtained when the methodology is applied, especially in terms of accuracy and precision. For this reason, the study of the effect that such matrices can produce in the application of the developed methodology is of great interest. With this aim, matrix effect was evaluated in all cases following the Matuszewski method [34]. For this purpose, five replicates of each matrix were extracted by the QuEChERS method once spiked at three different concentration levels (low level: 1.88 μg/kg, except in cow kefir where they were 4 μg/kg for ZAN and 2.56 μg/kg for natural and synthetic estrogens and formononetin and prunetin; medium level: 17.5 μg/kg and high level: 37.5 μg/kg) at the end of the process and analyzed by UHPLC-QqQ-MS/MS. Then, matrix effect was calculated as the percentage of the ratio of the spiked sample and the standard area at the same concentration as proposed by Matuszewski et al. [34]. As can be seen in Table 2, the values of the percentage of matrix effect vary considerably between matrices and analytes, finding percentages lower

Table 1
Instrumental calibration data of the selected compounds.

Analyte	Retention time (min)	Calibration data (n = 7)			
		Range of concentration studied(μg/L)	Slope	Intercept	R^2
Phytoestrogens					
Daidzein	3.01	0.50–750	$8.06 \cdot 10^{-4} \pm 2.67 \cdot 10^{-5}$	$1.62 \cdot 10^{-3} \pm 7.85 \cdot 10^{-3}$	0.9992
Enterodiol	3.09	0.50–750	$4.05 \cdot 10^{-4} \pm 2.64 \cdot 10^{-5}$	$4.81 \cdot 10^{-3} \pm 9.53 \cdot 10^{-3}$	0.9970
Glycitein	3.12	0.50–750	$1.06 \cdot 10^{-3} \pm 6.60 \cdot 10^{-5}$	$6.79 \cdot 10^{-3} \pm 2.06 \cdot 10^{-2}$	0.9971
Enterolactone	3.29	0.50–750	$1.21 \cdot 10^{-3} \pm 2.57 \cdot 10^{-5}$	$3.72 \cdot 10^{-3} \pm 9.28 \cdot 10^{-3}$	0.9997
Genistein	3.39	0.50–750	$1.56 \cdot 10^{-3} \pm 5.10 \cdot 10^{-5}$	$8.37 \cdot 10^{-3} \pm 1.79 \cdot 10^{-2}$	0.9992
Formononetin	4.09	1.0–750	$5.32 \cdot 10^{-1} \pm 1.04 \cdot 10^{-5}$	$-3.80 \cdot 10^{-3} \pm 3.64 \cdot 10^{-3}$	0.9997
Prunetin	4.57	1.0–750	$1.61 \cdot 10^{-1} \pm 1.83 \cdot 10^{-4}$	$2.63 \cdot 10^{-2} \pm 6.60 \cdot 10^{-2}$	0.9999
Biochanin A	4.66	0.50–750	$2.57 \cdot 10^{-2} \pm 9.14 \cdot 10^{-4}$	$5.80 \cdot 10^{-2} \pm 3.29 \cdot 10^{-1}$	0.9991
Mycoestrogens					
β-ZAL	3.99	0.50–750	$2.17 \cdot 10^{-3} \pm 2.34 \cdot 10^{-5}$	$2.62 \cdot 10^{-3} \pm 8.44 \cdot 10^{-3}$	0.9999
β-ZEL	4.16	0.50–750	$1.04 \cdot 10^{-3} \pm 4.29 \cdot 10^{-5}$	$7.06 \cdot 10^{-3} \pm 1.47 \cdot 10^{-2}$	0.9980
α-ZAL	4.51	0.50–750	$6.42 \cdot 10^{-3} \pm 1.45 \cdot 10^{-4}$	$3.22 \cdot 10^{-2} \pm 5.05 \cdot 10^{-2}$	0.9996
α-ZEL	4.64	0.50–750	$8.90 \cdot 10^{-4} \pm 4.32 \cdot 10^{-5}$	$1.01 \cdot 10^{-2} \pm 1.48 \cdot 10^{-2}$	0.9980
ZAN	4.72	0.50–750	$4.40 \cdot 10^{-2} \pm 1.96 \cdot 10^{-3}$	$6.23 \cdot 10^{-1} \pm 6.69 \cdot 10^{-1}$	0.9980
ZEN	4.82	0.50–750	$3.84 \cdot 10^{-3} \pm 5.30 \cdot 10^{-5}$	$1.37 \cdot 10^{-3} \pm 1.92 \cdot 10^{-2}$	0.9999
Synthetic estrogens					
EE_2	3.88	5.0–750	$1.58 \cdot 10^{-3} \pm 5.18 \cdot 10^{-5}$	$-1.49 \cdot 10^{-2} \pm 1.87 \cdot 10^{-2}$	0.9990
DES	4.13	5.0–750	$8.61 \cdot 10^{-3} \pm 5.00 \cdot 10^{-4}$	$3.67 \cdot 10^{-2} \pm 1.75 \cdot 10^{-1}$	0.9975
DS	4.34	5.0–750	$1.35 \cdot 10^{-2} \pm 1.56 \cdot 10^{-4}$	$-4.87 \cdot 10^{-2} \pm 4.62 \cdot 10^{-2}$	0.9999
HEX	4.34	5.0–750	$1.83 \cdot 10^{-2} \pm 1.03 \cdot 10^{-3}$	$-2.61 \cdot 10^{-2} \pm 3.60 \cdot 10^{-1}$	0.9976
Natural estrogens					
E_3	2.43	5.0–750	$1.88 \cdot 10^{-3} \pm 8.11 \cdot 10^{-5}$	$-5.80 \cdot 10^{-3} \pm 2.98 \cdot 10^{-2}$	0.9983
17β-E_2	3.68	5.0–750	$1.53 \cdot 10^{-3} \pm 9.84 \cdot 10^{-5}$	$-3.46 \cdot 10^{-3} \pm 3.10 \cdot 10^{-2}$	0.9968
17α-E_2	3.88	5.0–750	$1.99 \cdot 10^{-3} \pm 8.83 \cdot 10^{-5}$	$-3.34 \cdot 10^{-2} \pm 3.10 \cdot 10^{-2}$	0.9982
E_1	3.92	5.0–750	$1.02 \cdot 10^{-2} \pm 2.10 \cdot 10^{-4}$	$-5.11 \cdot 10^{-2} \pm 6.21 \cdot 10^{-2}$	0.9997

R^2: Determination coefficient.

than 80% for the majority of cases with RSD lower than 19%, except for DES, DS, HEX, 17β-E_2 and E_1 in goat kefir, E_3 in cow kefir and enterodiol in whole cheese for which the matrix effect percentage was higher than 80%. These results indicated a clear ion suppression effect of the matrices for almost all cases and, consequently, it brought about the necessity of taking such aspect into account for the rest of the study.

The QuEChERS-UHPLC–MS/MS method was then applied and validated for the analysis of whole and skimmed cow cheese as well as kefir with cow and goat origin. For this purpose, calibration curves were prepared in order to evaluate the linearity of the methodology in the range of concentration of interest. Due to the results obtained from the matrix effect study, matrix-matched calibration curves were developed in each case. With this aim the samples where spiked at the end of the process and the curves were prepared based on the ratio between analyte and IS peak areas chosen for each group of analytes, by injecting 7 different levels of concentrations in quadruplicate (n = 7). Results, including the studied linear range with the lowest calibration level, are shown in Table 3. As can be seen, determination coefficients (R^2) obtained in all cases were higher than 0.9905. LOQs of the method, defined as the lowest matrix matched calibration concentration which provided a signal to noise ratio higher than 10 for the quantification transition and at least 3 for the confirmation transition (if it was available) were in the range 0.025–0.250 μg/kg for skimmed cheese, 0.050–0.500 μg/kg for whole cheese and in the ranges 0.050–2.50 μg/kg and 0.050–0.500 μg/kg for cow and goat kefir, respectively.

With the aim of studying the reproducibility of the procedure, recovery studies were developed for all samples at three levels of concentration carrying out 5 replicates at each level (see Table 4). A blank matrix of each type was also extracted and spiked at the same concentration level at the end of the extraction procedure. No interferences were found in any of them when blanks of the matrices were evaluated. Fig. 1 shows the quantification transition obtained for each analyte when cow kefir was analyzed. Similar chromatograms were also obtained for the other three types of dairy products. However, the presence of some of the target analytes could be determined when the blank matrices were analyzed. As can be seen in Fig. 2 for the whole cheese matrix, daidzein, glycitein, enterolactone and genistein were detected in some of the studied matrices. Consequently, and in order to carry out the correct validation of the procedure for all analytes, their areas were subtracted during the study. Relative recovery values were calculated taking into account the matrix effect, that is to say, comparing samples spiked at the beginning and at the end of the methodology. The obtained results, which are shown in Table 4, demonstrated the excellent reproducibility as well as the good efficiency of the extraction procedure applied in this case, since relative recovery values were in the range 78–119%, 70–119%, 73–119%, 71–118% for skimmed and whole cheese and cow and goat kefir, respectively, with RSD values lower than 14% for all samples.

3.3. Analysis of real samples

Once the methodology was validated and taking into account the excellent results obtained, a group of 8 real samples from diverse commercial brands, purchased in different supermarkets of Tenerife, were analyzed using the developed QuEChERS-UHPLC-QqQ-MS/MS method. This group included 2 different samples of each type of matrix studied. Their content of carbohydrates, proteins and fats are shown in Table S2 of the Supplementary material.

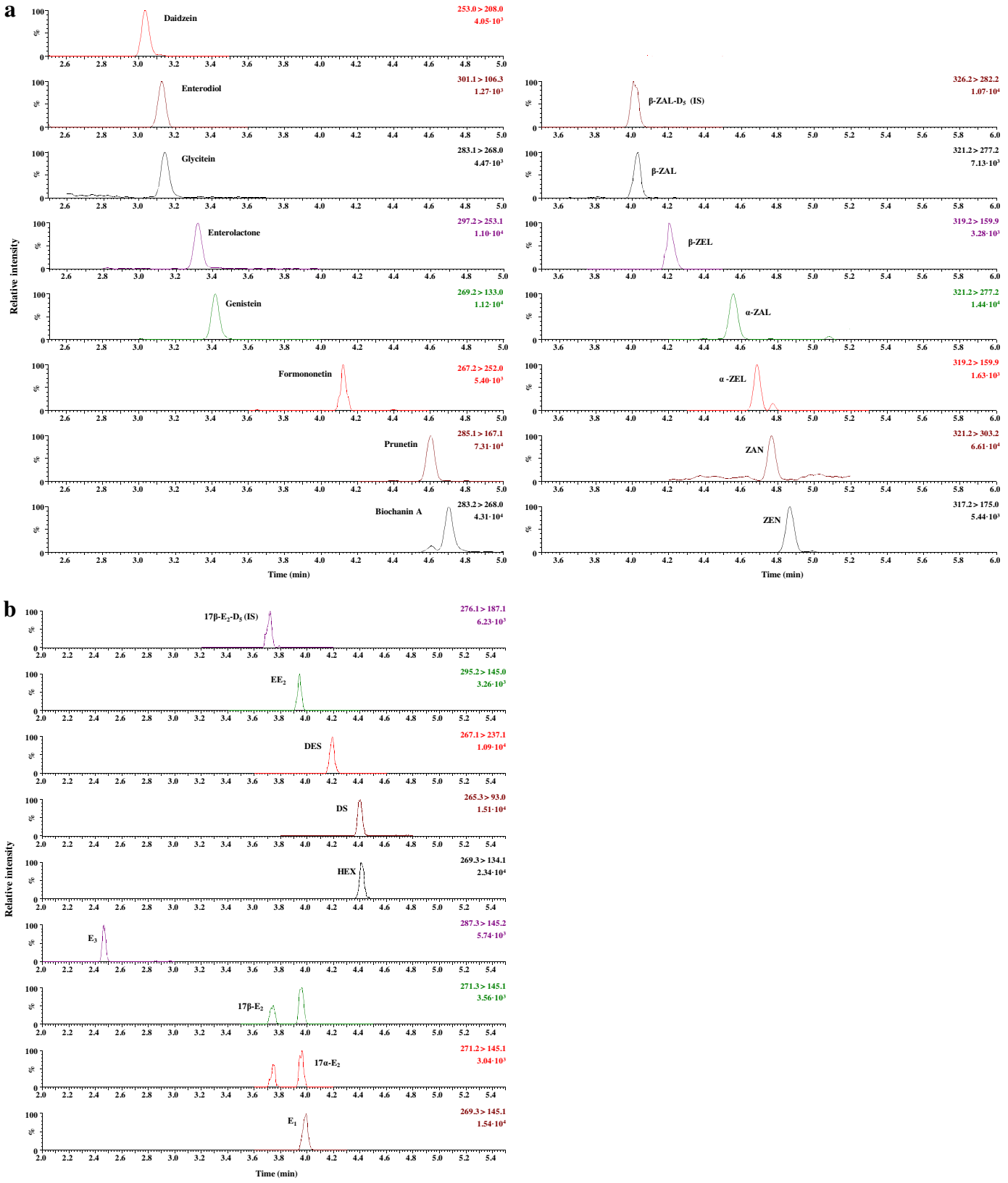

Fig. 1. a) UHPLC–MS/MS chromatograms of phytoestrogens, mycoestrogens and β-ZAL-D_5 (IS); b) UHPLC–MS/MS chromatograms of natural and synthetic estrogens and 17β-E_2-D_5 (IS) of a spiked goat kefir sample after the QuEChERS procedure. Injection volume: 5 μL. Sample dissolved in 500 μL of 50/50 (v/v) MeOH/water. Separation at 40 °C. Concentration in the sample: 25 μg/kg of IS and 17.5 μg/kg of the target analytes.

Table 2
Average results of the matrix effect study (n = 15) of the QuEChERS-UHPLC–MS/MS method for the selected compounds in the different matrices at three levels of concentration.

Analyte	Type of matrix	ME[a,b] %	RSD, %	Analyte	Type of matrix	ME[a,b] %	RSD, %
Daidzein	Skimmed cheese	62	4	α-ZEL	Skimmed cheese	30	17
	Whole cheese	49	17		Whole cheese	28	8
	Cow kefir	33	11		Cow kefir	29	12
	Goat kefir	20	8		Goat kefir	19	5
Enterodiol	Skimmed cheese	70	11	ZAN	Skimmed cheese	63	19
	Whole cheese	82	5		Whole cheese	26	19
	Cow kefir	33	2		Cow kefir	33	18
	Goat kefir	18	4		Goat kefir	16	5
Glycitein	Skimmed cheese	54	9	ZEN	Skimmed cheese	25	9
	Whole cheese	66	17		Whole cheese	14	18
	Cow kefir	43	12		Cow kefir	20	9
	Goat kefir	18	14		Goat kefir	16	7
Enterolactone	Skimmed cheese	19	6	EE_2	Skimmed cheese	60	8
	Whole cheese	33	10		Whole cheese	43	1
	Cow kefir	45	8		Cow kefir	67	5
	Goat kefir	28	18		Goat kefir	77	18
Genistein	Skimmed cheese	51	15	DES	Skimmed cheese	48	5
	Whole cheese	58	8		Whole cheese	31	6
	Cow kefir	39	7		Cow kefir	61	15
	Goat kefir	21	6		Goat kefir	92	4
Formononetin	Skimmed cheese	39	19	DS	Skimmed cheese	52	3
	Whole cheese	39	6		Whole cheese	34	4
	Cow kefir	34	18		Cow kefir	65	5
	Goat kefir	24	4		Goat kefir	87	3
Prunetin	Skimmed cheese	54	18	HEX	Skimmed cheese	48	3
	Whole cheese	27	17		Whole cheese	30	6
	Cow kefir	34	17		Cow kefir	65	4
	Goat kefir	37	11		Goat kefir	82	4
Biochanin A	Skimmed cheese	23	5	E_3	Skimmed cheese	56	11
	Whole cheese	14	4		Whole cheese	72	8
	Cow kefir	23	8		Cow kefir	90	5
	Goat kefir	22	9		Goat kefir	78	14
β-ZAL	Skimmed cheese	42	16	17β-E_2	Skimmed cheese	47	3
	Whole cheese	43	9		Whole cheese	43	4
	Cow kefir	32	17		Cow kefir	72	3
	Goat kefir	14	13		Goat kefir	87	18
β-ZEL	Skimmed cheese	46	10	17α-E_2	Skimmed cheese	60	5
	Whole cheese	39	17		Whole cheese	42	3
	Cow kefir	24	6		Cow kefir	70	4
	Goat kefir	17	11		Goat kefir	80	9
α-ZAL	Skimmed cheese	26	16	E_1	Skimmed cheese	62	7
	Whole cheese	28	3		Whole cheese	44	17
	Cow kefir	31	4		Cow kefir	67	8
	Goat kefir	21	17		Goat kefir	87	6

[a] Results obtained as an average of each analyte at the same concentration level of the recovery studies.
[b] Calculated following the Matuszewski method [34].

Results, which are presented in Table 5, show the presence of some phytoestrogens (daidzein, glycitein, enterolactone and genistein) in the analyzed samples. Besides, some of them could be even quantified since they are present at concentrations above the LOQ of the method (in the range 1.14–46.7 μg/kg), while the presence of mycoestrogens, natural or synthetic estrogens was not observed in any of the samples.

As indicated in the introduction section, the content of phytoestrogens in kefir samples has not been reported up to now since these compounds have not been previously analyzed in such matrices. Regarding cheese samples, the obtained results, in the range of 1.76–46.7 μg/kg for daidzein, glycitein and enterolactone, are comparable to the content previously determined by Křížová et al. [7] who reported values around 11.7–30.5 μg/kg for daidzein, genistein and glycitein, and slightly lower that the data reported by Kuhnle et al. [6] who found enterolactone in concentrations of 30–230 μg/kg in different type of cheese products. As it was demonstrated by Křížová et al. [7], these differences can be associated with the animal diet as well as the type of process applied to each product which can modify considerably the initial level of phytoestrogens present in the raw material used for its preparation.

These results demonstrate the suitability of the methodology for the analysis of the target compounds as well as the importance of their development for the evaluation of the presence of such compounds in samples extensively consumed by the population like milk derivatives.

3.4. Comparison with other methodologies

As it was indicated in the introduction section, only a few studies have been published regarding the evaluation of estrogenic compounds in milk or milk derivatives [6,7,17–19,26–29] except for kefir samples, which has not been previously analyzed. Concerning cheese samples, it should be indicated that it has only been previously analyzed to determine phyto-, natural and synthetic estrogens, but not mycoestrogens [6,7,17,18]. In this sense, Guoliang et al. applied a simple solvent extraction procedure followed by HPLC-FD with a previous derivatization with ethyl-acridine-sulfonyl chloride for the determination of E_1, E_2 and E_3 among other type of compounds. However, no data related with the sensitivity of the methodology were indicated for this specific matrix. A similar approach was carried out by Chavaliere et al. [18] who applied an ultrasound assisted solvent extraction using 1 g of cheese sample and a mixture of 5 mL of MeOH/water (80:20, v/v) containing 1% (v/v) trifluoroacetic acid as extraction solvent, followed by a SPE clean-up step using graphitized carbon black, for the extraction of

Table 3
Matrix matched calibration data of the selected compounds in the different matrices.

Analyte	Type of matrix	Calibration data (n = 7)				Analyte	Type of matrix	Calibration data (n = 7)			
		Range of concentration studied (μg/L)	Slope	Intercept	R^2			Range of concentration studied (μg/L)	Slope	Intercept	R^2
Daidzein	Skimmed cheese	0.5–750	$1.43 \cdot 10^{-3} \pm 6.53 \cdot 10^{-5}$	$-9.08 \cdot 10^{-3} \pm 2.01 \cdot 10^{-2}$	0.9984	α-ZEL	Skimmed cheese	5–750	$8.29 \cdot 10^{-4} \pm 4.08 \cdot 10^{-5}$	$-1.73 \cdot 10^{-3} \pm 1.26 \cdot 10^{-2}$	0.9982
	Whole cheese	1–750	$1.54 \cdot 10^{-3} \pm 3.57 \cdot 10^{-5}$	$3.17 \cdot 10^{-4} \pm 1.10 \cdot 10^{-2}$	0.9996		Whole cheese	5–750	$5.70 \cdot 10^{-4} \pm 2.46 \cdot 10^{-5}$	$-1.98 \cdot 10^{-3} \pm 7.80 \cdot 10^{-3}$	0.9986
	Cow kefir	1–750	$1.67 \cdot 10^{-3} \pm 1.47 \cdot 10^{-4}$	$1.71 \cdot 10^{-2} \pm 4.54 \cdot 10^{-2}$	0.9942		Cow kefir	5–750	$6.44 \cdot 10^{-4} \pm 1.99 \cdot 10^{-5}$	$-7.15 \cdot 10^{-3} \pm 7.20 \cdot 10^{-3}$	0.9993
	Goat kefir	1–750	$1.31 \cdot 10^{-3} \pm 8.49 \cdot 10^{-5}$	$1.46 \cdot 10^{-2} \pm 2.91 \cdot 10^{-2}$	0.9968		Goat kefir	10–750	$6.39 \cdot 10^{-4} \pm 3.66 \cdot 10^{-5}$	$3.08 \cdot 10^{-3} \pm 1.09 \cdot 10^{-2}$	0.9975
Enterodiol	Skimmed cheese	5–750	$1.07 \cdot 10^{-3} \pm 7.59 \cdot 10^{-5}$	$-1.60 \cdot 10^{-2} \pm 2.74 \cdot 10^{-2}$	0.9962	ZAN	Skimmed cheese	5–750	$4.91 \cdot 10^{-2} \pm 4.16 \cdot 10^{-3}$	$-5.65 \cdot 10^{-1} \pm 1.28 \cdot 10$	0.9946
	Whole cheese	5–750	$1.09 \cdot 10^{-3} \pm 9.47 \cdot 10^{-6}$	$6.08 \cdot 10^{-4} \pm 2.80 \cdot 10^{-3}$	0.9999		Whole cheese	5–750	$1.51 \cdot 10^{-2} \pm 4.03 \cdot 10^{-4}$	$7.51 \cdot 10^{-2} \pm 1.28 \cdot 10^{-1}$	0.9995
	Cow kefir	10–750	$1.18 \cdot 10^{-3} \pm 3.92 \cdot 10^{-5}$	$-4.26 \cdot 10^{-3} \pm 1.25 \cdot 10^{-2}$	0.9992		Cow kefir	50–750	$8.69 \cdot 10^{-3} \pm 1.18 \cdot 10^{-3}$	$7.52 \cdot 10^{-1} \pm 4.70 \cdot 10^{-1}$	0.9905
	Goat kefir	5–750	$6.81 \cdot 10^{-4} \pm 3.72 \cdot 10^{-5}$	$-7.64 \cdot 10^{-3} \pm 1.34 \cdot 10^{-2}$	0.9977		Goat kefir	5–750	$6.20 \cdot 10^{-2} \pm 2.27 \cdot 10^{-3}$	$5.97 \cdot 10^{-2} \pm 7.75 \cdot 10^{-1}$	0.9990
Glycitein	Skimmed cheese	1–750	$2.26 \cdot 10^{-3} \pm 5.50 \cdot 10^{-5}$	$8.28 \cdot 10^{-3} \pm 1.69 \cdot 10^{-2}$	0.9995	ZEN	Skimmed cheese	1–750	$6.53 \cdot 10^{-3} \pm 7.04 \cdot 10^{-4}$	$-9.70 \cdot 10^{-2} \pm 2.16 \cdot 10^{-1}$	0.9913
	Whole cheese	5–750	$2.34 \cdot 10^{-3} \pm 9.40 \cdot 10^{-5}$	$2.22 \cdot 10^{-2} \pm 2.98 \cdot 10^{-2}$	0.9988		Whole cheese	5–750	$1.90 \cdot 10^{-3} \pm 8.65 \cdot 10^{-5}$	$-1.75 \cdot 10^{-2} \pm 2.64 \cdot 10^{-2}$	0.9984
	Cow kefir	10–750	$2.39 \cdot 10^{-3} \pm 1.47 \cdot 10^{-4}$	$4.06 \cdot 10^{-2} \pm 5.31 \cdot 10^{-2}$	0.9971		Cow kefir	5–750	$2.73 \cdot 10^{-3} \pm 1.34 \cdot 10^{-4}$	$-3.59 \cdot 10^{-2} \pm 4.69 \cdot 10^{-2}$	0.9982
	Goat kefir	10–750	$2.90 \cdot 10^{-3} \pm 2.03 \cdot 10^{-4}$	$2.53 \cdot 10^{-2} \pm 7.35 \cdot 10^{-2}$	0.9963		Goat kefir	5–750	$4.67 \cdot 10^{-3} \pm 1.40 \cdot 10^{-4}$	$-2.39 \cdot 10^{-2} \pm 4.80 \cdot 10^{-2}$	0.9993
Enterolactone	Skimmed cheese	1–750	$3.30 \cdot 10^{-3} \pm 1.28 \cdot 10^{-4}$	$8.75 \cdot 10^{-2} \pm 3.93 \cdot 10^{-2}$	0.9988	EE_2	Skimmed cheese	5–750	$3.39 \cdot 10^{-3} \pm 4.78 \cdot 10^{-5}$	$-2.38 \cdot 10^{-2} \pm 1.47 \cdot 10^{-2}$	0.9997
	Whole cheese	5–750	$2.70 \cdot 10^{-3} \pm 1.50 \cdot 10^{-4}$	$6.78 \cdot 10^{-2} \pm 5.52 \cdot 10^{-2}$	0.9971		Whole cheese	5–750	$1.42 \cdot 10^{-3} \pm 1.16 \cdot 10^{-4}$	$-3.11 \cdot 10^{-2} \pm 3.42 \cdot 10^{-2}$	0.9950
	Cow kefir	5–750	$8.83 \cdot 10^{-3} \pm 8.29 \cdot 10^{-5}$	$3.25 \cdot 10^{-2} \pm 2.90 \cdot 10^{-2}$	0.9999		Cow kefir	10–750	$2.06 \cdot 10^{-3} \pm 2.10 \cdot 10^{-4}$	$-6.47 \cdot 10^{-2} \pm 6.24 \cdot 10^{-2}$	0.9922
	Goat kefir	5–750	$3.98 \cdot 10^{-3} \pm 2.43 \cdot 10^{-4}$	$8.33 \cdot 10^{-2} \pm 7.22 \cdot 10^{-2}$	0.9972		Goat kefir	5–750	$2.07 \cdot 10^{-3} \pm 9.61 \cdot 10^{-5}$	$-3.18 \cdot 10^{-2} \pm 2.96 \cdot 10^{-2}$	0.9984
Genistein	Skimmed cheese	1–750	$2.97 \cdot 10^{-3} \pm 3.26 \cdot 10^{-4}$	$-1.99 \cdot 10^{-2} \pm 1.14 \cdot 10^{-1}$	0.9910	DES	Skimmed cheese	5–750	$1.24 \cdot 10^{-2} \pm 7.25 \cdot 10^{-4}$	$-8.96 \cdot 10^{-2} \pm 2.29 \cdot 10^{-1}$	0.9974
	Whole cheese	5–750	$2.23 \cdot 10^{-3} \pm 1.11 \cdot 10^{-4}$	$-1.65 \cdot 10^{-2} \pm 3.29 \cdot 10^{-2}$	0.9981		Whole cheese	5–750	$7.20 \cdot 10^{-3} \pm 4.93 \cdot 10^{-4}$	$-1.23 \cdot 10^{-1} \pm 1.46 \cdot 10^{-1}$	0.9965
	Cow kefir	5–750	$4.91 \cdot 10^{-3} \pm 9.97 \cdot 10^{-5}$	$-2.29 \cdot 10^{-2} \pm 2.95 \cdot 10^{-2}$	0.9997		Cow kefir	5–750	$7.12 \cdot 10^{-3} \pm 1.79 \cdot 10^{-4}$	$-7.76 \cdot 10^{-2} \pm 5.66 \cdot 10^{-2}$	0.9995
	Goat kefir	5–750	$4.05 \cdot 10^{-3} \pm 1.23 \cdot 10^{-4}$	$-8.00 \cdot 10^{-3} \pm 3.66 \cdot 10^{-2}$	0.9993		Goat kefir	5–750	$1.26 \cdot 10^{-2} \pm 8.31 \cdot 10^{-4}$	$-1.25 \cdot 10^{-1} \pm 2.56 \cdot 10^{-1}$	0.9967
Formononetin	Skimmed cheese	5–750	$9.91 \cdot 10^{-4} \pm 1.07 \cdot 10^{-4}$	$2.11 \cdot 10^{-2} \pm 3.36 \cdot 10^{-2}$	0.9913	DS	Skimmed cheese	5–750	$1.60 \cdot 10^{-2} \pm 4.97 \cdot 10^{-4}$	$-1.34 \cdot 10^{-1} \pm 1.75 \cdot 10^{-1}$	0.9992
	Whole cheese	5–750	$6.75 \cdot 10^{-4} \pm 2.02 \cdot 10^{-5}$	$-3.43 \cdot 10^{-3} \pm 6.24 \cdot 10^{-3}$	0.9993		Whole cheese	5–750	$1.02 \cdot 10^{-2} \pm 7.38 \cdot 10^{-4}$	$-2.21 \cdot 10^{-1} \pm 2.34 \cdot 10^{-1}$	0.9960
	Cow kefir	5–750	$1.36 \cdot 10^{-3} \pm 3.32 \cdot 10^{-5}$	$-1.27 \cdot 10^{-2} \pm 1.05 \cdot 10^{-2}$	0.9995		Cow kefir	5–750	$9.03 \cdot 10^{-3} \pm 2.55 \cdot 10^{-4}$	$-5.62 \cdot 10^{-2} \pm 8.07 \cdot 10^{-2}$	0.9994
	Goat kefir	5–750	$1.97 \cdot 10^{-3} \pm 8.21 \cdot 10^{-5}$	$-2.19 \cdot 10^{-2} \pm 2.60 \cdot 10^{-2}$	0.9990		Goat kefir	5–750	$1.35 \cdot 10^{-2} \pm 9.39 \cdot 10^{-4}$	$-1.77 \cdot 10^{-1} \pm 2.98 \cdot 10^{-1}$	0.9963
Prunetin	Skimmed cheese	5–750	$3.69 \cdot 10^{-2} \pm 3.33 \cdot 10^{-3}$	$-5.44 \cdot 10^{-1} \pm 1.03 \cdot 10$	0.9940	HEX	Skimmed cheese	5–750	$2.34 \cdot 10^{-2} \pm 5.47 \cdot 10^{-4}$	$-1.72 \cdot 10^{-1} \pm 1.73 \cdot 10^{-1}$	0.9996
	Whole cheese	10–750	$7.67 \cdot 10^{-3} \pm 2.81 \cdot 10^{-4}$	$-4.40 \cdot 10^{-2} \pm 8.68 \cdot 10^{-2}$	0.9990		Whole cheese	5–750	$1.28 \cdot 10^{-2} \pm 9.54 \cdot 10^{-4}$	$-2.26 \cdot 10^{-1} \pm 3.01 \cdot 10^{-1}$	0.9958
	Cow kefir	15–750	$8.21 \cdot 10^{-3} \pm 4.16 \cdot 10^{-4}$	$-5.83 \cdot 10^{-2} \pm 1.50 \cdot 10^{-1}$	0.9981		Cow kefir	5–750	$1.52 \cdot 10^{-2} \pm 5.70 \cdot 10^{-4}$	$-1.22 \cdot 10^{-1} \pm 1.80 \cdot 10^{-1}$	0.9989
	Goat kefir	5–750	$2.69 \cdot 10^{-2} \pm 9.44 \cdot 10^{-4}$	$-3.01 \cdot 10^{-2} \pm 3.00 \cdot 10^{-1}$	0.9993		Goat kefir	5–750	$1.73 \cdot 10^{-2} \pm 1.40 \cdot 10^{-3}$	$-1.70 \cdot 10^{-1} \pm 4.90 \cdot 10^{-1}$	0.9951
Biochanin A	Skimmed cheese	5–750	$1.80 \cdot 10^{-2} \pm 1.50 \cdot 10^{-3}$	$-3.14 \cdot 10^{-1} \pm 5.41 \cdot 10^{-1}$	0.9948	E_3	Skimmed cheese	5–750	$3.47 \cdot 10^{-3} \pm 1.73 \cdot 10^{-4}$	$-3.48 \cdot 10^{-2} \pm 5.92 \cdot 10^{-2}$	0.9982
	Whole cheese	5–750	$8.17 \cdot 10^{-3} \pm 4.31 \cdot 10^{-4}$	$-3.76 \cdot 10^{-2} \pm 1.33 \cdot 10^{-1}$	0.9980		Whole cheese	5–750	$3.69 \cdot 10^{-3} \pm 3.71 \cdot 10^{-4}$	$-7.39 \cdot 10^{-2} \pm 1.10 \cdot 10^{-1}$	0.9924
	Cow kefir	5–750	$1.58 \cdot 10^{-2} \pm 1.05 \cdot 10^{-3}$	$-5.52 \cdot 10^{-2} \pm 3.78 \cdot 10^{-1}$	0.9967		Cow kefir	5–750	$3.16 \cdot 10^{-3} \pm 8.76 \cdot 10^{-5}$	$-2.35 \cdot 10^{-2} \pm 3.06 \cdot 10^{-2}$	0.9994
	Goat kefir	5–750	$1.77 \cdot 10^{-2} \pm 1.26 \cdot 10^{-3}$	$8.20 \cdot 10^{-2} \pm 3.87 \cdot 10^{-1}$	0.9962		Goat kefir	5–750	$2.63 \cdot 10^{-3} \pm 1.45 \cdot 10^{-4}$	$-1.75 \cdot 10^{-2} \pm 4.28 \cdot 10^{-2}$	0.9977
β-ZAL	Skimmed cheese	5–750	$3.38 \cdot 10^{-3} \pm 2.50 \cdot 10^{-4}$	$-2.75 \cdot 10^{-2} \pm 8.73 \cdot 10^{-2}$	0.9959	$17\beta\text{-}E_2$	Skimmed cheese	5–750	$2.37 \cdot 10^{-3} \pm 1.54 \cdot 10^{-4}$	$-4.92 \cdot 10^{-2} \pm 4.87 \cdot 10^{-2}$	0.9968
	Whole cheese	5–750	$2.60 \cdot 10^{-3} \pm 6.07 \cdot 10^{-5}$	$-1.11 \cdot 10^{-2} \pm 2.02 \cdot 10^{-2}$	0.9997		Whole cheese	5–750	$1.66 \cdot 10^{-3} \pm 1.61 \cdot 10^{-4}$	$-4.20 \cdot 10^{-2} \pm 5.82 \cdot 10^{-2}$	0.9930
	Cow kefir	10–750	$3.14 \cdot 10^{-3} \pm 1.07 \cdot 10^{-4}$	$5.63 \cdot 10^{-4} \pm 3.40 \cdot 10^{-2}$	0.9991		Cow kefir	5–750	$1.87 \cdot 10^{-3} \pm 8.43 \cdot 10^{-5}$	$-3.04 \cdot 10^{-2} \pm 2.95 \cdot 10^{-2}$	0.9985
	Goat kefir	5–750	$3.08 \cdot 10^{-3} \pm 1.35 \cdot 10^{-4}$	$-2.60 \cdot 10^{-2} \pm 4.15 \cdot 10^{-2}$	0.9986		Goat kefir	5–750	$2.12 \cdot 10^{-3} \pm 1.44 \cdot 10^{-4}$	$-4.45 \cdot 10^{-2} \pm 4.55 \cdot 10^{-2}$	0.9965
β-ZEL	Skimmed cheese	5–750	$1.69 \cdot 10^{-3} \pm 8.48 \cdot 10^{-5}$	$-1.84 \cdot 10^{-2} \pm 2.51 \cdot 10^{-2}$	0.9977	$17\alpha\text{-}E_2$	Skimmed cheese	5–750	$3.24 \cdot 10^{-3} \pm 1.40 \cdot 10^{-4}$	$-5.56 \cdot 10^{-2} \pm 4.93 \cdot 10^{-2}$	0.9986
	Whole cheese	5–750	$1.22 \cdot 10^{-3} \pm 2.06 \cdot 10^{-5}$	$2.06 \cdot 10^{-3} \pm 6.51 \cdot 10^{-3}$	0.9998		Whole cheese	5–750	$2.02 \cdot 10^{-3} \pm 1.87 \cdot 10^{-4}$	$-5.54 \cdot 10^{-2} \pm 5.54 \cdot 10^{-2}$	0.9936
	Cow kefir	10–750	$1.25 \cdot 10^{-3} \pm 6.81 \cdot 10^{-5}$	$-1.38 \cdot 10^{-2} \pm 2.46 \cdot 10^{-2}$	0.9978		Cow kefir	5–750	$2.14 \cdot 10^{-3} \pm 1.53 \cdot 10^{-4}$	$-4.37 \cdot 10^{-2} \pm 4.84 \cdot 10^{-2}$	0.9961
	Goat kefir	5–750	$1.60 \cdot 10^{-3} \pm 6.45 \cdot 10^{-5}$	$-4.61 \cdot 10^{-3} \pm 1.99 \cdot 10^{-2}$	0.9988		Goat kefir	5–750	$2.48 \cdot 10^{-3} \pm 1.29 \cdot 10^{-4}$	$-3.16 \cdot 10^{-2} \pm 3.98 \cdot 10^{-2}$	0.9980
α-ZAL	Skimmed cheese	1–750	$6.96 \cdot 10^{-3} \pm 3.34 \cdot 10^{-4}$	$-5.84 \cdot 10^{-2} \pm 1.02 \cdot 10^{-1}$	0.9983	E_1	Skimmed cheese	5–750	$1.66 \cdot 10^{-2} \pm 3.74 \cdot 10^{-4}$	$-1.26 \cdot 10^{-1} \pm 1.31 \cdot 10^{-1}$	0.9996
	Whole cheese	5–750	$3.65 \cdot 10^{-3} \pm 1.89 \cdot 10^{-4}$	$-2.67 \cdot 10^{-2} \pm 5.82 \cdot 10^{-2}$	0.9980		Whole cheese	5–750	$1.03 \cdot 10^{-2} \pm 9.87 \cdot 10^{-4}$	$-2.40 \cdot 10^{-1} \pm 3.13 \cdot 10^{-1}$	0.9931
	Cow kefir	10–750	$4.87 \cdot 10^{-3} \pm 8.45 \cdot 10^{-5}$	$2.94 \cdot 10^{-2} \pm 2.97 \cdot 10^{-2}$	0.9998		Cow kefir	5–750	$1.13 \cdot 10^{-1} \pm 6.14 \cdot 10^{-4}$	$-1.40 \cdot 10^{-1} \pm 2.16 \cdot 10^{-1}$	0.9978
	Goat kefir	5–750	$4.18 \cdot 10^{-3} \pm 2.76 \cdot 10^{-4}$	$4.21 \cdot 10^{-2} \pm 8.71 \cdot 10^{-2}$	0.9967		Goat kefir	5–750	$1.07 \cdot 10^{-2} \pm 5.05 \cdot 10^{-4}$	$-6.92 \cdot 10^{-2} \pm 1.49 \cdot 10^{-1}$	0.9983

R^2: Determination coefficient.

Table 4
Results of the recovery study (n = 5) of the QuEChERS-UHPLC–MS/MS method for the selected compounds in the different matrices at three levels of concentration.

Analyte	Type of matrix	Level 1[a] (n = 5) Recovery% (RSD, %)	Level 2[b] (n = 5) Recovery% (RSD, %)	Level 3[c] (n = 5) Recovery% (RSD, %)	LOQ_{method}[d] (μg/kg)	Analyte	Type of matrix	Level 1[a] (n = 5) Recovery% (RSD, %)	Level 2[b] (n = 5) Recovery% (RSD, %)	Level 3[c] (n = 5) Recovery% (RSD, %)	LOQ_{method}[d] (μg/kg)
Daidzein	Skimmed cheese	109 (5)	94 (10)	92 (4)	0.025	α-ZEL	Skimmed cheese	96 (8)	112 (8)	93 (8)	0.250
	Whole cheese	109 (8)	84 (6)	90 (3)	0.050		Whole cheese	83 (11)	83 (9)	80 (2)	0.250
	Cow kefir	119 (13)	119 (4)	114 (7)	0.050		Cow kefir	93 (11)	73 (5)	94 (7)	0.250
	Goat kefir	100 (8)	103 (3)	99 (5)	0.050		Goat kefir	91 (4)	78 (11)	80 (4)	0.500
Enterodiol	Skimmed cheese	97 (10)	94 (9)	91 (4)	0.250	ZAN	Skimmed cheese	78 (9)	119 (4)	99 (8)	0.250
	Whole cheese	90 (14)	80 (6)	90 (7)	0.250		Whole cheese	84 (9)	78 (6)	88 (8)	0.250
	Cow kefir	103 (6)	93 (8)	99 (5)	0.500		Cow kefir	111 (9)	74 (6)	84 (8)	2.50
	Goat kefir	107 (1)	84 (6)	91 (8)	0.250		Goat kefir	110 (7)	86 (6)	84 (5)	0.250
Glycitein	Skimmed cheese	102 (9)	100 (8)	101 (3)	0.050	ZEN	Skimmed cheese	97 (10)	116 (3)	112 (9)	0.050
	Whole cheese	106 (6)	81 (6)	90(7)	0.250		Whole cheese	98 (15)	71 (3)	78 (3)	0.250
	Cow kefir	98 (11)	109 (10)	97 (7)	0.500		Cow kefir	93 (7)	77 (9)	82 (5)	0.250
	Goat kefir	106 (7)	96 (6)	101 (8)	0.500		Goat kefir	102 (9)	76 (8)	55 (8)	0.250
Enterolactone	Skimmed cheese	109 (10)	115 (7)	110 (2)	0.050	EE_2	Skimmed cheese	80 (6)	102 (8)	103 (6)	0.250
	Whole cheese	98 (7)	119 (7)	113 (10)	0.250		Whole cheese	97 (6)	90 (12)	102 (9)	0.250
	Cow kefir	108 (6)	118 (6)	106 (10)	0.250		Cow kefir	93 (7)	92 (8)	88 (8)	0.500
	Goat kefir	109 (11)	112 (6)	78 (11)	0.250		Goat kefir	85 (13)	100 (9	87 (4)	0.250
Genistein	Skimmed cheese	109 (8)	98 (9)	92 (2)	0.050	DES	Skimmed cheese	87 (9)	86 (7)	97 (10)	0.250
	Whole cheese	102 (6)	78 (6)	82 (4)	0.250		Whole cheese	105 (5)	108 (9)	86 (9)	0.250
	Cow kefir	100 (9)	107 (12)	108 (11)	0.250		Cow kefir	101 (10)	80 (7)	79 (6)	0.250
	Goat kefir	100 (8)	118 (8)	82 (8)	0.250		Goat kefir	81 (8)	83 (6)	91 (8)	0.250
Formononetin	Skimmed cheese	88 (9)	96 (15)	92 (4)	0.250	DS	Skimmed cheese	85 (9)	90 (9)	107 (2)	0.250
	Whole cheese	87 (10)	70 (3)	77 (12)	0.250		Whole cheese	86 (7)	97 (10)	83 (9)	0.250
	Cow kefir	103 (10)	103 (9)	100 (10)	0.250		Cow kefir	88 (4)	81 (6)	75 (6)	0.250
	Goat kefir	112 (9)	112 (9)	74 (8)	0.250		Goat kefir	81 (14)	88 (3)	84 (5)	0.250
Prunetin	Skimmed cheese	99 (7)	90 (9)	96 (8)	0.250	HEX	Skimmed cheese	100 (5)	108 (5)	114 (2)	0.250
	Whole cheese	79 (11)	76 (5)	70 (5)	0.500		Whole cheese	75 (9)	106 (7)	93 (9)	0.250
	Cow kefir	77 (10)	77 (4)	86 (7)	0.750		Cow kefir	109 (5)	96 (8)	81 (6)	0.250
	Goat kefir	102 (8)	94 (3)	78 (4)	0.250		Goat kefir	84 (13)	101 (4)	95 (4)	0.250
Biochanin A	Skimmed cheese	97 (6)	89 (8)	92 (10)	0.250	E_3	Skimmed cheese	109 (10)	96 (9)	103 (13)	0.250
	Whole cheese	78 (4)	82 (9)	92 (10)	0.250		Whole cheese	89 (12)	97 (4)	85 (8)	0.250
	Cow kefir	94 (5)	83 (3)	85 (4)	0.250		Cow kefir	102 (6)	119 (7)	105 (6)	0.250
	Goat kefir	105 (14)	71 (11)	76 (6)	0.250		Goat kefir	96 (14)	100 (4)	96(2)	0.250
β-ZAL	Skimmed cheese	90 (7)	112 (6)	92 (2)	0.250	17β-E_2	Skimmed cheese	92 (8)	114 (6)	94 (9)	0.250
	Whole cheese	94 (11)	92 (1)	84 (3)	0.250		Whole cheese	92 (10)	80 (10)	84 (13)	0.250
	Cow kefir	98 (10)	99 (6)	99 (6)	0.500		Cow kefir	77 (9)	93 (6)	96 (8)	0.250
	Goat kefir	86 (10)	103 (5)	94 (3)	0.250		Goat kefir	95 (9)	86 (9)	91 (4)	0.250
β-ZEL	Skimmed cheese	98 (8)	111 (8)	89 (3)	0.250	17α-E_2	Skimmed cheese	107 (9)	110 (5)	100 (8)	0.250
	Whole cheese	84 (10)	71 (3)	81 (4)	0.250		Whole cheese	81 (11)	95 (12)	93 (12)	0.250
	Cow kefir	73 (4)	93 (10)	99 (6)	0.500		Cow kefir	99 (6)	103 (10)	97 (5)	0.250
	Goat kefir	78 (9)	81 (11)	76 (6)	0.250		Goat kefir	96 (4)	89 (6)	92 (3)	0.250
α-ZAL	Skimmed cheese	84 (10)	106 (9)	97 (6)	0.050	E_1	Skimmed cheese	112 (6)	107 (3)	101 (8)	0.250
	Whole cheese	90 (8)	78 (5)	71 (5)	0.250		Whole cheese	96 (9)	100 (6)	91 (5)	0.250
	Cow kefir	102 (11)	90 (9)	106 (8)	0.500		Cow kefir	96 (5)	109 (9)	99 (5)	0.250
	Goat kefir	100 (4)	89 (1)	109 (9)	0.250		Goat kefir	85 (10)	103 (2)	99(3)	0.250

[a] Concentrations of the analytes in the samples: 1.88 μg/kg except in cow kefir where they were 4 μg/kg for ZAN and 2.56 μg/kg for natural and synthetic estrogens and formononetin and prunetin.
[b] Concentrations of the analytes in the samples: 17.5 μg/kg.
[c] Concentrations of the analytes in the samples: 37.5 μg/kg.
[d] Defined as the lowest matrix matched calibration concentration which provided a signal noise ratio higher than 10 for the quantification transition and at least 3 for the confirmation transition (if it was available).

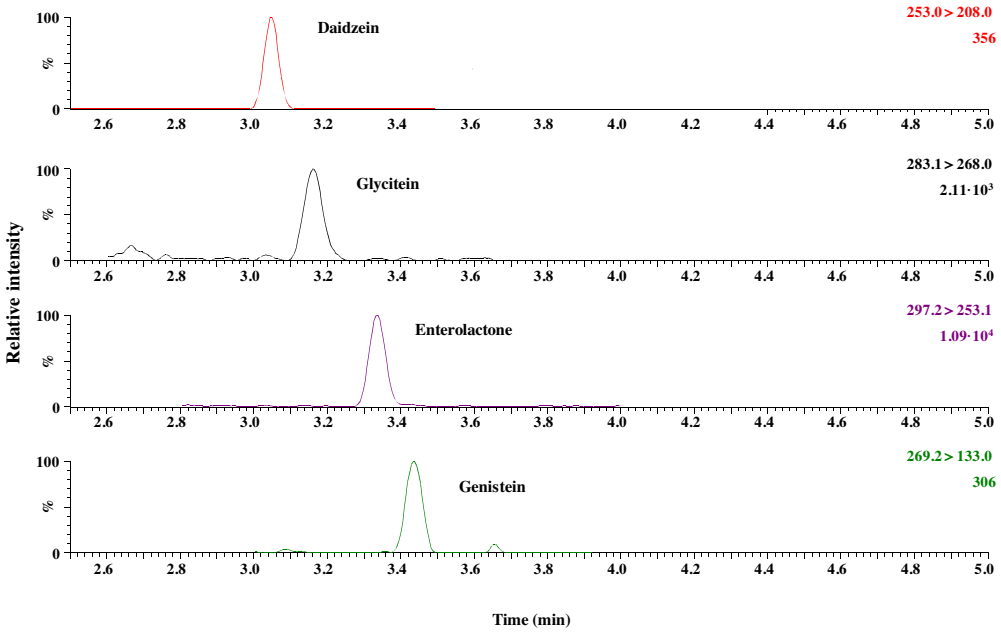

Fig. 2. UHPLC–MS/MS chromatograms of the analytes found in a blank whole cheese sample after the QuEChERS procedure. Injection volume: 5 μL. Sample dissolved in 500 μL of 50/50 (v/v) MeOH/water. Separation at 40 °C.

Table 5
Analysis of real samples using the developed QuEChERS-UHPLC–MS/MS method.

Analytes	Concentration of analyte (μg/kg)[a,b]							
	SC1	SC2	WC1	WC2	CK1	CK2	GK1	GK2
Daidzein	1.8 ± 0.9	3.3 ± 0.9	<LOQ	<LOQ	n.d.	n.d.	<LOQ	4.8 ± 1.3
Glycitein	13.6 ± 0.4	25.1 ± 0.5	1.9 ± 0.8	3.4 ± 0.8	n.d.	n.d.	5.9 ± 1.4	11.4 ± 1.4
Enterolactone	34.7 ± 0.7	46.7 ± 0.8	23.3 ± 1.0	24.5 ± 1.1	8.8 ± 0.2	n.d.	<LOQ	4.0 ± 1.1
Genistein	<LOQ	<LOQ	<LOQ	<LOQ	n.d.	n.d.	<LOQ	1.1 ± 0.6

[a] Results obtained as an average of two analyses for each product.
[b] n.d.: not detected. SC: skimmed cheese; WC: whole cheese; CK: cow kefir; GC: goat kefir.

four natural estrogens prior their determination by UHPLC–MS/MS obtaining LOQs in the range 0.77–237 μg/kg, while in the article reported by our group [17] a HF-LPME-HPLC-DAD/FD method was applied for the determination of four natural and four synthetic estrogens in 3 g of cheese samples using octanol as extraction solvent and resulting LOQs from 2.3 to 5.4 μg/kg. With respect to phytoestrogens analysis, Kuhnle et al. [6] applied a LLE using 2 mL of MeOH in sodium acetate to 100 mg of sample followed by a hydrolysis with β-glucuronidase/α-glucosidase and a SPE in C_{18} cartridges for the determination of twelve phytoestrogens by HPLC-QTrap-MS/MS, obtaining a LOQ for all analytes of 50 μg/kg. Křížová et al. [7] used the same hydrolysis reactant and ethyl acetate for the LLE of four phytoestrogens from cheese samples prior their analysis by HPLC-IT-MS/MS. In this case the LOQs were around 6–17 μg/kg. As can be seen, LOQs preciously obtained are considerably higher than the ones achieved in this work for cheese (between 0.025 and 0.50 μg/kg) which demonstrates the great sensitivity of the developed methodology and their suitable application for the analysis of estrogenic compounds whose occurrence has been reported at very low concentration in dairy products [1,6].

4. Conclusions

In this work, a methodology based on the analysis by UHPLC-QqQ-MS/MS after the application of the QuEChERS method has been applied for the determination of a group of 22 endocrine disruptors including 8 phytoestrogens, 6 mycoestrogens as well as 4 natural and 4 synthetic estrogens in complex dairy products samples such as skimmed and whole cheese and kefir with cow and goat origin. Validation of the whole method was carried out in terms of matrix effect, linearity, repeatability and reproducibility obtaining excellent results as well as recovery values in the range 70–119% and low LOQs (between 0.025 and 0.50 μg/kg for cheese and between 0.05 and 2.5 μg/kg for kefir samples). The developed procedure constitutes an environmental friendly method which combines the advantages that QuEChERS extraction provides in terms of simplicity and low cost, together with the great specificity, reproducibility and sensitivity of the UHPLC-QqQ-MS/MS system to determine the selected estrogenic compounds. According to the good results obtained, the methodology was successfully applied for the analysis of real samples finding the presence of some phytoestrogens in the majority of the analyzed products. Therefore, this method presents a clear alternative for the determination of this group of endocrine disruptors in very complex samples in which their determination and control presents a great relevance due to the hormonal disorders and diseases that can produce in consumers of this type of commercial products.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2017.03.034.

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