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Development of analytical methodologies for the determination of compounds with oestrogenic activity

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AUTOR/A

BARBARA	SOCAS	RODRIGUEZ

DIRECTOR/A

MIGUEL ANGEL	RODRIGUEZ	DELGADO

CODIRECTOR/A

JAVIER	HERNANDEZ	BORGES

DEPARTAMENTO O INSTITUTO UNIVERSITARIO

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Development of analytical Methodologies for the Determination of Compounds with Oestrogenic Activity

Bárbara Socas Rodríguez

Supervisors

Miguel Ángel Rodríguez Delgado
Javier Hernández Borges

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D. MIGUEL ÁNGEL RODRÍGUEZ DELGADO, CATEDRÁTICO DE UNIVERSIDAD, Y D. JAVIER HERNÁNDEZ BORGES, PROFESOR TITULAR DEL DEPARTAMENTO DE QUÍMICA DE LA FACULTAD DE CIENCIAS DE LA UNIVERSIDAD DE LA LAGUNA,

INFORMAN:

Que Dña. Bárbara Socas Rodríguez, Licenciada en Química por la Universidad de La Laguna, ha realizado bajo nuestra dirección los trabajos conducentes a la realización de su Tesis Doctoral titulada DEVELOPMENT OF ANALYTICAL METHODOLOGIES FOR THE DETERMINATION OF COMPOUNDS WITH OESTROGENIC ACTIVITY.

Revisado el trabajo, autorizamos su presentación, para que se pueda proceder a su lectura y defensa pública, y optar al grado de Doctora en Química con Mención Internacional por esta Universidad.

Y para que así conste, firmamos el presente en San Cristóbal de La Laguna, a veinticinco de mayo de dos mil diecisiete.

Fdo: Dr. Miguel Ángel Rodríguez Delgado Fdo: Dr. Javier Hernández Borges

FACULTAD DE CIENCIAS

DEPARTAMENTO DE QUÍMICA

DEVELOPMENT OF ANALYTICAL METHODOLOGIES FOR THE DETERMINATION OF COMPOUNDS WITH OESTROGENIC ACTIVITY

MEMORIA PARA LA OBTENCIÓN DEL GRADO DE DOCTORA

BÁRBARA SOCAS RODRÍGUEZ

SAN CRISTÓBAL DE LA LAGUNA, TENERIFE

MAYO 2017

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A mis padres

A mis hermanos

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INTRODUCTION

I.- INTRODUCTION

I.1.- The endocrine system

The endocrine system is, together with the nervous system, responsible of the metabolism coordination of mammalians. Both of them regulate the internal environment of the organism maintaining it in a homeostasic state (physiological balance) in which the system is modified only according to its requirements. The activity of the endocrine system is based on the release of chemical messengers (*hormones*) from the endocrine glands and specialised tissues when a new signal is received. These hormones are transported through the bloodstream until they reach the target cells in which they trigger the metabolic process they were designed for (Debuse, 2001). Among the different functions that are coordinated by the endocrine system, the most relevant are: cell and tissue growth, heartbeat, blood pressure, renal function, gastrointestinal tract mobility, secretion of digestive enzymes as well as metabolism, breastfeeding and the activity of the reproductive system (Herrera, 1991).

The structure of the endocrine system has a hierarchic distribution constituted by diverse glands and tissues along the body, which are coordinated by the hypothalamus. This gland is responsible of releasing hypothalamic hormones which are sent to the adenohypophysis. In turn, the adenohypophysis synthesises tropic hormones which are transported through the bloodstream to the peripheral glands such as: adrenal, pineal and reproductive glands (ovaries and testes), the thyroid, the parathyroid and the pancreas. Finally, these glands generate specific hormones that are carried by the blood to their receptors which are on the surface or inside of the target cells (Koolman and Röhm, 2004; Nelson and Cox, 2013).

I.1.1.- Hormones

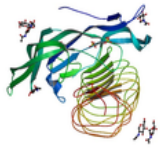
The classification of hormones can be developed according to different criteria. Among them, one of the most widely applied is based on their biochemical structure. In this sense, it is possible to distinguish between *peptidic*, *amino acid* and *steroid hormones* (see Figure I.1):

- *Peptidic hormones*. Most hormones belong to this class. It is comprised by peptides from 3 to more than 200 amino acids, including pancreatic hormones such as *insulin* and *glucagon* which regulate the level of glucose in blood, and all the hormones generated in

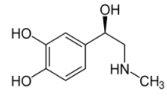
the hypothalamus and hypophysis as, for example, the glycoprotein *lutropin* that regulates the secretion of two specific hormones such as *testosterone* and *progesterone*.

- *Amino acid hormones*. This group is formed by hydrosoluble compounds synthesised in the cell cytoplasm and stored in secretion granules until their final release. Some of them are produced by the thyroid gland, as in the case of *thyroxine* and *triiodothyronine* which are more lipophilic than the rest and regulate the energetic metabolism, especially in liver and muscles. Others, called *catecholamines*, are generated by the adrenal gland, being *adrenaline* and *dopamine* (DA) some of the most relevant. Their main functions include: the increase of the heartbeat, the shrink of the blood vessels, the stimulation of the metabolism of *glucagon* in the liver and muscles and the dilatation of the bronchus.
- *Steroid hormones*. This group is formed by lipids derived from *cholesterol* and synthesised in the mitochondria and the smooth endoplasmic reticulum. Due to their hydrophobic nature, they need to be linked to plasmatic proteins to adequately flow through the bloodstream until they reach the target cells. This group is constituted by *corticosteroids* and sexual hormones. The first ones, which are synthesised by the adrenal cortex, can be classified into *glucocorticoids* as, for example, *cortisol*, and *mineralocorticoids* like *aldosterone*. With respect to the second group, their secretion is carried out by the testes (this is the case of *androgens* like *testosterone*) and by ovaries (the case of *progestogens* like *progesterone* and *oestrogens* as, for example, *estradiol*, E_2). Apart from *corticosteroids* and sex hormones, there are other steroids that can be included in this group since it shares a great number of features with the other two: the derivatives of vitamin D, like *calcitriol*, which has as principal activity the stimulation of the absorption of Ca^{2+} in the intestine and the increase of its concentration in blood.

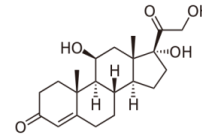
In addition to the three groups described above, there is another type of compounds that, although they are not synthesised in the same way as the rest of endocrine hormones, they can be consider as such due to their endocrine activity (see Figure I.1). They are known as *eicosanoid hormones* and are generated from *arachinodic acid* in the cell membrane. Their function is developed in closed cells due to their instability and insolubility. Among them, three relevant types can be found: *prostaglandins* and *leukotrienes*, which are involved in muscle constriction, as well as *thromboxanes*, which control blood coagulation (Herrera, 1991; Koolman and Röhm, 2004; Nelson and Cox, 2013).



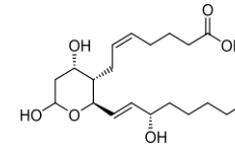
Lutropin (peptide)



Adrenaline (amino acid)



Cortisol (steroid)



Thromboxane (eicosanoid)

Figure I.1.- Some examples of different hormone structures.

Hormone levels in blood are normally extremely low (in the range 10^{-7}-10^{-12} mol/L) and vary considerably depending on the physiological cycles (Koolman and Röhm, 2004). An important aspect related to this variation is the type of secretion and transport mode of the hormone. In this sense, when they are released in an *endocrine* mode, they are secreted to the blood and should go across the organism until they reach the target cell. In some occasions, they can go through distances of one metre and their biological effects may need minutes or even hours to be accomplished. On the contrary, there are other hormones that act in a *paracrine* mode, which means that they are discharged in the extracellular space and their activity is developed in the neighbouring cells, or in an *autocrine* mode in which they act in the same cell where they are secreted by their linking to the receptors that are present in the cellular surface. Apart from such secretion modes, some authors include in this description the ones known as *pheromones* that have been found in other species such as insects, fungus or algae since their effects unleash a response in another individual of the same species. However, in general terms, pheromones are not currently included in the group of hormones since they are considered as their ancestors in the evolutionary system (Herrera, 1991; Nelson and Cox, 2013).

Another relevant aspect that has an important effect in the hormone levels in blood, is their activity on the target cell. In this sense, it is possible to distinguish two different behaviours between the hormone and the specific protein receptor of the cell (Figure I.2): hormones that bond to extracellular proteins or G proteins which are on the cell membrane generating a fast response with the modification of one or more enzymes activity, and those that interact with intracellular proteins that are located in the cytoplasm or in the cellular nucleus resulting in a slower response in which the genetic expression is usually modified (Debuse, 2001; Nelson and Cox, 2013). The first behaviour is developed by hydrosoluble hormones such as *peptidic* and *amino acid* hormones which bond to the receptor present in the

cell membrane undergoing a conformational change that results in the production of an intracellular secondary messenger that transfers the information to another molecular system or enzyme inside of the cell. The second case is associated with *steroid* and *thyroid* hormones, which bond to steroid receptors and *thyrosine kinase*, respectively. The lipophilic character of these two enables them to go through the plasmatic membrane and to bond to the receptor inside the cell. In this case, the hormone-receptor complex carries the messenger itself, stimulating the synthesis of proteins and, consequently, modifying the expression of specific genes in the cell nucleus. Despite the fact that these two action systems are completely different, the same hormone can act in both modes depending on the cell in which it develops its activity since it is totally determined by the genetic information contained in such cell (Debuse, 2001).

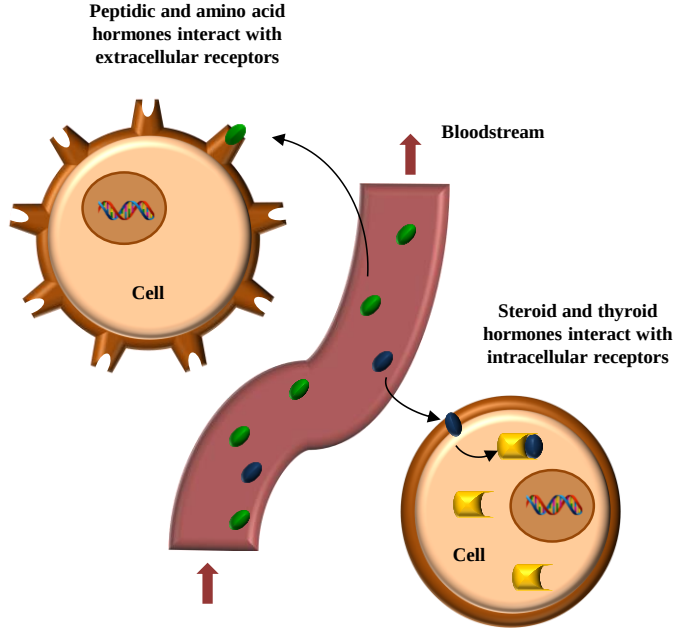


Figure I.2.- Principal interaction mechanisms between hormones and target cells.

I.1.1.1.- Steroid hormones

As it has been previously indicated, steroid hormones are compounds with a lipid nature derived from cholesterol coming from the diet or synthesised by the cells that secret steroid hormones. As a consequence, all of them have a similar chemical structure constituted

by a nucleus of cyclopentaperhydro[a]phenanthrene, also called *gonane*, formed by four rings of carbon atoms with different unsaturation, substituents and lateral chains. Taking all these aspects into account, it is possible to distinguish three different types of steroids hormones based on their primary skeleton (Figure I.3): *oestrogens*, with an *estrane* structure, which are the responsible of the development of the secondary sex characters of females, the development of their organs, the induction of enzymes responsible of the metabolism of carbohydrates and lipids, the control of intestine mobility, blood coagulation and the retention of Na^+ and water by kidney; *androgens*, that have an *androstane* structure and that control the development of the secondary sex characters of males as well as their organs; and, finally, those with a *pregnane* framework in which *progestagens* are included, which are in charge of preparing the endometrium for pregnancy and participate in the synthesis of the rest of steroids hormones, as well as *corticosteroids*. Concerning this last group of hormones, there can be distinguished between *glucocorticoids*, that regulate the metabolism of carbohydrates (Nelson and Cox, 2013), and *mineralocorticoids*, that have different functions such as the control of electrolites concentration in blood, the improvement of Na^+ reabsorption in the kidney, K^+ removal from the urine or the regulation of Ca^{2+} and phosphate concentrations that constitutes the mineral part of bones and teeth (Koolman and Röhm, 2004; Nelson and Cox, 2013).

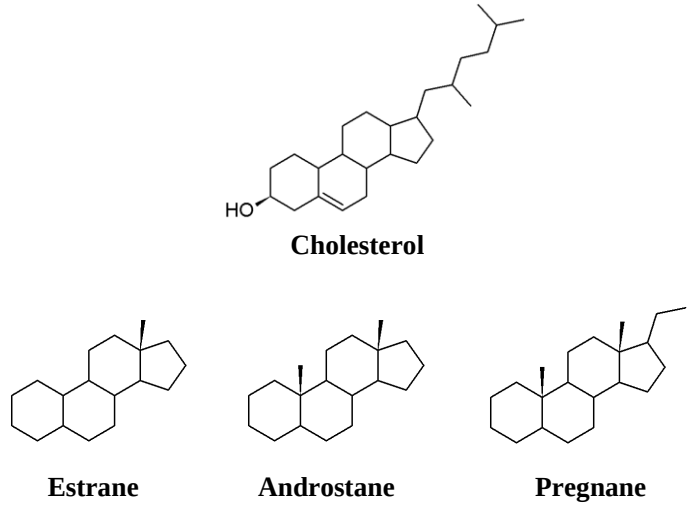


Figure I.3.- Basic structures of steroid hormones derived from the cholesterol molecule.

Concerning the cholesterol necessary for the synthesis of steroid hormones, it is stored in ester lipid drops in the cytoplasm. When a stimulus triggers the secretion of these compounds, cholesterol is discharged from the drops into the mitochondria where it is transformed into *pregnolone* by the enzymatic degradation of its lateral chain, a process that controls the velocity of the steroids genesis. Then, the newly synthesised *pregnolone* is hydroxilated and subsequently oxidised in C_3 resulting in the production of *progesterone*. This hormone is the precursor of the rest of steroid hormones that are generated in the mitochondria or in the smooth endoplasmic reticulum and that are later released to the bloodstream through the cell membrane. Such process is carried out in the endocrine glands responsible of the synthesis of each group of steroid hormones, although their distribution is not completely strict. In general terms, *corticosteroids* are secreted in the adrenal cortex, *oestrogens* and *progestagens* in the ovaries whereas *androgens* are produced in the testes. However, ovaries and testes are able to synthesise *androgens* and *oestrogens*, respectively, and all glands that generate steroid hormones can produce progesterone although they are not able to secrete it (Debuse, 2001; Herrera, 1991).

Once their activity in the organism is over, the metabolism of steroid hormones is carried out almost completely in the liver and kidneys. It is based on a combination of chemical reactions that transform them into more polar compounds such as sulfate esters or gluco-esters that can be removed by the urine and, to a lesser degree, in the bile. The evaluation and study of the concentration of these metabolites in the urine serve as a base of the clinic research of hormonal metabolism (Koolman and Röhm, 2004).

I.2.- Endo and exoestrogens

As it has been previously indicated, *oestrogens* are compounds included in the group of the steroid hormones of mammalians together with *corticosteroids*, *progestagens* and *androgens*. Their chemical structure is, in fact, an *estrane* skeleton of eighteen carbons with an aromatic ring and a hydroxyl group in C_3. Since they are biologically synthesised by the organism, they are called *endoestrogens*. The free forms of endoestrogens are *estrone* (E_1), *17α-estradiol* (17α-E_2), *17β-estradiol* (17β-E_2) and *estriol* (E_3) but there are also a great number of methoxylated, hydroxylated, glucuronated and sulfated metabolites as a result of the different metabolic processes developed by the organism (Tso and Aga, 2010; Zhao et al., 2015).

Apart from endoestrogens, there is another important group of compounds with oestrogenic activity called *exoestrogens* (Figure I.4), which is constituted by a great number of families of compounds with either a synthetic or a natural origin. Also known as endocrine disrupting compounds (EDCs), exoestrogens can act as oestrogen-mimicking substances enhancing or suppressing the oestrogenic activity. Some of them are pesticides, polychlorinated biphenyls (PCBs), bisphenols, phthalates, etc. Among them, there should be highlighted *ethynylestradiol* (EE_2), a synthetic derivative of E_2 which is a human contraceptive, or synthetic stilbenes such as *dienestrol* (DS), *hexestrol* (HEX) or *diethylstilbestrol* (DES) which are commonly used as anabolic steroids (Noppe et al., 2008; Rena et al., 2017; Shao et al., 2005). Other families of oestrogens of natural origin are also included in this group. This is the case of *mycoestrogens*, which are mycotoxins produced by different fungal species of the gender *Fusarium*. Zearalenone (ZEN) and its metabolites *zearalanone* (ZAN), *α-zearalenol* (α-ZEL), *β-zearalenol* (β-ZEL), *α-zearalanol* (α-ZAL) and *β-zearalanol* (β-ZAL) belong to this class of compounds. In fact, they are the only group of mycotoxins that have an oestrogenic effect. Another important family of oestrogenic compounds with a natural origin is constituted by *phytoestrogens*, which are non-steroidal compounds synthesised by plants that have a protective effect against several herbivore pathogens. Their metabolites also have oestrogenic or anti-oestrogenic effects. In this group, several isoflavones, lignans or cumestrans are included (Jarošová et al., 2015).

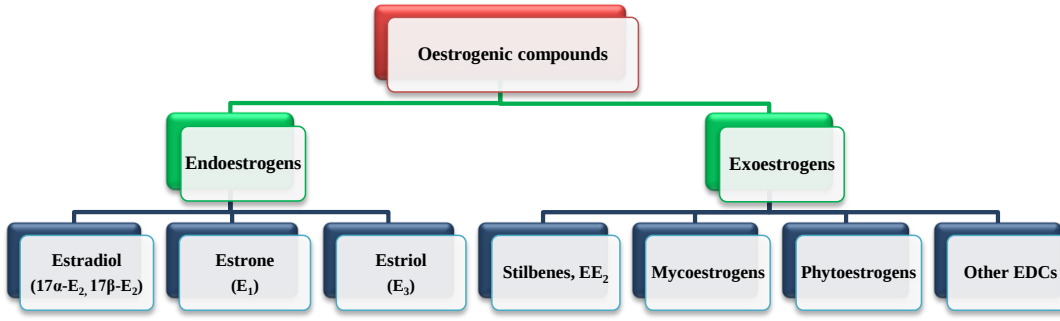


Figure I.4.- General classification of oestrogenic compounds.

I.3.- Selected compounds with oestrogenic activity

As discussed in the previous section, the variety of compounds with oestrogenic activity is considerably large, not only those that are biologically synthesised by the

mammalian organism but also those that have an external origin and that are able of mimicking their functions.

I.3.1.- Natural oestrogens

The most remarkable natural oestrogens or endoestrogens are, as previously indicated, the four forms E_1, 17α-E_2, 17β-E_2 and E_3 which present the structures shown in Table I.1 in which are also indicated their main characteristics. For each of them, the synthetic route is different. 17β-E_2 is principally synthesised in the ovaries as well as E_1, although it is also the product of the metabolism of *androstenedione* in the adipose tissue, which is also secreted by the ovaries and the adrenal cortex. 17β-E_2 is also produced by the uterus of pregnant mammalians. In contrast, E_3 is generated by the oxidation of the other two (17β-E_2 and E_1) by the action of hydroxylases and due to the intestinal reabsorption in the liver and the small intestine (Serrano et al., 2001). In general, 17β-E_2, E_1 and E_3 are the oestrogens most commonly found in blood. 17β-E_2 is also the most abundant oestrogen in tissues, although its isomer, 17α-E_2, has also been found in plasma and urine of pre-partum females as a possible product of the aromatisation of *epitestosterone*. E_3 is the predominant oestrogen during the pregnancy period while in postmenopausal females the level of E_1 is considerably higher respect to the others since the synthesis of this compound in peripheral tissues, such as the adipose tissue, is favoured, especially in obese individuals. However, other peripheral tissues such as the adrenal or breast glands also increase the secretion of other sex steroids hormones during this period. (Alsayari et al., 2017; Hobe et al., 2002; Jouan et al., 2006; U.S. National Toxicology Program, 2016).

Concerning the oestrogenic activity of endoestrogens, the most remarkable is that of 17β-E_2 since it is twelve times higher than that of E_1 and eighty times larger than that of E_3, being 17α-E_2 the one that has the weakest activity (Malekinejad et al., 2006). This aspect is closely related to their affinity with the ligand binding domains of nuclear oestrogenic receptors, ERα (the most dominant) and ERβ, to which they are linked previously to the modification of the genetic expression. Both receptors have similar structures and they only differ in the chromosomal location and the sequence of amino acids. Their location is also different: while ERα is present in adipocytes and in the circulatory, immune and female reproductive systems, ERβ is located in the prostate, ovaries, testes, bladder, urethra, lungs and vascular system. However, it is ERα the one that has a predominant role in the oestrogen

physiology. In fact, an increase of the oestrogens levels brings about an over-activation of this receptor that can result in the development of cancer related to breast and endometrial tissues (Bronowicka-Kłys et al., 2016). This fact evidences the importance of the interaction hormone-receptor as well as its regulation and correct development (Bronowicka-Kłys et al., 2016; Mungenast and Thalhammer, 2014).

Natural oestrogens metabolites are usually methoxylated, hydroxylated, glucuronated and sulfated forms. Although they are supposed to be oestrogenically inactive, some studies have demonstrated their close relation with the development of certain cancer diseases (Tso and Aga, 2010; Zhao et al., 2015). Among them, 2-hydroxyestradiol (2-OHE_2), obtained by the hydroxylation of E_2, is one of the most important biologically synthesised metabolites. Its possible interaction with oestrogen receptors and relation with cancer proliferation has awakened the interest of researchers. However, no conclusive results have been obtained. While some authors attributed the interaction of quinone derivatives of such hydroxylated form of E_2 with DNA to the production of oncogenic mutations and breast cancer (Huang et al., 2012), others suggest their possible inhibiting effects on the proliferation of endometric cells, proposing it as promising treatment against endometrial cancer (Samartzis et al., 2016).

I.3.2.- Synthetic oestrogens

Synthetic oestrogens are compounds designed to have a similar skeleton to that of endoestrogens and, consequently, they can act like them since they also link to oestrogenic receptors. Among them, EE_2 and stilbene derivatives (DES, DS and HEX) are the most relevant. One of the main structural features of oestrogenic compounds is the distance between the carbons 3 and 16-17 and, as can be seen in Table I.1, synthetic oestrogens maintain this proportion in their structure in spite of not having a steroid based nucleus (Serrano et al., 2001).

From a structural point of view, EE_2 is the anthropogenic exoestrogen most similar to endoestrogens. In fact, it presents an estrane framework with an aromatic ring that has a hydroxyl group in C_3 and hydroxyl and alkyl substituents in the cyclopentane ring. This compound has been widely applied in hormone replacement therapy and as treatment for ovulation and alopecia control, and prostate and breast cancer because it is more oral active than natural oestrogens (Noppe et al., 2008; Rena et al., 2017). However, it has also been illegally used as growth promoter in calves (Shao et al., 2005).

Table I.1.- Structure and properties of the most important natural and synthetic estrogens.

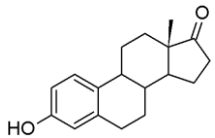
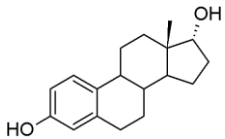
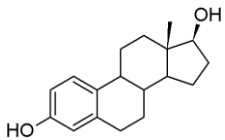
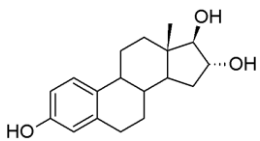
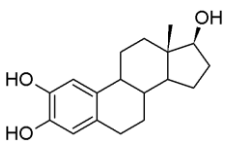
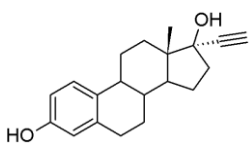
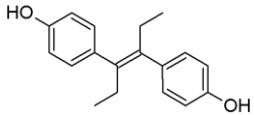
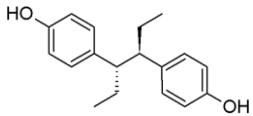
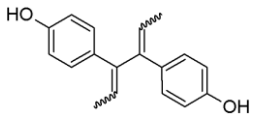
Name	Structure	Formula[a)]	MW[b)] (g/mol)	Water solubility[a), b), c)] (g/L, 25 °C)	Vapour Pressure[a), b), c)] (mmHg, 25 °C)	Log K_{ow}[a), b)]	Melting point[b)] (°C)	pK_a[b)]
Estrone (E_1)		$C_{18}H_{22}O_2$	270.37	$3.0 \cdot 10^{-2}$	$2.49 \cdot 10^{-10}$	3.13	258-260	10.45 ± 0.40
17α-Estradiol (17α-E_2)		$C_{18}H_{24}O_2$	272.39	$3.9 \cdot 10^{-3}$	$9.82 \cdot 10^{-9}$	4.15	216-219	10.27 ± 0.60
17β-Estradiol (17β-E_2)		$C_{18}H_{24}O_2$	272.39	$3.6 \cdot 10^{-3}$	$6.38 \cdot 10^{-9}$	4.01	173-179	10.27 ± 0.60
Estriol (E_3)		$C_{18}H_{24}O_3$	288.39	$2.6 \cdot 10^{-2}$	$9.93 \cdot 10^{-12}$	2.45	281-283	10.25 ± 0.70
2-Hydroxyestradiol (2-OHE_2)		$C_{18}H_{24}O_3$	288.39	-	-	-	190-191	10.12 ± 0.60
17α-Ethynylestradiol (EE_2)		$C_{20}H_{24}O_2$	296.40	$4.8 \cdot 10^{-3}$	$6.00 \cdot 10^{-9}$	4.20	141-146	10.24 ± 0.60

Table I.1.- (Continued).

Name	Structure	Formula[a)]	MW[b)] (g/mol)	Water solubility[a), b), c)] (g/L, 25 °C)	Vapour Pressure[a), b), c)] (mmHg, 25 °C)	Log K_{ow}[a), b)]	Melting point[b)] (°C)	pK_a[b)]
Diethylstilbestrol (DES)		$C_{18}H_{20}O_2$	268.36	$1.2 \cdot 10^{-2}$	$1.41 \cdot 10^{-8}$	5.07	169-172	10.18 ± 0.26
Hexestrol (HEX)		$C_{18}H_{22}O_2$	270.37	$1.2 \cdot 10^{-2}$	$1.17 \cdot 10^{-8}$	5.60	185-188	9.80 ± 0.26
Dienestrol (DS)		$C_{18}H_{18}O_2$	266.34	$1.2 \cdot 10^{-2}$	$4.29 \cdot 10^{-9}$	5.90	227-228	9.21 ± 0.15

[a)] Taken from SciFinder® (https://scifinder.cas.org). [b)] Taken from the National Center for Biotechnology Information. PubChem Compound database (http://pubchem.ncbi.nlm.nih.gov). [c)] Taken from the Interactive PhysProp database (http://esc.srcinc.com).

MW: Molecular weight; K_{ow}: Octanol/water partition-coefficient; K_a: Acidity constant.

In contrast, stilbene derivatives do not have a steroid based skeleton but they are able to mimic oestrogen activity due to their particular conformation and to the presence of the hydroxyl group in the aromatic ring since such aspect is decisive in the affinity of the analyte for the oestrogenic receptors (Serrano et al., 2001). This group of oestrogenic compounds that includes DES, DS and HEX, has been largely used as veterinary drugs to increase the weight gain of animals and for the treatment of oestrogen deficiency disorders (Liu et al., 2010; Shao et al., 2005).

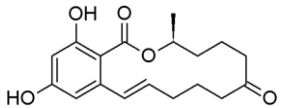
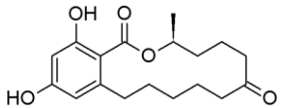
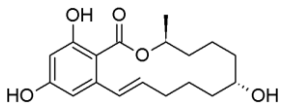
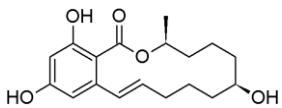
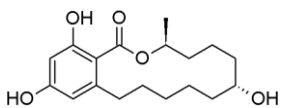
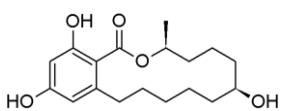
I.3.3.- Mycoestrogens

Another group of oestrogenic compounds widely known are the resorcycle lactones ZEN, ZAN, α-ZEL, β-ZEL, α-ZAL and β-ZAL (see Table I.2), which are secondary metabolites of different *Fusarium* species that usually contaminate cereal grains, corn silage and hay (Pittet, 1998). Their partial similarity with endoestrogens provides them with a high affinity for oestrogen receptors. Consequently, they have a strong oestrogenic capacity which brings about the development of important hormonal disorders in animals and humans such as, hyperestrogenism and severe reproductive and fertility problems (Laganà et al., 2001). Among them, ZEN has been one of the most studied due to their probed hepatotoxic, hematotoxic, immunotoxic, genotoxic, teratogenic and carcinogenic effects in mammalians (Belhassen et al., 2015). Apart from that, ZEN can be bio-transformed by different oxidation and reduction processes carried out in the liver, stomach and intestine into five main metabolites (Figure I.5).

Of special relevance is its conversion to the alpha isomers α-ZEL and α-ZAL, since they both present an affinity nine times higher than the rest of the derivatives for positive human breast cancer receptors and four times stronger than ZEN. In fact, their oestrogenic capacity is equivalent to that of 17β-E_2, the most important endoestrogen (Benzoni et al., 2008; Meucci et al., 2011).

Besides their natural origin, mycoestrogens have also been synthesised for their use as growth promoters of cattle although such application is currently forbidden in the European Union (EU) (Noppe et al., 2008).

Table I.2.- Structure and properties of mycoestrogens.

Name	Structure	Formula[a)]	MW[a)] (g/mol)	Water solubility[a), b)] (g/L, 25 °C)	Vapour Pressure[a), b)] (mmHg, 25 °C)	Log K_{ow}[a), b)]	Melting point[a)] (°C)	pK_a[a)]
Zearalenone (ZEN)		$C_{18}H_{22}O_5$	318.36	$2.7 \cdot 10^{-2}$	$1.07 \cdot 10^{-11}$	3.58	164-165	7.58 ± 0.40
Zearalanone (ZAN)		$C_{18}H_{24}O_5$	320.38	$2.6 \cdot 10^{-1}$	$6.65 \cdot 10^{-14}$	4.28	191-193	7.83 ± 0.40
α-Zearalenol (α-ZEL)		$C_{18}H_{24}O_5$	320.38	1.9	$3.40 \cdot 10^{-15}$	3.18	169-170	7.61 ± 0.60
β-Zearalenol (β-ZEL)		$C_{18}H_{24}O_5$	320.38	1.9	$3.40 \cdot 10^{-15}$	3.18	173-174	7.61 ± 0.60
α-Zearalanol (α-ZAL)		$C_{18}H_{26}O_5$	322.40	$5.5 \cdot 10^{-1}$	$4.16 \cdot 10^{-14}$	4.65	146-148	8.08 ± 0.60
β-Zearalanol (β-ZAL)		$C_{18}H_{26}O_5$	322.40	$5.5 \cdot 10^{-1}$	$4.16 \cdot 10^{-14}$	4.65	156-158	8.08 ± 0.60

[a)] Taken from SciFinder® (https://scifinder.cas.org). [b)] Taken from the Interactive PhysProp database (http://esc.srcinc.com).

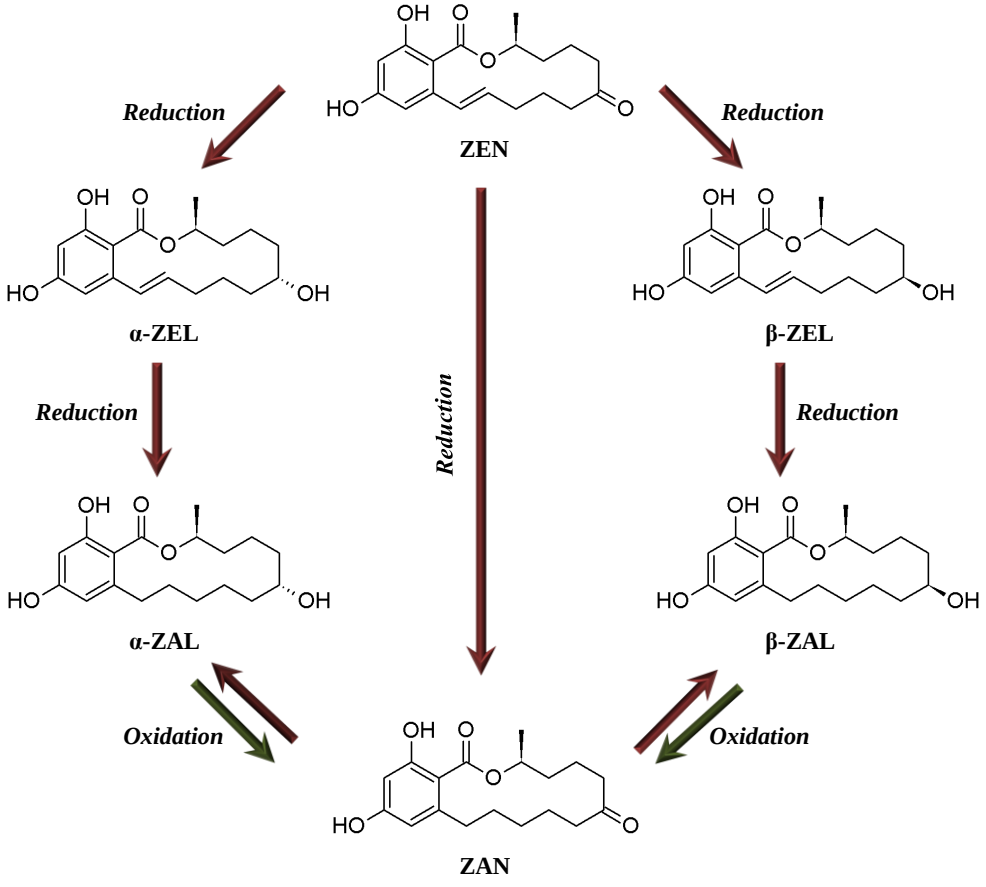


Figure I.5.- Bio-transformation of ZEN in its derivatives. Redrawn from (Belhassen et al., 2015).

I.3.4.- Phytoestrogens

Phytoestrogens are non-steroid compounds that can act as EDCs and that are produced by different types of plants. They have a protection effect against the pathogens of herbivores. As it is shown in Table I.3, this group of compounds includes isoflavones such as *biochanin A*, *daidzein*, *genistein*, *equol*, *glycitein*, *formononetin* and *prunetin* that, together with *coumestrol*, are present in edible plants, especially in soya and legumes, as well as the lignans *enterodiol* and *enterolactone* which come from seeds that are activated by the intestinal flora when they are ingested by animals (Jarošová et al., 2015; Serrano et al., 2001).

Table I.3.- Structure and properties of some phytoestrogens.

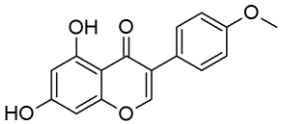
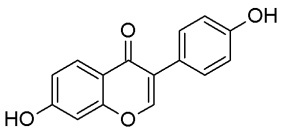
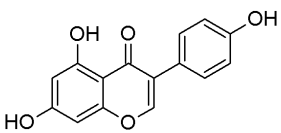
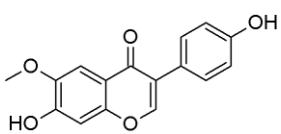
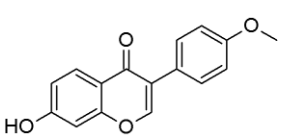
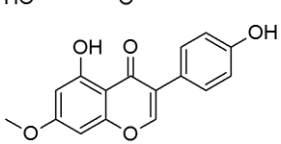
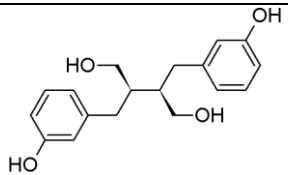
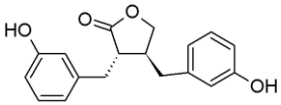
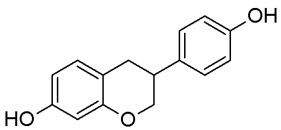
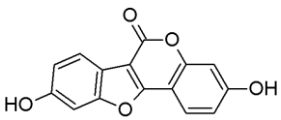
Name	Structure	Formula[a)]	MW[a)] (g/mol)	Water solubility[a), b)] (g/L, 25 °C)	Vapour Pressure[a), b)] (mmHg, 25 °C)	Log K_{ow}[a), b)]	Melting point[a)] (°C)	pK_a[a)]
Biochanin A		$C_{16}H_{12}O_5$	284.26	$5.4 \cdot 10^{-2}$	$2.25 \cdot 10^{-11}$	3.34	189-191	6.50 ± 0.20
Daidzein		$C_{15}H_{10}O_4$	254.24	$1.2 \cdot 10^{-2}$	$2.77 \cdot 10^{-9}$	2.55	323	7.01 ± 0.20
Genistein		$C_{15}H_{10}O_5$	270.24	$9.2 \cdot 10^{-3}$	$5.18 \cdot 10^{-12}$	2.84	302	6.51 ± 0.20
Glycitein		$C_{16}H_{12}O_5$	284.26	$9.7 \cdot 10^{-2}$	$1.36 \cdot 10^{-12}$	2.55	178-180	7.03 ± 0.20
Formonetin		$C_{16}H_{12}O_4$	268.26	$4.8 \cdot 10^{-2}$	$8.17 \cdot 10^{-10}$	2.86	257	6.99 ± 0.20
Prunetin		$C_{16}H_{12}O_5$	284.26	$2.8 \cdot 10^{-2}$	$1.49 \cdot 10^{-12}$	4.03	240	6.35 ± 0.20

Table I.3.- (Continued).

Name	Structure	Formula[a)]	MW[a)] (g/mol)	Water solubility[a), b)] (g/L, 25 °C)	Vapour Pressure[a), b)] (mmHg, 25 °C)	Log K_{ow} [a), b)]	Melting point[a)] (°C)	pK_a [a)]
Enterodiol		$C_{18}H_{22}O_4$	302.36	$8.8 \cdot 10^{-1}$	$3.79 \cdot 10^{-13}$	1.28	555	9.68 ± 0.10
Enterolactone		$C_{18}H_{18}O_4$	298.33	-	-	-	-	9.93 ± 0.10
Equol		$C_{15}H_{14}O_3$	242.27	$1.6 \cdot 10^{-1}$	$2.05 \cdot 10^{-8}$	2.77	158	9.94 ± 0.40
Coumestrol		$C_{15}H_8O_5$	268.22	$1.1 \cdot 10^{-1}$	$2.58 \cdot 10^{-7}$	2.82	285	8.25 ± 0.20

[a)] Taken from SciFinder® (https://scifinder.cas.org). [b)] Taken from the Interactive PhysProp database (http://esc.srcinc.com).

There currently exists some controversy concerning their oestrogenic or antioestrogenic activity. Some authors defend that their similarity with *estradiol* allows them to link with the oestrogenic receptors of reproductive organ tissues and also those of bones, liver, heart and brain by developing a preventive effect against several hormone-dependent diseases such as osteoporosis, high level of cholesterol in blood, hypertension or cancer (Fayed, 2015). Others attribute them an endocrine disrupting activity. In this sense, they have been associated with several diseases related to the hormonal balance in humans including ovaries, uterus and oviduct deformities as well as premature puberty, irregular menstrual cycles in the adulthood (Fayed, 2015; Patisaul and Jefferson, 2010) or even the development of breast cancer, although evidences of their mitigating effect on this disease have been reported in the literature (Dewi et al., 2016; Fayed, 2015). Furthermore, several in vitro tests have indicated that this agonist and antagonist duality can be related to the levels of these compounds in blood. In this sense, Dees et al. (Dees et al., 1997) demonstrated that when these analytes are present in small doses they develop an oestrogenic effect by stimulating mammal cells proliferation and the expression of oestrogenic dependant genes. However, when the doses increase, the same phytoestrogens can act against the oestrogenic activity, an aspect that is also regulated by the levels of endoestrogens in the individual (Serrano et al., 2001). Regarding their oestrogenic activity, it has been evaluated by their comparison with 17β-E_2, which was taken as model, finding that coumestrol is among those that present the greatest activity with a relative potency of 10^{-3} (Procházková et al., 2017).

I.4.- Importance of the analysis of the selected compounds with oestrogenic activity in water, milk and dairy products

As previously mentioned, biologically synthesised oestrogens play an important role in the endocrine system of humans by executing important tasks regarding not only the regulation of different sexual functions but also controlling other important aspects such as mineral, fat, sugar, protein and cholesterol metabolisation, blood coagulation, etc. However, an excess of oestrogenic compounds caused by an over-exposition originated from the environment or the diet, can bring about important endocrine disorders which, additionally, can be cumulative and also appear in successive generations (Shi et al., 2011). Such disorders are frequently related to poor semen quality (low mobility and inadequate morphology), sexual differentiation organs abnormalities or menarche at young age, among others (de Almeida Ferreira Braga and Borges, 2002; Ganmaa et al., 2012; Rasier et al., 2006; Toppari, 2002). Moreover, the association of

high levels of oestrogens in humans with the development of a good number of cancers has also been widely reported both in males and females, including breast, ovaries, endometrial, testicular and prostatic cancer (Farlow et al., 2009; Ganmaa et al., 2001; Tso and Aga, 2010).

The main routes of exposition to such contaminants are the dietary intake of contaminated food and environmental pathways, due to the presence of these compounds in soil and water as a result of human or animal wastes.

On the one hand, the presence of oestrogens in water samples is principally caused by the increase of the production of synthetic substances and the subsequent waste generation. Besides, the disposal of excrements of animal and humans that have been treated with oestrogens or that have ingested contaminated food by natural sources or as a result of plastic packaging transference, also plays an important role in this sense (Shi et al., 2011). In fact, it is well known that the world's human population discharges around 30000 kg of natural oestrogens per year and at least 700 kg/year of synthetic oestrogens coming uniquely from the use of birth control pills (Adeel et al., 2017). However, birth control pills constitute only a small part in comparison with the release associated with livestock. It should also be mentioned that diverse studies have reported the occurrence of these hormones in water samples including pond and lake water (Procházková et al., 2017), farm wastewater as well as sewage treatment plants and groundwater (Adeel et al., 2017; Kolpin et al., 2014), finding concentrations around 0.6-3270 ng/L. In general terms, E_1 followed by 17α-E_2 and 17β-E_2 were the oestrogens most commonly found at higher concentrations although it is known that EE_2 has a larger persistence than endoestrogens in natural environments (Adeel et al., 2017).

Despite the aforementioned studies, there are important shortages regarding the knowledge about the oestrogenic contamination in the environment and, especially, in water samples. Furthermore, it has been demonstrated that oestrogens can affect the morphology, metabolism and the correct reproduction of aquatic animals producing feminisation, alteration of the sex ratio or impaired gonadal development in several species as well as the inhibition of amphibian metamorphosis (Procházková et al., 2017). Besides, they can also affect root, branches and flowering development as well as germination processes of plants (Adeel et al., 2017; Procházková et al., 2017). That is way the assessment of the occurrence of oestrogens using sensitive techniques that allow their study at the low concentrations at which they appear in such matrices as well as inter-disciplinary studies that evaluate their ecological, environmental and human impact have gained great attention in the last years.

On the other hand, the diet is the principal source of oestrogens intake not only from food of animal origin such as milk, dairy products, meat, eggs, etc., but also from others of vegetal and fungal origin that constitute an important via of exposition of phytoestrogens and mycoestrogens, respectively. However, it should be highlighted that, among them, milk and dairy products are the main source of the intake of these compounds.

The high levels of these hormones in milk are closely associated with the current practices carried out by farmers who continue milking livestock in the last period of pregnancy when the oestrogen levels are markedly elevated, especially in the case of E_1 concentration which rises during pre-partum stage and the first days of lactation (Jouan et al., 2006). A noteworthy example is the case of the Holstein cows that are original from Germany and the Netherlands. These cows, which are frequently fed with a combination of grass and concentrate of grain, proteins and by-products, are artificially inseminated only three months after calving. This breeding procedure allows that the animals continue lactating for almost the whole pregnancy period, around 305 days per year versus the usual 150 days of conventional practices (Ganmaa et al., 2012). Apart from that, the use of some oestrogens as anabolic steroids in cattle has influenced considerably the levels of such compounds in milk since, although their use is forbidden in most countries, they have been illegally used in order to increase the production of milk as well as for growth promotion purposes (Noppe et al., 2008).

Additionally, the lipophilic character of oestrogens (most of them have octanol/water partition-coefficients (K_{ow}) around 2-6) facilitates their transference to milk through the mammal glands when their levels in blood increase as a result of the facts previously indicated. Besides, the worldwide consumption of milk and dairy products as well as their high content in fat, has brought about that these products constitute the 60-70 % of the total oestrogen intake of humans (Ganmaa et al., 2012), which is of special concern due to the important health risk associated with them.

I.4.1.- Current legislation

The use and commercialisation of EDCs for anabolic and therapeutic purposes in the livestock sector as well as others such as aquaculture, has become an issue of great concern due to the introduction of these compounds in the food chain and into the environment as a result of such practices. In fact, the EU assumed in the Parliament resolution of 14 March 2013 on the protection of public health from endocrine disrupters (2012/2066(INI)), that EDCs with

oestrogenic or anti-oestrogenic activity can affect the functions of the reproductive female system. Such resolution also indicated that they alter hormone levels and the menstrual cycle, that they favour the development of uterine diseases such as fibroids and endometriosis, alter breast growth and lactation as well as produce premature puberty, miscarriage and breast cancer in women.

Regarding the legislation concerning water samples, although the Environmental Quality Standards Directive 2008/105/EC, later amended by Directive 2013/39/EU, proposed the need to provide high-quality monitoring data on the concentrations of potentially polluting substances in the aquatic environment in order to design new actions and strategies to control the presence of contaminants in water, maximum residue limits (MRLs) have not been established for EDCs in such matrices. However, since 2011, in the Proposal Directive COM (2011) 876, both 17β-E_2 and EE_2 were included in the list of priority pollutants that must be controlled in surface water due to their potential harmful effects and based on the evidences of their risk on the health of humans established initially by the Water Framework Directive (Council Directive 2000/60/EC).

Concerning milk and dairy products, MRLs have not been established yet neither for endoestrogens nor for exoestrogens, including synthetic, myco and phytoestrogens. However, the use of oestrogens and other sexual hormones in the livestock sector have been widely regulated in the EU. In fact, MRLs for hormones in meat have been set from 2010 by Commission Regulation 37/2010. In this sense, Council Directive 81/602/EEC, published in 1981, banned the commercialisation of stilbenes, stilbenes derivatives, their salts and esters for their administration to any animal as well as substances with thyrostatic, oestrogenic, androgenic and gestagenic effect in farming animals, with some exceptions for certain therapeutic applications. This Directive and its successive modifications were amended by Council Directive 96/22/EC in which it was specifically prohibited the administration of 17β-E_2 and α-ZAL, among other hormones, for animal growth promotion purposes. Later on, in 2003, after the advice of the Scientific Committee on Veterinary Matters relating to Public Health about the carcinogenic effect of 17β-E_2, the use of this hormone was permanently banned by Council Directive 2003/74/EC, excluding its use in particular circumstances that could be a risk for animal health such as foetus maceration or mummification as well as pyometra in cattle. Finally, the last modification of Council Directive 96/22/EC published in 2008 (Council Directive 2008/97/CE) banned any use of 17β-E_2 in food-producing animals for

therapeutic purposes since there exist other alternatives as, for example, prostaglandins, which are already extensively used by veterinarians of the Member States.

For its part, the European Food Safety Authority (EFSA) established in 2014 in the Panel on Contaminants in the Food Chain a tolerable daily intake of 0.25 µg/kg body weight for ZEN but no data regarding similar compounds has been found in this respect. Regarding phytoestrogens, biochanin A and formononetin were included in the list of substances that interfere in the hormonal system of humans and wildlife by EU Communication COM (2001) 262. Moreover, in the following communication (SEC (2007) 1635), biochanin A was included in the list of substances with an evident endocrine disrupting effect.

As can be seen along this section, in spite of the important concern regarding EDCs and the fact that the disorders associated with the hormonal system have suffered a sharp increase in the last twenty years, the current legislation is still insufficient, basically, due to the lack of reliable data of their real effect and persistence. In fact, in one of the last resolutions published on this respect (European Parliament Resolution 2012/2066(INI)), the European Parliament highlighted that there was no scientific basis for setting a limit value, since they have an effect even at extremely low concentrations. For this reason, it should be considered that ECDs are substances without a clear threshold, that is to say, any exposure to them may entail a risk. Besides, in such resolution the Commission and Member States were urged to register any reproductive disorder reported with the aim of providing more data and carrying out an exhaustive revision of the legislation in this respect. Such regular update of the database of active EDCs will also support targeted research projects and emphasise their adverse effects at low concentrations, including the improvement or development of analytical methods.

I.5.- Analytical methods for the determination of the selected compounds with oestrogenic activity

As it has been previously indicated, the concentration of oestrogenic compounds in biological fluids such as blood or urine is very low. Moreover, these compounds can appear in environmental and food samples at levels in the ng/L or ng/kg range, which, together with the fact that they can affect the human organism at small concentrations, gives rise to the need of developing new and efficient methodologies for their determination and quantification in such complex matrices.

The detection of these compounds has been commonly carried out by different types of

immunoassays such as enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) which allow carrying out the evaluation of a large number of oestrogenic active substances. However, the troublesome cross-reactions associated with the similar structure of oestrogens have clearly limited their application (Tomšíková et al., 2012). Contrary, the use of chromatographic techniques, including gas chromatography (GC) and liquid chromatography (LC) and, though to a less degree, electrophoretic techniques like capillary electrophoresis (CE), has emerged as interesting alternatives since they do allow the individual analysis of each oestrogenic compound in the samples of interest (Szultka et al., 2013; Tomšíková et al., 2012). Apart from that, the application of sensors in which electrochemistry is combined with nanotechnology and molecular biology for the sensitive and specific determination of oestrogenic compounds has also been largely developed in the last years (Gunatilake et al., 2016).

I.5.1.- Gas chromatography determination

Although both GC and LC are the techniques most commonly applied for the analysis of oestrogenic compounds, GC has been less used due to the limitation associated with the non-volatility of such group of analytes. In this sense, and although the determination of native oestrogens has also been directly carried out by GC (Yilmaz and Kadioglu, 2012), the majority of the applications involve a previous derivatisation step to increase their volatility and their thermal stability. As previously demonstrated in such applications, their derivatisation also allows decreasing their polarity and improving their determination by mass spectrometry (MS) by the production of more favourable fragmentation patterns (Capriotti et al., 2013).

Among the reagents customarily used for oestrogens derivatisation, it should be remarked N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) and N-O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA), although others such as N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) or pentafluoropropionic anhydride (PFPA) have also been used due to their facility to silylate the hydroxyl groups of oestrogens. The main problem of this process is the possible reaction with more than one hydroxyl group resulting in more than one derivative and, consequently, decreasing the sensitivity and selectivity of the determination. In order to avoid this problem, some catalysts are added to the reaction medium to improve the efficiency of the process and to achieve the complete silylation of all hydroxyl groups. Different substances are commonly used with this aim as, for example,

trimethylchlorosilane (TMCS) or pentafluorobenzyl bromide (PFBBr) to BSTFA silylation or trimethyliodosilane (TMIS) and NH_4I to MSTFA derivatisation, although in this last case the addition of small amounts of dithioerythritol (DTE) or 2-mercaptoethanol (BME) which prevent the oxidative degradation of the reagent are commonly necessary (Capriotti et al., 2013).

As it is shown in Table I.4 in which some examples of the application of GC to the analysis of oestrogenic compounds have been compiled, conventional GC is the main working mode. However, two dimensional GC (GCxGC) also has been occasionally applied in this field (Kopperi et al., 2013) in its comprehensive mode. With respect to the detectors used, most applications are based on the coupling of GC with MS or tandem mass spectrometry (MS/MS) whereas the use of others like a flame ionisation detector (FID) or an electron capture detector (ECD) has been less extended despite the fact that they are more simple and fairly inexpensive (Migowska et al., 2012). MS detectors and, in particular, MS/MS, offer higher sensitivity as well as a reliable identification of the analytes supported by the precursor and product ions selection and their ratio. Regarding MS application, the combination of non-polar columns together with the use of BSTFA as derivatisating reagent and electron ionisation (EI) as ionisation source is preferred, using either ion trap (IT) (Ribeiro and Tiritan, 2015), simple quadrupole (Q) (González et al., 2017), triple quadrupole (QqQ) (Albero et al., 2014) or time of flight (TOF) (Kopperi et al., 2013) analysers.

I.5.2.- Liquid chromatography determination

LC is probably the most appropriate technique for the analysis of the oestrogenic compounds previously commented since this technique does not present the limitations associated with their low volatility and thermal stability and also allows the determination of conjugated and non-conjugated oestrogens without a previous hydrolysis step, as it will be later shown.

As can be seen in Table I.5, different modalities of LC have been used for this purpose including high-performance liquid chromatography (HPLC), ultra-high-performance liquid chromatography (UHPLC) and others less common such as capillary-liquid chromatography (CLC) or nano-liquid chromatography (nano-LC) using, in most occasions, reverse-phase columns. In particular, UHPLC offers a great number of advantages respect to HPLC since the use of stationary phases with particles smaller than 2 μm increases the number of theoretical plates of the column, decreases the analysis time and improves the resolution of the peaks.

Table I.4.- Some examples of the application of GC for the analysis of oestrogenic compounds.

Analytes	Matrix	Column	Derivatising agent	Analytical technique	Reference
E_1, 17β-E_2, E_3, DES	River and wastewater	5 % diphenyl-95 % dimethylpolysiloxane and 100 % dimethylpolysiloxane	PFPA	GC-ECD	Migowska et al., 2012
E_1, 17β-E_2, E_3, EE_2	Wastewater	5 % phenyl-95 % methylpolysiloxane and 50 % phenyl-50 % methylsiloxane	BSTFA/TMCS	GCxGC-MS* (TOF)	Kopperi et al., 2013
E_1, 17β-E_2, E_3, EE_2, DES, HEX, α-ZEL	Biosolid and poultry manure	5 % phenyl-arylene-95 % dimethylpolysiloxane	MSTFA/TMIS/DTE	GC-MS/MS* (QqQ)	Albero et al., 2014
Daidzein, genistein, formononetin, equol, coumestrol, enterodiol, enterolactone, ZEN, α-ZEL, β-ZEL	Environmental water	5 % phenyl-95 % methylpolysiloxane	MSTFA/NH_4I/BME	GC-MS/MS* (IT)	Ribeiro and Tiritan, 2015
17α-E_2, 17β-E_2, E_3, EE_2, 2-$MeOE_2$, DS, HEX, ZEN, α-ZAL, β-ZAL, α-ZEL, β-ZEL	Milk and yogurt	5 % diphenyl-arylene-95 % dimethylpolysiloxane	BSTFA/TMCS	GC-MS/MS* (QqQ)	D'Orazio et al., 2016b
E_1, 17β-E_2, EE_2	Wastewater	5 % phenyl-95 % methylpolysiloxane	BSTFA/TMCS	GC-MS/MS* (Q)	González et al., 2017

*EI has been used as ionisation technique. 2-$MeOE_2$: 2-methoxyestradiol.

Concerning miniaturised LC systems, although the number of applications is reduced, both CLC and nano-LC have demonstrated to provide several improvements in terms of low consumption of solvents, low time of analysis and the possibility of using low volumes of sample (Pröfrock, 2010). As it happened in GC applications, the use of two dimensional LC (LCxLC) has also been occasionally used for the analysis of oestrogens (Nguyena et al., 2011).

Table I.5.- Some examples of the application of LC for the analysis of oestrogenic compounds.

Analytes	Matrix	Column	Ionisation source (mode)	Analytical technique	Reference
17β-E_2, E_3, EE_2	Lake water	C_{18}	-	HPLC-DAD/FD	Wen et al., 2006
E_1, E_2, E_3, conjugated oestrogens	Milk	C_{18}	ESI (+)	CLC-MS/MS (QqQ)	Farlow et al., 2009
E_1, 17α-E_2, 17β-E_2, E_3, EE_2, DES, DS, α-ZAL, β-ZAL	Muscle tissue	C_{18}	APCI (+/-)	UHPLC-MS/MS (QqQ)	Vanhaecke et al., 2011
E_1, 17β-E_2, E_3, EE_2, DES	Milk and meat	C_{18}	ESI (-)	UHPLC-MS/MS (QTOF)	Li et al., 2013a
E_1, 17α-E_2, 17β-E_2, E_3, EE_2, 2-$MeOE_2$, DS, ZEN, α-ZAL, β-ZAL, α-ZEL	Milli-Q and mineral water	Phenyl	ESI (-)	Nano-LC-MS (IT)	D'Orazio et al., 2016a
E_1, 17α-E_2, 17β-E_2, ZEN, ZAN, α-ZAL, β-ZAL, α-ZEL, β-ZEL, daidzein, glycitein, genistein, coumestrol, equol, formononetin, biochanin A	River water	C_{18}	ESI (-)	UHPLC-MS/MS (QqQ)	Capriotti et al., 2016b

C_{18}: Octadecylsilane.

The combination of LC with diode array detection (DAD) and fluorescence detection (FD) has been reported in diverse occasions due to the simplicity and low cost of these systems and the great sensitivity of the second of them. Apart from that, its hyphenation with MS has also been largely carried out for the determination of oestrogenic compounds using different analysers. In this sense, IT (D'Orazio et al., 2016a), Q (Yan et al., 2009), QqQ (Farlow et al., 2009), TOF (Labadie and Hill, 2007) or quadrupole-time of flight (QTOF) (Li et al., 2013a) have been applied in MS or MS/MS (depending on the analyser). Electrospray ionisation (ESI), working in negative mode, is the ionisation source which has offered the most suitable results in terms of sensitivity (Capriotti et al., 2013), although others such as atmospheric pressure chemical ionisation (APCI) (Vanhaecke et al., 2011) or atmospheric pressure photoionisation (APPI) (Viglino et al., 2008) have also been used. In fact, APCI has shown a higher decrease of the matrix effect respect to ESI which is more prone to produce ion suppression phenomena. The main problem associated with the use of atmospheric pressure ionisation (API) sources for the analysis of oestrogens is that these analytes present a poor ionisation capacity. For this

reason, the use of chemical derivatisation or the addition of specific additives is necessary, in some cases. The principal strategies in this sense are the incorporation of moieties with high proton or electron affinity in the case of APCI sources and the production of permanently charged or easily protonable or deprotonable groups for ESI mode (Capriotti et al., 2013). The most common of these strategies is the use of dansyl chloride to produce a derivative that considerably increases the sensitivity (Malekinejad et al., 2006). Besides, the addition of modifiers such as triethylamine (TEA) (Ronan and McHugh, 2013), NH_4OH (Sodré et al., 2010) or formic acid (Matějíček, 2011) to the mobile phase, which is usually constituted by mixtures of methanol (MeOH)/H_2O or acetonitrile (ACN)/H_2O, also produce a sensitivity enhancement, though their post column addition have also provided good results (Laganà et al., 2004).

I.5.3.- Capillary electrophoresis determination

Although in a less degree than LC or GC, CE has also been applied for the determination of oestrogenic compounds since, despite offering several advantages such as low analysis time, high selectivity, low solvent volumes and small sample amount, the technique presents important sensitivity drawbacks related to the low injection volumes commonly used, which require the use of very sensitive detectors as well as the use of on-line preconcentration techniques (Koel and Kaljurand, 2011). Due to the high pK_a values of oestrogenic compounds, especially in the case of endoestrogens and mycoestrogens which present pK_a higher than 7.58, their analysis has been based on capillary zone electrophoresis (CZE) and electrokinetic chromatography (EKC) (See Table I.6). In the last case, micelles (micellar electrokinetic chromatography, MEKC) or micro-emulsions (micro-emulsion electrokinetic chromatography, MEEKC) have been used as pseudo-stationary phases. In addition, capillary electrochromatography (CEC) has also been occasionally reported (D'Orazio et al., 2015, 2016a; Kuehnbaum and Britz-McKibbin, 2011; Sirén et al., 2008) although its hyphenation with sensitive detectors such as MS is still in a research stage (D'Orazio et al., 2016a; Starkey et al., 2002). In the case of MEKC, apart from the buffer media, also ionic surfactants such as dodecyl sodium sulfate (SDS) (Sirén et al., 2008) or cetyltrimethylammonium bromide (CTAB) (Chang et al., 2008) are necessary to generate pseudo-phases. Besides, some authors have also indicated the necessity of including other additives in the background electrolyte (BGE). In this sense, hydroxypropyl-β-cyclodextrin (HP-β-CD) (Zhang et al., 2007) or taurocholate (Sirén et al., 2008) have been added with the aim of favouring the transport of the

analytes. In the same way, also organic solvents such as ethanol or MeOH have been used in order to increase the solubility of the analytes in the BGE and to improve the resolution and selectivity of the separation (Zhang et al., 2007).

Table I.6.- Some examples of the application of CE for the analysis of oestrogenic compounds.

Analytes	Matrix	BGE	Analytical technique	Reference
Biochanin A, formononetin, genistein, daidzein	Red clover	30 mM $Na_2B_4O_7$, 20 mM SDS, 4 mg/mL HP-β-CD 5 % (v/v) ethanol, pH 10.1	MEKC-UV	Zhang et al., 2007
17β-E_2	Urine	20 mM $C_2H_7NO_2$, 29.5 mM SDS, 36.8 mM taurocholate, pH 9.61	MEKC-ESI[a)]-MS/MS (QqQ)	Sirén et al., 2008
E_1, 17β-E_2, E_3, conjugated oestrogens	Urine	50 mM CH_5NO_3, pH 9.5	CZE-ESI[b)]-MS (TOF)	Kuehnbaum and Britz-McKibbin, 2011
E_1, 17β-E_2, E_3	Fishpond, waterwork and river water	60 mM NaOH, pH 12.8	CZE-ED	Li et al., 2013b
E_1, 17α-E_2, 17β-E_2, E_3, EE_2, 2-MeOE_2, ZEN, α-ZAL, β-ZAL, α-ZEL, β-ZEL	Milk and yogurt	45 mM APFO 10 % (v/v) MeOH, pH 9.0	MEEKC-ESI[a)]-MS (IT)	D'Orazio et al., 2015
HEX, DES, DS	Tap, lake and seawater	10 mM $Na_2B_4O_7 \cdot 10H_2O$, 20 mM SDS, pH 10.8	MEKC-DAD	Liu et al., 2016

[a)] Positive mode. [b)] Negative mode. APFO: Ammonium perfluorooctanoate; ED: Electrochemical detector.

As can be seen in Table I.6, CE has been coupled with different detection systems including UV and diode array detectors as well as EDs or MS. Although UV and diode array are the most common, the use of sensitive EDs offers an important advantage of the technique respect to LC systems since the combination of LC with EDs is very complicated as a result of the presence of organic phases (Vacek et al., 2008). With respect to MS, TOF (Kuehnbaum and Britz-McKibbin, 2011), QqQ (Sirén et al., 2008) and IT (D'Orazio et al., 2015) have been used as analysers not only with a quantification goal but also for structural determination as it occurs with the majority of CE-MS applications devoted to the analysis of phytoestrogens (Vacek et al., 2008). In general terms, ESI is the interface preferably chosen; however, some problems

are usually associated with this type of coupling due to the generation of high currents and low flows in the range of nL/min. In the systems developed so far, the use of a sheath liquid is necessary, which can be an organic solvent or an aquo-organic mixture that provides the required electrical connection and that favours the ionisation of the analytes. Moreover, in the case of MEKC, the ionic surfactants used can contaminate the interface during the determination (Somsen et al., 2010). In this sense, the alternative use of semi-volatile surfactants such as APFO has allowed the coupling of MEKC with MS which has been used in the analysis of oestrogens (D'Orazio et al., 2015).

I.5.4.- Sample preparation approaches

The low concentrations at which oestrogenic compounds are found in food, environmental and biological samples, as well as the great complexity of such matrices, have brought about the necessity of applying efficient extraction, preconcentration and clean up strategies in order to solve the challenging task of obtaining clean extracts without matrix interferences, a fact that is also related to the achievement of a good sensitivity.

In the particular case of food and biological analysis, the determination of conjugated oestrogens is also of great interest. In fact, an important number of methodologies based on different chemical and enzymatic hydrolysis have been developed before the final extraction step to generate their free forms. Concerning chemical hydrolysis, solvolysis is the most common process although the use of enzymes is more extended using either *E. coli β-glucuronidase* or *Helix pomatia* juice, which is constituted by a mixture of *β-glucuronidase* and *arylsulfatase*. Reaction time, pH and temperature conditions are usually optimised for each particular case with the aim of avoiding the generation of side products that could diminish the reproducibility of the process (Noppe et al., 2008). Another important aspect related to the sample pretreatments applied for the analysis of oestrogens in food samples is that concerning the presence of proteins, since they reduce the extraction capacity of the methodologies as a result of the high affinity of oestrogens for these molecules (Kinsell et al., 2009). Besides, proteins are able to interact in an irreversible way with the stationary phases of LC systems decreasing their durability. The use of organic solvents such as ACN, MeOH, acetone or ethyl acetate together with the addition of salts and different acids or buffers are the procedures commonly applied for the deproteinisation of the samples (Kinsell et al., 2009).

Solid-liquid extraction (SLE), liquid-liquid extraction (LLE) and, particularly, solid-

phase extraction (SPE), have been the sample pretreatment techniques mostly applied for the analysis of oestrogenic compounds, preceded by grinding, freeze-drying and homogenising in the case of solid samples (Gunatilake et al., 2016; Noppe et al., 2008). However, different modifications have been incorporated (also for the analysis of these compounds) to these classical techniques in order to reduce solvent consumption, extraction time and to improve the efficiency of the process. In this sense, several methods have been applied as, for example, pressurized liquid extraction (PLE) (Salgueiro-González et al., 2013), supercritical fluid extraction (SFE) (Xu et al., 2006) or microwave-assisted extraction (MAE) (Kumirska et al., 2015), among others. With respect to SPE, the incorporation of molecularly imprinted polymers (MIPs) as sorbents provides higher extraction selectivity (González-Sálamo et al., 2015). Moreover, another procedure in which the basis of LLE or SLE and SPE are combined and that has also been applied for the determination of oestrogenic compounds is the QuEChERS method (standing for *quick*, *easy*, *cheap*, *effective*, *rugged* and *safe*). Initially developed for the analysis of pesticides, the method has shown excellent results for the extraction of a great number of compounds including endoestrogens and exoestrogens, although almost exclusively from food samples (González-Curbelo et al., 2015).

I.6.- New trends in sample preparation

In the last years, the new trends in sample preparation, also those related to the analysis of oestrogenic compounds in different matrices, have been focused on the application of Green Analytical Chemistry principles in order to develop more eco-friendly methodologies. Simplicity, miniaturisation, automation as well as the reduction of extraction time and organic solvents amount used are the main features that should be considered in order to decrease the contamination level of any analytical methodology, but without diminishing its efficiency. In this respect the use of miniaturised techniques, the application of new materials and the development of alternatives to the use of organic solvents are the principal strategies carried out in the field of contaminants analysis and, particularly, in the determination of oestrogenic compounds as it will be shown below.

I.6.1.- Use of new extraction materials

The introduction of new sorbent materials in the field of sample preparation has allowed the development of novel methodologies with particular characteristics. In this sense, and as it is shown in Table I.7, high specific materials used in miniaturised techniques such as

dispersive SPE (dSPE), magnetic-dSPE (m-dSPE), micro-SPE (μ-SPE) or solid-phase microextraction (SPME) have been suitably applied in the field of Analytical Chemistry and, particularly, for oestrogen analysis. Among the most common materials, there should be remarked the use of MIPs (Zacs et al., 2016), nanoparticles (NPs) (Capriotti et al., 2016b), metal-organic frameworks (MOFs) (Hu et al., 2013) and carbonaceous nanomaterials such as carbon nanotubes (CNTs) (Zhu et al., 2014) or graphene (Nainga et al., 2016), as well as different combinations of them (Lan et al., 2014b). Since both CNTs and NPs have been applied in this PhD Thesis, a more extensive description of them and their applications will be carried out.

Table I.7.- Some examples of the application of new extraction materials for the analysis of oestrogenic compounds.

Analytes	Matrix	Extraction material	Analytical method	LOQs	Reference
E_1, 17β-E_2, EE_2, DES	Water	Graphene	μ-SPE-HPLC-UV	0.8-1.7 ng/L	Nainga et al., 2016
17β-E_2, EE_2	Tap water	MIPs	SPE-GC-MS/MS	0.08 ng/L	Zacs et al., 2016
E_1, 17β-E_2, E_3	Rain, lake and river water	MWCNTs	dSPE-HPLC-DAD	0.16-0.25 μg/L	Zhu et al., 2014
E_1, 17β-E_2, E_3, EE_2	Fish Pork	Fe_3O_4@ZIF-8@MIP	SPME-HPLC-DAD	1.4-5.5 μg/kg	Lan et al., 2014b
Genistein	Water	MWCNTs	dSPE-HPLC-UV	0.97 μg/L	Xu et al., 2015

I.6.1.1.- Carbon nanotubes

CNTs, firstly reported by Sumio Iijima (Iijima, 1991) in 1991, are allotropic forms of graphitic carbon constituted by graphene sheets rolled up in the shape of a cylinder shape that can have open or close ends, depending on the synthetic procedure used for their production. The preparation of CNTs can be carried out by different methods including chemical vapour deposition (CVD), arc discharge and laser vaporisation or ablation. Both single-walled (SWCNTs) and multi-walled CNTs (MWCNTs) structures (see Figure I.6) of different dimensions, type of torsion (zigzag, armchair and chiral) and diameters around 0.4-3 nm in the

case of SWCNTs and 1.4-100 nm for MWCNTs can be obtained (Ravelo-Pérez et al., 2010).

The main feature of CNTs that distinguishes them from other conventional carbon sorbents is their small size together with their large surface area which provide them with particular properties such as excellent tensile strength, great resilience, semiconducting and conducting electrical nature, outstanding thermal conductivity, stability, etc. Such properties make them highly interesting for their use in the biotechnology, pharmacy, electronic, scientific or industrial fields (Bhadra and Mitra, 2013).

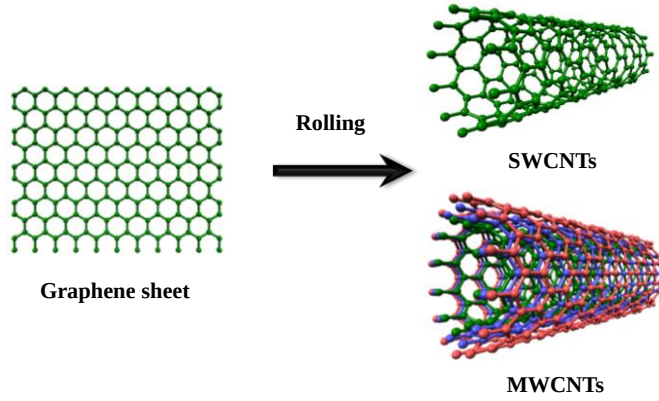


Figure I.6.- CNTs structures.

Particularly, their applications in Analytical Chemistry are very varied. In fact, they have been used for the construction of sensors, as stationary phases in chromatography and pseudo-stationary phases in CE, as matrices in matrix-assisted laser desorption/ionisation (MALDI) or as sorbents in sample preparation which constitutes one of the most important uses of these materials. SPE and their different approaches (Ying et al., 2013) as well as SPME (Sarafraz-Yazdi et al., 2013), stir bar sorptive extraction (SBSE) (Es'haghi et al., 2011) or matrix-solid phase dispersion (MSPD) (Su et al., 2011) comprise the sorbent-based techniques in which CNTs have been most commonly applied. In this sense, they have been used under their pristine form but also functionalised, linked or aggregated to other materials modifying their chemical and physical properties. The main objective of such functionalisation is to increase their specificity, their extraction capacity and to improve their solubility since it is usually poor in the majority of the solvents due to the existing van der Waals interactions between the nanotubes. Regarding the modification procedures applied, there are, basically,

two types: those that result in a covalent bonding, which are based on an initial oxidation with strong acids at high temperatures; and those in which a non-covalent functionalisation is produced due to the tendency of these material to form aggregates via van der Waals forces, stacking interactions, hydrogen bonds, electrostatic forces and hydrophobic interactions. Moreover, depending on the application, they can be disposed under different formats as, for example, cartridges, fibres, stir bars, solid suspensions, etc. (all of them laboratory made, since they are not commercialised in such ways) for the analysis of a great variety of organic and inorganic analytes in food, environmental or biological samples (Ravelo-Pérez et al., 2010).

Concerning oestrogens analysis, the use of CNTs as extraction materials has been focused on the determination not only of natural and synthetic oestrogens (Ding et al., 2011; Guan et al., 2010; Kumar and Mohan, 2012; Su et al., 2011; Teixeira et al., 2013; Zhu et al., 2014) but also for the extraction of mycoestrogens (Jiang et al., 2017; Moreno et al., 2016; Ying et al., 2013) and phytoestrogens (Xu et al., 2015) in different types of environmental and food samples including water, honey, milk or butter. In general terms, SPE and, especially, its dSPE and m-dSPE modalities have been the procedures most commonly applied although MSPD (Su et al., 2011) and SPME (Yu et al., 2007) have also been used with the same aim. In most cases, the CNTs employed have been MWCNTs combined with magnetic-NPs (m-NPs) (Guan, 2010; Makkliang et al., 2015) while the use of SWCNTs, MWCNTs, MWCNTs-NH_2, MWCNTs-COOH and the possible interaction between the sorbent and the analytes have also been evaluated (Ding et al., 2011).

I.6.1.2.- Nanoparticles

NPs are nano-size structures with a diameter in the 1-100 nm range which are usually constituted by non-magnetic inorganic components (i.e. SiO_2) or magnetic inorganic substrates which are the most common. In this case, NPs are made of metals such as cobalt, chromium, nickel, gold or silver, alloys such as $CoPt_3$ and FePt or metallic oxides as, for example, the iron oxides magnetite (Fe_3O_4) and maghemite (γ-Fe_2O_3) which have been the ones most widely applied (Li et al., 2012b; Tian et al., 2013). They can present either ferromagnetism (permanent magnetism) or superparamagnetism (if they can be attracted by a magnetic field without remaining any residual magnetism when it is eliminated) (González-Sálamo et al., 2016a).

With respect to the synthesis of this kind of materials, there exist a great number of processes depending on the type of NPs but, in general terms, and especially in the case of m-

NPs, there are two important drawbacks that should be considered before their final synthesis. First of all, they have a high tendency to form aggregates and, as a result, they lose their huge surface-to-volume ratio. Secondly, they present a high chemical reactivity that brings about a decrease or loss of magnetism. For these reasons, and with the additional aim of increasing their extraction selectivity, they are usually coated with inorganic and organic layers during the preparation process. The type of coating or modification of their surface is closely associated with their subsequent application. In this sense, the most common combinations include the use of polymeric materials, ionic liquids (ILs) or surfactants as well as their functionalisation with different chemical groups (González-Sálamo et al., 2016b). One of the most usual is the application of polymers due to their great stability in a wide range of pH. The most recurrent of these coating procedures include the use of MIPs to provide high selectivity, though others like polypyrrole (Gao et al., 2011; Zhao et al., 2013a), have also been applied. Apart from that, the functionalisation of NPs with suitable groups, molecules or biological receptors which can act as ligands allowing a more selective procedure is also of high interest. Most applications of this type of modifications have been focused on the extraction of metallic ions (Asgharinezhad et al., 2014). Apart from the coating of NPs with specific protecting materials they have also been combined with other nanomaterials such as MOFs (Maya et al., 2015), CNTs (Makkliang et al., 2015) or graphene (Es'haghi et al., 2014), among others.

Like the rest of nanomaterials, NPs present particular features that make them very interesting for their use in numerous fields. In fact, and concerning chemical applications, they have been used as chemosensors, in catalysis, in magnetic resonance imaging, drug delivery and as stationary phases in chromatographic techniques and sample preparation (Li et al., 2012b). Specifically in the field of sample preparation, m-NPs, and specially iron oxides, have a great relevance as a result of the existence of simple and fast procedures of fabrication which can also be carried out at large scale. Furthermore, they have an easy modifiable surface as a consequence of the hydroxyl groups present on it, they can usually be reused, have good dispersability in aqueous solutions, small size and huge surface area, low toxicity and easy manipulation when an external magnet is used which simplifies the overall procedure (González-Sálamo et al., 2016a; Li et al., 2012b).

Regarding oestrogens analysis, NPs have been applied for the analysis of food (Ding et al., 2011; Gao et al., 2011; Ma et al., 2011; Wang et al., 2011a, 2011b, 2016; Yuan et al., 2012), environmental (Capriotti et al., 2016a, 2016b; Huang and Lee, 2015; Tahmasebi and

Yamini, 2014; Xu et al., 2011) and biological (Reyes-Gallardo et al., 2017; Wang et al., 2012) samples, including the determination of natural and synthetic oestrogens as well as myco and phytoestrogens. Although different extraction techniques have been used as, for example, SPME (Lan et al., 2014a), the majority of publications are based on dSPE (Ma et al., 2011; Reyes-Gallardo et al., 2017; Wang et al., 2011b; Yuan et al., 2012) and, particularly, on m-dSPE (Capriotti et al., 2016a, 2016b; Ding et al., 2011; Gao et al., 2011; Huang and Lee, 2015; Tahmasebi and Yamini, 2014; Wang et al., 2011a, 2012, 2016; Xu et al., 2011). The employment of non-magnetic-NPs has been mainly carried out using SiO_2 NPs whereas Fe_3O_4 have been the most common for magnetic applications. In almost all cases, NPs were combined with other materials like graphitised carbon (Capriotti et al., 2016a), MWCNTs (Ding et al., 2011), surfactants (Wang et al., 2016), polymers (Capriotti et al., 2016b; Gao et al., 2011; Huang and Lee, 2015; Reyes-Gallardo et al., 2017; Xu et al., 2011) and, especially, with MIPs in order to increase the selectivity of the extraction processes using 17β-E_2 (Ma et al., 2011; Wang et al., 2011a), E_3 (Yuan et al., 2012) or EE_2 (Wang et al., 2011b, 2012) as templates. Even though, functionalised NPs have also been used for their analysis (Tahmasebi and Yamini, 2014).

I.6.2.- Use of ionic liquids

ILs are salts constituted by an organic cation and an inorganic or organic anion with melting points below 100 °C, which are usually liquids at room temperature. The first IL stable was synthesised in 1992 by Wilkes and Zaworotko (Wilkes and Zaworotko, 1992). It was 1-ethyl-3-methylimidazolium but nowadays there exists a great number of ILs with a different molecular structure. The cation derives from a Lewis base as, for example, imidazolium, pyrrolidinium, pyridinium, tetraalkyl ammonium, tetraalkyl phosphonium or sulfonium, while the anion or poly-anion might be inorganic like tetrafluoroborate, hexafluorophosphate, bromide, etc., or organic like trifluoromethylsulfonate $[CF_3SO_3]^-$, bis[(trifluoromethyl)sulfonyl]imide $[(CF_3SO_2)_2N]^-$ and trifluoroethanoate $[CF_3CO_2]^-$. They have particular characteristics which are closely related to their structure and the ionic combination. In fact, their low melting point is associated with the relatively large size of the ions and the asymmetry of the structure. Among the rest of characteristics, their low volatility, high thermal stability, tuneable viscosity and solubility, reusability, non-flammability and good electronic conductivity should be highlighted (Fontanals et al., 2012; Sun and Armstrong, 2010).

All this features have attracted a huge interest for these materials as "green solvents" for diverse applications such as electrolytes in batteries, in solar and fuel cells, as lubricants or as heat-transfer fluids. However they are not as "green" as they were initially thought to, since, as a consequence of their high chemical and thermal stability and non-volatility, they present a high persistence in terrestrial and aquatic environments. In addition, it has been demonstrated that several ILs also present an important toxicological activity affecting different types of organisms (Thuy Phamet al., 2010).

Particularly, their applications in Chemistry have undergone a sharp increase in organic catalysis, inorganic synthesis and in Analytical Chemistry where they have been applied as stationary phases modifiers in LC and GC, capillary wall coatings in CE, MALDI matrices, modifying supports in sensors and in extraction methods where they act as substitutes of conventional solvents (Fumes et al., 2015; Krossing et al., 2006). In particular, their uses have been mainly focused on several extraction techniques including LLE (Absalan et al., 2008) and liquid-phase microextraction (LPME) where they act as novel extraction solvents (An et al., 2017) as well as in SPME (Pei et al., 2017) and SPE (Tian and Row, 2011) by modification or coating of the fibres and solid sorbents.

Regarding the analysis of oestrogens, ILs have been used for detection purposes in diverse applications. In particular, they have been used as part of sensors by the preparation of a nanocomposite constituted of palladium NPs and a conductive IL supported on a glassy carbon electrode, increasing the surface area of the electrode and, consequently, the active sites provided for the target analytes adsorption (Afzali and Fathirad, 2016). Besides, they have also been used for increasing the sensitivity of their detection by the formation of a three dimensional inclusion complex constituted by the target oestrogen, β-cyclodextrins and the IL, which has a quenching effect enhancing the fluorescence of the complex (Wang et al., 2014). However, their current main field of application is sample preparation. In this sense, ILs have acted as modifiers in the preparation of a polymeric monolithic cake in an improved version of the SBSE known as stir cake sorptive extraction (SCSE), in which the sorbent is located inside of a special holder avoiding their contact with the bottom of the vessel (Chen et al., 2016), and SPE (Aftafa et al., 2014) procedures, as alternative solvents in conventional (Cao et al., 2014) or assisted (Magiera and Sobik, 2017; Zhang et al., 2014) LLE, as extraction solvents in dispersive liquid-liquid microextraction (DLLME) (Bozkurt and Işik, 2015; Jiang et al., 2015; Soares Emídio et al., 2015; Wang et al., 2015; Wu et al., 2012) and, without any doubt, as

coatings in SPME procedures (usually by the synthesis of polymeric ILs) (Feng et al., 2015, 2016; Mei et al., 2015; Sun et al., 2016). These applications not only have been focused on the extraction of natural and synthetic oestrogens, but also on myco (Bozkurt and Işik, 2015; Soares Emídio et al., 2015; Wang et al., 2015; Zhang et al., 2014) and phytoestrogens (Cao et al., 2014; Magiera and Sobik, 2017; Zhang et al., 2014) and their determination in environmental and food matrices. However, the number of applications is still reduced.

I.6.3.- Miniaturisation of the sample treatment

Sample preparation constitutes one of the most laborious steps of any analytical method. Depending on the application, it can have a high cost, it can be time consuming and, if not properly managed, it can also have a negative impact on the environment. In this context, it is essential the search and development of new, simpler, faster, cost-effective and environmentally friendly procedures, which allow avoiding or minimising these types of problems. To achieve this aim, a constant tendency in the Analytical Chemistry field over the last years, has been the miniaturisation of sample pretreatment procedures (Ribeiro et al., 2014). Based on that, different modifications and improvements of the conventional extraction procedures have been carried out from the beginning of the 90's, resulting in the implementation of new methodologies in which only a few mg or µg of sorbent in the case of sorbent based extractions and only some µL of solvent in LLE and SLE are necessary. Since the introduction of SPME in 1990 by Arthur and Pawliszyn (Arthur and Pawliszyn, 1990), the number of miniaturised techniques and their applications has considerably increased. In addition, their combination with different analytical techniques has also suffered great improvements. Among these extraction techniques, there should be highlighted the use of SPME, micro-dSPE (µ-dSPE) or SBSE as sorbent based extraction procedures and the array of well-established LPME methods that include single-drop microextraction (SDME), hollow-fibre-LPME (HF-LPME) and DLLME.

I.6.3.1.- Micro-dispersive solid-phase extraction

At some stage, any analytical procedure or technique suffers certain important changes that can finally lead to its transformation or evolution. During the last decade, as previously commented, many of these changes have been focused on miniaturisation and that is what has happened to SPE which has also been used under its dSPE modality, which has been later transformed to µ-dSPE when much smaller amounts of sorbents (less than 100 mg) are used. In

this case, the sorbent is dispersed in a liquid sample or extract directly or assisted by shaking, ultrasounds or any other mechanism. Then, the phases are separated by decantation, centrifugation or by the application of an external magnet, in the case of magnetic sorbents. Finally, while the liquid phase is discharded, the analytes retained in the sorbent are eluted with an organic solvent as it is shown in Figure I.7. Among the advantages of this technique, it should be remarked its simplicity and rapidity. Moreover, the fact that only very small amounts of sorbents are used, allow consuming low volumes of elution solvent reinforcing the environmentally friendly character of the technique (Płotka-Wasylka et al., 2015; Ribeiro et al., 2014).

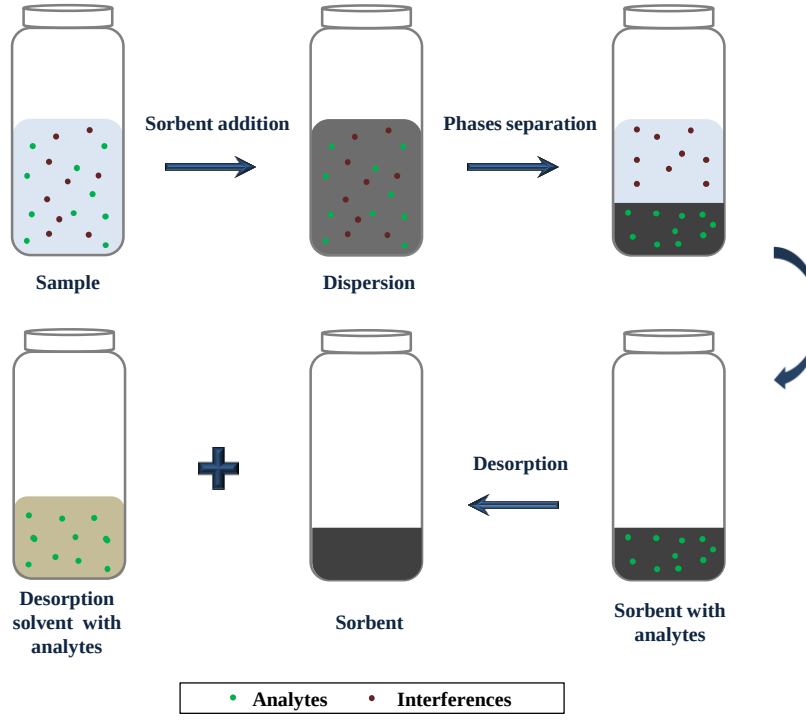


Figure I.7.- Scheme of the performance of µ-dSPE.

An important aspect for the development or improvement of sorbent-based miniaturised techniques and, particularly, µ-dSPE, is the application of the innovations introduced in the field of nanotechnology and, in particular, the introduction of new nanomaterials, as previously commented. Their huge surface areas and excellent extraction

capacities allow their use as µ-dSPE sorbents. In this sense, CNTs (Xu et al., 2016), graphene (Yu et al., 2013), MOFs (Lirio et al., 2016), NPs (Hassanpoor et al., 2015) or combinations of them (Bahar and Karami, 2015) have been largely used in the last years in µ-dSPE applications.

It is noteworthy to mention once more the use of magnetic or magnetisable NPs as extraction sorbents since in this case the use of an external magnetic field is enough to separate the sorbent with the retained analytes after the extraction step. The technique, named magnetic-µ-dSPE (m-µ-dSPE), allows avoiding the centrifugation step, simplifying considerably the µ-dSPE procedure and reducing the extraction time (Khezeli and Daneshfar, 2017).

This approach has been widely applied for the analysis of inorganic and organic substances, including oestrogenic compounds, in a great variety of samples (González-Sálamo et al., 2016b). In this sense, both m-µ-dSPE (González et al., 2017; Hashemi et al., 2014; Wang et al., 2016; Zhao et al., 2013b) and µ-dSPE (Reyes-Gallardo et al., 2017) have been applied, using modified NPs in the majority of cases, and also their combination with MOFs (González et al., 2017), for the determination of such compounds in biological, food and environmental samples prior to their analysis by GC or LC.

I.6.3.2.- Liquid-phase microextraction techniques

LPME techniques emerged as a result of the search of miniaturised LLE techniques. They allow the simplification of the procedure, they are less time-consuming and also avoid the frequent use of large amounts of toxic organic solvent volumes commonly applied in conventional LLE (Asensio-Ramos et al., 2011b).

In these techniques, the extraction is carried out between a few microlitres of a water immiscible solvent (acceptor phase) and several millilitres of an aqueous phase that contains the analytes (donor phase). As a consequence of the use of very low amounts of the extractant, a high preconcentration factor is frequently obtained, which considerably increases the sensitivity of the methodology. Moreover, the fact that the extraction and preconcentration processes can be carried out in only one step, allows simplifying the procedure and reducing the extraction time as well as the cost. This group of techniques comprises three different main approaches: SDME, HF-LPME and DLLME. The main differences among them are associated with the operational mode which depends on the type of sample and analytes (Ribeiro et al., 2014).

SDME, introduced in 1996 by Jeannot and Cantwell (Jeannot and Cantwell, 1996), was the first of the LPME techniques developed. It is based on the distribution of the analytes between a single microdrop of an acceptor organic solvent, formed on the tip of a syringe, and the aqueous donor phase that is in contact with it. Once the extraction is accomplished, the microdrop is retracted back into the syringe for its subsequent analysis in a chromatographic or electrophoretic system. Depending on the operational mode it is possible to distinguish: SDME in *two phases*, when the analytes go directly from the donor phase to the organic acceptor, or SDME in *three phases*, if the analytes are transferred to a third phase before being extracted by the acceptor solvent. Likewise, each mode comprises different approaches (Figure I.8). On the one hand, there can be distinguished those based on SDME in two phases. Among them, there can be highlighted direct immersion-SDME (DI-SDME), when the microdrop is immersed in the donor phase supported on the tip of a syringe; directly suspended droplet microextraction (DSDME), if the microdrop is floating in the donor phase without the support of a syringe; drop-to-drop microextraction (DDME), when it is immerse in a very small volume of sample; and, finally, continuous flow microextraction (CFME), if the drop is in middle of a donor phase that flows continuously.

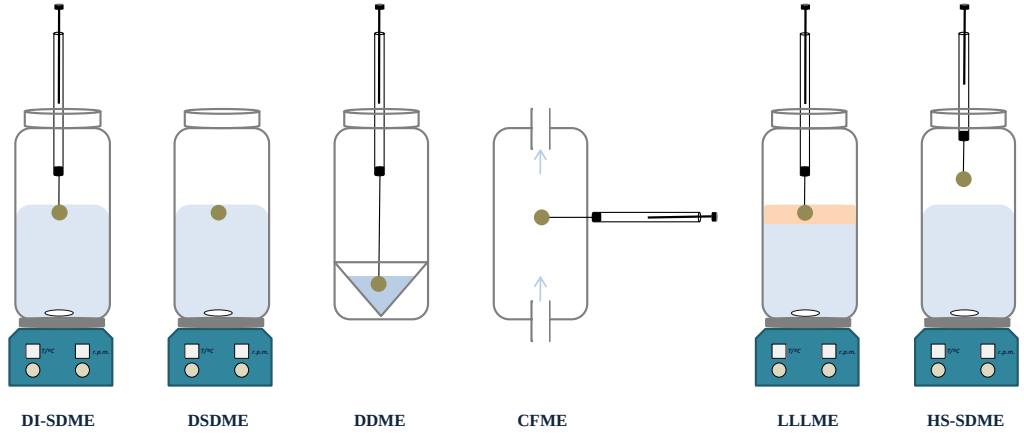


Figure I.8.- Scheme of the different operational modes of SDME.

On the other hand, there are also those based on three phases SDME among which it can be highlighted liquid-liquid-liquid microextraction (LLLME), if the third phase is another organic solvent, and headspace SDME (HS-SDME), when the microdrop is maintained in the

headspace by means of a syringe. Despite the advantages offered by this technique in terms of simplicity, environmental compatibility and specificity (Romero et al., 2007), there exist several drawbacks that limit its application. First of all, SDME has a low extraction capacity that decreases considerably the efficiency of the procedure as well as a poor reproducibility. Besides, the drop, that is usually formed with toluene, hexane, octanol, decane or even ILs, can be easily dislodged and an essential and careful control of the extraction time, stirring speed or temperature is necessary (Asensio-Ramos et al., 2011b).

With the aim of solving the stability problems of the drops in SDME, Pedersen-Bjergaard and Rasmussen (Pedersen-Bjergaard and Rasmussen, 1999) introduced in 1999 a variation called HF-LPME. In this case, the process is based on an initial extraction in a supported liquid membrane (SLM) maintained into the pores of a hollow fibre (HF), commonly made of polypropylene (PP), and then into an acceptor phase located in the lumen of the HF. Once the extraction is finished, the acceptor phase can be retracted back into the syringe or the analytes can be re-extracted from the fibre into another solvent compatible with the analytical technique. As it happens with SDME, it is possible to distinguish between HF-LPME in *two phases*, when the acceptor phase inside the pores and lumen is constituted by a solvent immiscible with water (generally octanol, dihexylether or toluene), or *three phases* HF-LPME, in which the membrane pores are impregnated with an immiscible organic solvent and the acceptor phase is constituted by an acid or basic aqueous solution located in the lumen of the membrane as can be seen in Figure I.9. The nature of the acceptor solution depends on the type of analytes in order to favour their transference from the organic solvent. In the first case, one of the ends of the HF is attached to a microsyringe while the other can be closed or not, whereas in three phases HF-LPME the second end should be closed to avoid the transference of the acceptor phase to the sample. Moreover, the technique can be developed in a dynamic mode in which the acceptor phase is continuously renewed. In this case, both ends should be attached to the acceptor flow system (Asensio-Ramos et al., 2011b).

Since HF-LPME can be carried out under non-equilibrium conditions, the extraction efficiency is considerably low compared with other miniaturised approaches. In order to solve this problem, an exhaustive evaluation of the extraction conditions (pH and ionic strength of the donor phase, temperature, extractant solvent type, extraction time, etc.) should be carried out taking into account the analytes and samples in each application. This aspect can also improve the specificity of the technique together with other modifications introduced as, for

example, the application of an electrical potential between the donor and acceptor phase, called electromembrane microextraction (EME) (Pedersen-Bjergaard and Rasmussen, 2006) or the use of ultrasounds (Shrivas and Patel, 2011), which favour the transference of analytes, as well as the inclusion of solid sorbents in the extractant phase (Yang et al., 2012).

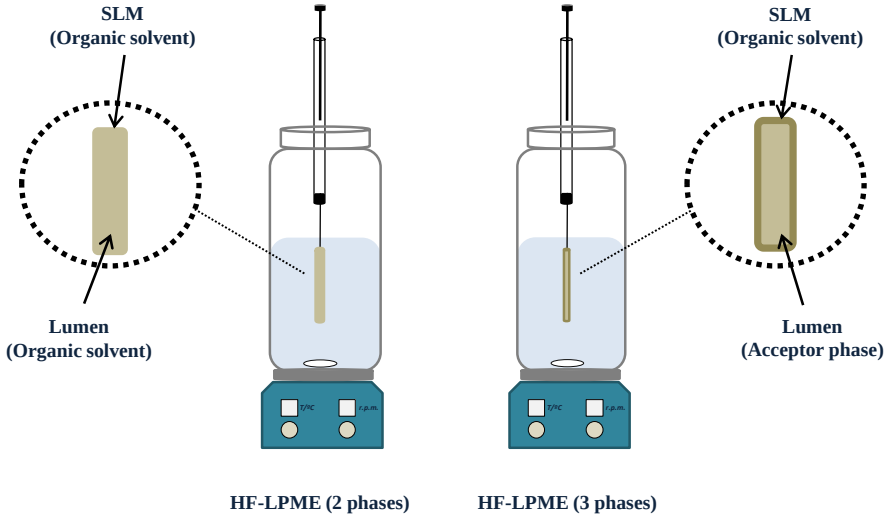


Figure I.9.- Scheme of the different operational modes of HF-LPME.

Finally, in 2006 Rezaee et al. (Rezaee et al., 2006) introduced the last of the LPME techniques developed: DLLME. It is a simple, quick and effective method for the extraction and preconcentracion of analytes based on the rapid injection of a mixture of a water inmiscible organic solvent (extractant) and a water miscible organic solvent (dipersant) in an aqueous sample which contain the analytes. The equilibrium is obtained very quickly, contrary to the rest of LPME techniques, due to the huge surface area of the fine droplets generated during the injection that increase significantly the contact between the donor and acceptor phase. Then, a drop of the extractant solvent containing the analytes, usually obtained by centrifugation, is collected with a syringe and injected in the chromatographic or electrophoretic system. Depending on the location of the extractant drop at the end of the extraction, it is possible to distinguish three different approaches that are shown in Figure I.10. If the extractant solvent has a higher density than the aqueous phase, as it occurs with chlorinated solvents (i.e. chlorobencene, dichloromethane (DCM), tetrachloromethane, etc.), the drop will be collected

at the bottom of the tube. In this case, the technique is called conventional DLLME. On the contrary, if the extractant solvent has a lower density than water as it happens, for example, with large chain alcohols, the drop is collected at the top of the sample and the approach is called floating organic-DLLME (FO-DLLME). Moreover, if the sample is cooled after the extraction it is possible to obtain the solidification of the organic drop (SFO-DLLME) boosting the separation of the phases. Following, increasing the temperature, the liquid phase is generated and the drop can be injected in the analytical instrument.

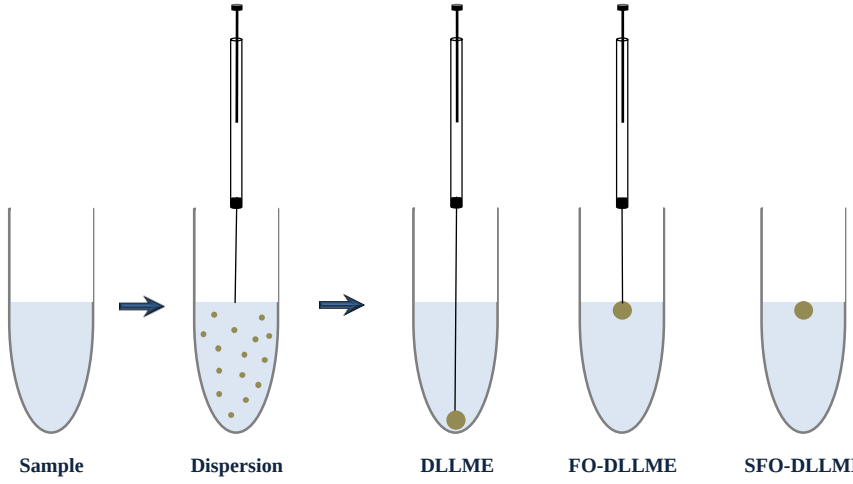


Figure I.10.- Scheme of the different operational modes of DLLME.

Since its introduction, DLLME has suffered numerous modifications in order to improve the procedure. In this sense, the incorporation of ultrasounds (Pizarro et al., 2012), vortex stirring (Zhang et al., 2012) or microwaves (Gao et al., 2010) assistance to favour the dispersion of the extractant cloud have been largely applied. Apart from that, in some occasions the use of the dispersant has been avoided with the aim of increasing the distribution coefficient of the analytes, however, in this case the use of ultrasounds or microwaves is essential to achieve an appropriate dispersion of the extractant. The introduction of alternative extractant solvents as ILs has also been tested. As it has been indicated in Section I.6.2., this type of solvents present a great number of advantages respect to volatile organic solvents conventionally used (Ocaña-González et al., 2016). Besides, ILs can be combined with magnetic materials avoiding the use of centrifugation steps and, consequently, simplifying the

procedure (Liu et al., 2017).

In addition to the main approaches previously discussed, there are a great number of variants respect to the three main LPME techniques that have been evaluated with the aim of improving their effectiveness. However, and despite the limitations of these approaches, they have been largely applied for the extraction and clean up of an overwhelming number of analytes in environmental (Ribeiro et al., 2014), food (Asensio-Ramos et al., 2011b) or biological matrices (Ocaña-González et al., 2016). In the particular case of oestrogenic compounds, the three modalities have been applied with excellent results as well as the use of some of the previously discussed modifications as, for example, ultrasounds and vortex assistance or the inclusion of solid sorbents (Bendicho et al., 2015; Gunatilake et al., 2016), being HF-LPME and DLLME the most commonly applied.

I.6.3.3.- Other miniaturised sample preparation procedures

Apart from the extensively discussed miniaturised techniques, others of great importance have also been applied for the analysis of oestrogens in several kinds of matrices. This is the case of SPME, in tube-SPME or SBSE, among others.

Without any doubt, SPME has been one of the most commonly studied, using glass fibres with polymeric coatings compatible with GC and LC systems due to the great simplicity of the technique which involves sampling, extraction, concentration, and sample analysis into a single step as well as the reusability of the fibre. However, SPME presents several limitations such as short lifetime and fragility of the fibre, high cost or carryover effects. The variety of matrices in which it has been applied is immense including environmental water, milk, fish, pork, etc. Apart from conventional SPME, also in tube-SPME has been applied in the analysis of oestrogens. In this case an open tubular fused-silica capillary with an inner surface coating is used and the analytes are extracted and concentrated onto the stationary phase by repeated draw/eject cycles or static sorption of the sample solution (Sosa-Ferrera et al., 2013). The automation of the process decreases the analysis time and provides better accuracy, precision as well as sensitivity than off-line manual techniques. In this case, the use of polymeric monolithic columns (Luo et al., 2017) is the most extended although conventional columns (Mitani et al., 2005) and others modified with ILs (Sun et al., 2016) have been used in environmental, food and biological sample analysis of oestrogenic compounds.

Finally, it is worth mentioning SBSE as another technique of interest in the analysis of

oestrogens. This approach is based on the principles of the SPME but in this case a magnetic stir bar frequently coated with polydimethylsiloxane (PDMS) -which is the only stationary phase commercialised- is the sorptive device. The technique has been applied for the analysis of oestrogens in a great number of matrices including milk, meat, water, plastics or urine using different stir bar coatings as, for example, the conventional PDMS or others such as MIPs (Qiu et al., 2016), MOFs (Hu et al., 2013) or other modifiers (Hu et al., 2012) and combined with LC and GC systems. In some occasions an in situ derivatisation prior GC analysis has also been developed (Kawaguchi et al., 2004).

CHAPTER II
OBJECTIVES

II.- OBJECTIVES

As it has been previously presented, oestrogens are natural hormones produced in mammalian organisms that have a key role in the development of female sexual characters and in the sexual behaviour. Apart from such naturally synthesised compounds, there also exist EDCs which mimic oestrogenic hormones activity with the subsequent hazards for human health when such an exposure originated from the environment or diet takes place. In particular, important endocrine disorders can be cited, including the development of cancer, even at very low exposure concentrations.

One of the main sources of oestrogens' occurrence in the environment and food is their use as growth promoters or with other veterinarian goals in the cattle industry. This fact results in contaminated livestock products including meat, milk and dairy products that are largely consumed by the population which brings about their respective harmful effects. Apart from that, they can also appear in the environment not only as a consequence of livestock practices but also due to the presence of other oestrogenic compounds of natural origin such as myco- and phytoestrogens with a fungi and a vegetable background, respectively. For this reason, the search and development of new analytical procedures which allow the determination of these types of compounds at the low concentrations at which they produce hazardous effects in humans is of great concern. In this sense, the use of new materials and novel methodologies with low organic solvents requirements, great selectivity, simplicity and rapidity could be presented as alternatives to achieve this goal.

In view of the foregoing, the main objective of this PhD Thesis is the development of simple, selective and environmentally friendly analytical methodologies for the determination of different oestrogenic compounds including natural and synthetic oestrogens as well as myco- and phytoestrogens in food matrices of animal origin, such as milk and dairy products, and environmental water samples, using novel extraction techniques combined with sensitive and rugged chromatographic systems. To achieve this goal, the following specific objectives have been established:

- The use of different modalities of LC, including HPLC and UHPLC, combined with conventional detectors such as DAD and FD systems as well as MS/MS for the appropriate separation and quantification of a wide group of oestrogenic compounds since they are the most adequate techniques for the suitable determination of this kind of analytes.

- The application of different LPME techniques including HF-LPME and DLLME for the selective extraction and preconcentration of the selected oestrogenic compounds in food and environmental samples. Likewise, the evaluation of ILs as alternative solvents in DLLME for the same purpose.
- The application of different miniaturised methods, based on the use of solid sorbents, such as μ-dSPE and m-μ-dSPE, for the extraction and preconcentration of oestrogenic compounds in milk, dairy products and different water samples.
- The evaluation of new nanomaterials such as pristine MWCNTs or m-NPs with a polymeric coating as selective extraction sorbents.
- The application of the QuEChERS method as extraction and clean up procedure for the analysis of milk and different dairy products.
- The validation of the developed methodologies in terms of repeatability, calibration, recovery, precision, accuracy, limits of detection (LODs) and limits of quantification (LOQs) with the aim of demonstrating their effectiveness in the determination of the selected oestrogenic compounds at the low levels at which they may appear in the analysed samples, as well as their capacity to obtain reliable and useful analytical data.
- The application of the developed analytical methodologies to the determination of natural, synthetic, myco- and phytoestrogens in different water samples, including tap, mineral, pond and wastewater; different types of milk including skimmed, semi-skimmed and whole milk with cow, goat, sheep and human origin as well as cheese, yogurt, probiotic products or kefir samples in order to demonstrate the applicability of such procedures as well as to determine the presence of oestrogenic residues in the selected matrices.

CHAPTER III
EXPERIMENTAL

III.- EXPERIMENTAL

III.1.- Analytical standards, solvents, reagents and solutions

- Analytical standards of 17α-E_2 (CAS 57-91-0), 17β-E_2 (CAS 50-28-2), 2-OHE_2 (CAS 362-05-0), biochanin A (CAS 491-80-5), coumestrol (CAS 479-13-0), daidzein (CAS 486-66-8), DES (CAS 56-53-1), DS (CAS 84-17-3), E_1 (CAS 53-16-7), E_3 (CAS 50-27-1), EE_2 (CAS 57-63-6), enterodiol (CAS 80226-00-2), enterolactone (CAS 78473-71-9), equol (CAS 94105-90-5), 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), ZEN (CAS 17924-92-4), α-ZAL (CAS 26538-44-3), α-ZEL (CAS 36455-72-8), β-ZAL (CAS 42422-68-4) and β-ZEL (CAS 71030-11-0) were purchased from Sigma-Aldrich Chemie and used without further purification (purity ≥ 95 %). 2-methoxyestradiol (2-$MeOE_2$) (CAS 362-07-2), 17β-estradiol-2,4,16,16,17-d_5 (17β-E_2-D_5) (CAS 221093-45-4) and chrysin (CAS 480-40-0) from Sigma-Aldrich Chemie, $^{13}C_{18}$-zearalenone ($^{13}C_{18}$-ZEN) from Biopure and β-zeralanol-10,10,11,12,12-d_5 (β-ZAL-D_5) from Witega Laboratorien Berlin-Adlershof GmbH were used as internal standards (ISs) without further purification (purity ≥ 95 %). Stock solutions of each analyte of 100 mg/L were precisely prepared in MeOH and stored in the darkness at -18 °C, except for natural oestrogens, for which concentration was 1000 mg/L. Working analyte mixtures were daily prepared by dilution with the appropriate volume of mobile phase.

- ACN, MeOH and acetone HPLC grade as well as ACN and MeOH HPLC-MS grade were purchased from Merck and VWR International while 1-octanol was purchased from Sigma-Aldrich Chemie. Tetrahydrofuran (THF), n-hexane and DCM were obtained from Panreac Química.

- Hydrochloric acid (HCl) 25 % (w/w) and acetic acid were purchased from Merck while sulphuric acid (H_2SO_4) 96 % (w/w) technical grade, formic acid 98 % (w/w) HPLC grade, ammonium hydroxide solution 25 % (v/v), 1-bromopentane and acetic acid 98 % (w/w) were provided by Panreac Química and Sigma-Aldrich Chemie.

- Sodium hydroxide (NaOH) and vegetal carbon were acquired from Panreac Química. Anhydrous magnesium sulfate ($MgSO_4$) (98 %), $MgSO_4 \cdot H_2O$ (97 %), sodium chloride (NaCl), sodium hexafluorophosphate ($NaPF_6$), celite, washed sea sand, silica gel, ammonium acetate (99.99 %), iron (II) sulfate hydrate ($FeSO_4 \cdot nH_2O$) and DA

hydrochloride were purchased from Sigma-Aldrich Chemie. Sodium hydride (NaH) was obtained from Janssen Chimica, imidazole and iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) were from Scharlau and sodium dihydrogen phosphate dehydrate ($NaH_2PO_4 \cdot 2H_2O$) and disodium hydrogen phosphate dehydrate ($Na_2HPO_4 \cdot 2H_2O$) were acquired from Merck.

- Milli-Q water was obtained from a Milli-Q gradient system A10 from Millipore.
- Supel™ QuE Z-Sep+ and pristine MWCNTs with an average diameter of 110-170 nm and 5-9 µm length were acquired from Sigma-Aldrich Chemie while octadecylsilane (C_{18}) was from Marcherey-Nagel.
- Buffer solutions of pH 4 and pH 7 for pH-meter calibration were purchased from VWR International.
- Crisolyt KCl 3 M solution for the correct maintenance of the pH-meter membrane was provided by Crison.
- KCl solution of 147 µS/cm, 1413 µS/cm and 12.88 mS/cm for the conductimeter calibration were obtained from Crison.
- Electrospray calibrant solution for IT-MS calibration was from Fluka.
- Xevo TQD standard solution for MS calibration was from Waters Chromatography.
- Nochromix® Cleaner for the cleaning of glass material was purchased from Sigma-Aldrich Chemie.

III.2.- Laboratory ware

- Graduated volumetric flasks, A class, of 5, 10, 25, 50, 100, 250, 500 and 1000 mL and beakers of 25, 50, 250, 500 and 1000 mL were from Afora.
- Graduated cylinders of 25, 100, 250 and 500 mL were from Proton.
- Grinding erlenmeyer flasks of 50 and 100 mL were from Alamo and Pasteur pipettes were acquired from VWR International.
- Glass bottles of 500 and 1000 mL with PP screw caps were from VWR International.
- Glass amber vials of 22 and 40 mL of capacity with solid caps and polytetrafluoroethylene (PTFE) liners were purchased from Supelco.

- Glass empty SPE columns of 6 mL of capacity, 7.5 cm length, 1.5 cm o.d. and 1.2 cm i.d. were acquired from Supelco.
- Hamilton syringes of 25 and 100 µL of capacity of borosilicate glass and stainless steel plunger were from Hamilton.
- Glass vials, B class, of 500 µL of capacity (40 mm length and 8 mm i.d.) with polyethylene (PE) caps for HPLC were from Waters Chromatography.
- TruView LC-MS certified clear glass 2 mL vials (12 x 32 mm) with screw neck, PTFE cap and preslit silicone septa were from Waters Chromatography.
- Insert glass conical vials of 0.25 mL of capacity (6 mm × 31 mm × 4.6 mm) were from Supelco.
- Replacement PTFE frits for SPE glass tubes with 20 µm of pore size, diameter of 11.5 mm and thickness of 3 mm were from Supelco.
- PTFE coated stirring bars of different sizes were from VWR International.
- Permanent disc magnet composed of Nd-Fe-B of 30 mm × 7 mm with a weight of 38 g and strength of 14 kg coated with Ni-Cu-Ni was acquired from Super-magnete.
- Accurel Q3/2 PP HF membrane (600 µm i.d., 200 µm wall thickness and 0.2 µm pore size) was purchased from Membrana GmbH.
- Polyvinylidene fluoride (PVFD) filter membranes Durapore® with a pore size of 0.22 µm and a diameter of 47 mm were from Sigma-Aldrich Chemie.
- Minisart SRP 15 PTFE syringe filter with PP housing and a pore size of 0.45 µm was from Sartorius.
- Polyethylene terephthalate (PET) syringe filters with a pore size of 0.20 and 0.45 µm and 25 mm diameter (Chromafil® Xtra PET-20/25; PET-45/25) and with a pore size of 0.20 µm and 15 mm diameter (Chromafil® Xtra PET-20/15) for polar and non-polar media were from Macherey-Nagel.
- Corning® Costar® Spin-X® cellulose acetate or nylon membrane PP centrifuge tube filters with a pore size of 0.22 µm were from Sigma-Aldrich Chemie.
- Norm-Ject® syringes of 12 and 50 mL of PP and polyethylene plungers were from Henke

Sass Wolf.

- PP microtubes of 1.5 mL were from Sarstedt.
- PP centrifuge tubes of 15 and 50 mL were from VWR International.
- Nova-Pak C_{18} column (150 mm × 3.9 mm, 4 µm) and Guard-Pak C_{18} pre-columns (4 µm) were from Waters Chromatography.
- X-Bridge C_{18} column (100 mm × 4.6 mm, 3.5 µm) and X-Bridge C_{18} pre-columns (20 mm × 4.6 mm, 3.5 µm) were from Waters Chromatography.
- Acquity UPLC BEH C_{18} columns (100 mm × 2.1 mm, 1.7 µm and 50 mm × 2.1 mm, 1.7 µm) with an Acquity UPLC BEH C_{18} VanGuard pre-column (5 mm × 2.1 mm, 1.7 µm) were from Waters Chromatography.

III.3.- Equipment

III.3.1.- Instrumentation

- AW-224 analytical balance with a maximum weighing capacity of 220 g and 0.1 mg of resolution was from Sartorius.
- CP2202S competence analytical balance with a maximum weighing capacity of 2200 g and 0.01 g of resolution was from Sartorius.
- Manual adjustable micropipettes with disposable plastic tips with different volume ranges were from Eppendorf.
- Manual adjustable Transferpette® S micropipettes with disposable plastic tips with different volume ranges were from Brand.
- pH-meter GLP 22 with a temperature sensor was from Crison.
- Conductimeter CM 35 with cell temperature control was from Crison.
- 7407 vibrating sample magnetometer (VSM) was from Lake Shore Cryotronics, Inc.
- X'Pert Pro diffractometer for X-ray diffraction (XRD) measurements was from PANalytical.
- S 4800 field emission scanning electron microscope was from Hitachi.

- JEM-2000EX and JEM 2100 microscopes for obtaining transmission electron micrographs were from Jeol.
- TriStar II analyser for obtaining nitrogen adsorption-desorption isotherms was from Micromeritics.
- Zetasizer Nano ZS for electrostic potential determination was from Malvern Instruments.
- FT/IR-6200 IRT-5000 spectrophotometer was from Jasco Inc.
- HR-800-UV microscope for Ramman measurements was from Horiba.
- Thermogravimetric balance, model Pyris Diamond TG/DTA, was from Perkin Elmer.
- VG-Escalab 210 spectrometer for X-ray photoelectron spectroscopy (XPS) measurements was from Thermo Scientific.

- ***HPLC separations***

  HPLC-DAD/FD analyses were carried out in a HPLC system equipped with a binary pump (model 1525), an autosampler (model 717 plus), a diode array detector (model 2998) and a fluorescence detector (model 2475 Multi λ) connected in series using a Nova-Pak C_{18} column and Guard-Pak C_{18} pre-column from Waters Chromatography.

  HPLC-IT-MS/MS analyses were performed using the same HPLC system hyphenated with an AmaZon SL IT-MS with an ESI as ionisation source from Bruker Daltonik GmbH using an X-Bridge C_{18} column and an X-Bridge C_{18} pre-column from Waters Chromatography.

- ***UHPLC separations***

  UHPLC-QqQ-MS/MS analyses were carried out in two different instruments:

  -An Acquity UPLC system equipped with a binary solvent manager and a sample manager with flow-through needle (FTN) coupled to a MS Xevo TQ-S QqQ detector using an Acquity UPLC BEH C_{18} column of 100 mm of length from Waters Chromatography.

  -An Acquity UPLC H-Class system equipped with a quaternary solvent manager and a sample manager FTN coupled to a MS Xevo QqQ detector using an Acquity UPLC BEH C_{18} column of 50 mm of length and an Acquity UPLC BEH C_{18} VanGuard Pre-column from Waters Chromatography.

III.3.2.- Apparatus

- Milli-Q gradient A10 system was from Millipore.
- Lab Dancer vortex with a fixed speed 2800 r.p.m. was from VWR International.
- T10 basic Ultra-Turrax with speed control was from IKA.
- Ultrasonic cleaner model 3510E-MT of 40 kHz with time control was from Branson.
- Ultrasonic cleaner model Ultrasons-512 of 50/60 kHz was from Selecta.
- 5702 centrifuge with time and speed control with a maximum velocity of 4400 r.p.m. (3000 x g) was from Eppendorf.
- 5415 D centrifuge with time and speed control with a maximum velocity of 13200 r.p.m. (16100 r.c.f.) was from Eppendorf.
- Rotavapor R-200 equipped with a V-800 vacuum controller and a V-500 vacuum pump were purchased from Büchi Labortechnik.
- Rotavapor RV-10 basic equipped with a thermostatic bath HB-10 from IKA and a CVC 3000 vacuum pump with a vacuum controller was from VWR International.
- Magnetic stirrer RCT Basic with temperature control (0-310 °C) and speed (0-1500 r.p.m.) was from IKA.
- Magnetic stirrer Agimatic-E 70002431 with a heater (50-350 °C) and a maximum speed of 1600 r.p.m. was from Selecta.
- Visiprep™ DL-SPE vacuum system with a capacity for 12 samples was from Supelco.
- Heater WTB model 7200 of 100 L of chamber capacity was from Binder.
- Furnace Carbolite CWF 11/13 of 13 L of chamber capacity and a maximum temperature of 1100 °C was from Carbolite.

III.3.3.- Software

- *Empower 2 v.6.0* programme from Waters Chromatography for performing control, data acquisition and chromatograms processing of the HPLC system.
- *Esquire NT* software from Bruker Daltonik for performing control, data acquisition and

chromatograms processing of the IT-MS system.

- *Masslynx*™ programme from Waters Chromatography for carrying out the UHPLC-MS instrument control and the data acquisition processing.
- *Microsoft Office Excel* 2003, 2007 and 2010 for the data processing including calibration curves preparation, recovery data, etc.
- *Microsoft Office Power Point* 2003, 2007 and 2010 for figures preparation including chromatograms presentation.

III.4.- Samples

In this PhD Thesis different environmental and food samples were analysed:

- *Section IV.1*:

  Whole, semi-skimmed and skimmed cow milk with the same content of proteins and carbohydrates and fat contents in the range 0.3-3.6 g per 100 mL of milk. Whole and skimmed yogurt, probiotic liquid product and white cheese of cow origin with protein content in the range 3.3-11.6 g, carbohydrates between 2.8 to 10 g and fats in the range 0.1-14 g per 100 g of product (values indicated in the commercial packaging). All samples were acquired in a local supermarket of Tenerife.

- *Section IV.2:*

  Mineral water (pH 7.8, conductivity 183.5 µS/cm at 25 °C) was acquired in a local supermarket from Tenerife. Wastewater (pH 8.7, conductivity 1440 µS/cm at 25 °C) was collected in a ravine (Valle de Guerra, La Laguna, Tenerife). Both samples were filtered through a Chromafil® Xtra PET-20/25 filter before extraction in order to remove any solid particle.

- *Section IV.3:*

  Mineral water (pH 6.5, conductivity 41 µS/cm at 25 °C) was acquired in a local supermarket of Tenerife while tap water (pH 8.6, conductivity 1791 µS/cm at 25 °C) was collected in our laboratory and wastewater (pH 8.0, conductivity 1420 µS/cm at 25 °C) was collected in a wastewater plant of the same island. Wastewater was filtered through a Chromafil® Xtra PET-45/25 filter before use.

- *Section IV.4:*

  Mineral water (pH 6.5, conductivity 43 µS/cm at 25 °C) was acquired in a local supermarket of Tenerife while pond water (pH 7.3, conductivity 214 µS/cm at 25 °C) was collected in a private pond of the North of Tenerife while the wastewater (pH 8.8, conductivity 1095 µS/cm at 25 °C) was collected in a wastewater treatment plant of the same island. Pond and wastewater were filtered through a Chromafil® Xtra PET-45/25 filter.

  Powdered infant milk, intended for feeding babies that are in the first 6 months of life, was acquired in a local supermarket of Tenerife and prepared in the laboratory following the indications of the manufacturer (the pH of the reconstituted sample was 6.7). The content of proteins, carbohydrates and fats was 9.6, 57.8 and 11.9 g per 100 g of sample, respectively, which were indicated on the commercial packaging of the product.

- *Section IV.5:*

  Cow and goat milk as well as natural yogurts of cow, goat and sheep origin with different fat (0.1-10.3 g per 100 g of sample), carbohydrate (2.8-4.9 g per 100 g of sample) and protein (2.8-4.9 g per 100 g of sample) content, were acquired in the Czech retail market and stored in the darkness at -20 °C until their use.

  Skimmed and whole cow cheese and kefir of cow and goat origin with protein (3.1-12 g per 100 g of sample), carbohydrate (3.5-4.4 g per 100 g of sample) and fat (0.2-10 g per 100 g of sample) content, were obtained in different supermarkets of Tenerife.

- *Section IV.6:*

  Cow, goat and sheep milk samples were bought in a local supermarket of Tenerife while human breast milk was kindly donated by a healthy woman 1 month after the childbirth. The content of proteins (3.0-5.4 g per 100 g of sample), carbohydrates (4.5-4.7 g per 100 g of sample) and fats (0.3-3.6 g per 100 g of sample) of the commercially acquired samples were indicated on the commercial packaging of each product.

All samples were stored in the darkness at 4 °C until their use unless otherwise indicated.

III.5.- Liquid chromatography analysis

III.5.1.- HPLC-DAD/FD analysis (Sections IV.1 and IV.2)

HPLC-DAD/FD chromatographic separation was carried out at 30 ºC following the gradient shown in Table III.1 and using 1 mM formic acid in ACN as mobile phase A and 1 mM formic acid aqueous solution at pH 3.50 as mobile phase B. The flow rate was established at 1 mL/min and the injection volume was 20 µL. For analytes detection both detectors were configured in multichannel mode to produce multiple chromatograms traces. Based on the absorption spectra obtained in the scan mode, the DAD system worked at 215 nm and 230 nm while the FD system was used setting two emission wavelengths at 310 nm and 320 nm and the same excitation wavelength at 280 nm, after obtaining the excitation and emission spectra of the target analytes.

Table III.1.- Gradient programme used for the HPLC-DAD/FD separation of Sections IV.1 and IV.2.

Time (min)	% A	% B	Curve*
0	15	85	-
1	28	72	6
5	37	63	6
22	37	63	6
23	100	0	6
28	100	0	6
29	15	85	6

* Curve 6 correlates with a linear gradient.

III.5.2.- HPLC-MS/MS analysis (Sections IV.3 and IV.4)

Chromatographic separation was carried out at 30 ºC following the gradient shown in Table III.2, considering ACN as mobile phase A and Milli-Q water as mobile phase B. The flow rate was 0.4 mL/min and the injection volume 20 µL. Regarding MS parameters, ESI was operated in negative mode under the following conditions: capillary voltage of 5500 V, end plate offset voltage of -600 V, nebulisation gas pressure of 20 psi, dry gas flow of 8 L/min, and temperature of 300 °C. For the IT, the ion charge control (ICC) was set at 60000, the maximum accumulation time at 200 ms with ten average scans per experiment and a rolling averaging of 5.

MS/MS experiments for confirmation studies were performed by fragmentation of the deprotonated molecule $[M-H]^-$, which was selected as the precursor ion. For these experiments, the set mass range was 70-350 m/z and the m/z width was set at 1. The fragmentation amplitude was individually optimised for each compound by the direct infusion of a 2 mg/L solution in A/B 50/50 (v/v).

Table III.2.- Gradient programme used for the HPLC-MS/MS separation of Sections IV.3 and IV.4.

Time (min)	% A	% B	Curve
0	50	50	-
2	90	10	6
6	90	10	6
7	50	50	6

* Curve 6 correlates with a linear gradient.

III.5.3.- UHPLC-MS/MS analysis (Section IV.5, analysis of milk and yogurt samples)

Chromatographic separation was carried at 40 °C considering MeOH as mobile phase A and Milli-Q water as mobile phase B for myco- and phytoestrogens analysis, while for natural and synthetic oestrogens MeOH/ACN 50/50 (v/v) was applied as mobile phase A and 2 mM ammonium hydroxide as mobile phase B. The gradient applied is shown in Table III.3. The MS system was operated in multiple reaction monitoring (MRM) mode using 1 precursor and 2 product ions as it is indicated in EU Council Directive 2002/657/EC. Ionisation source conditions were: capillary voltage of 2.6 kV, source temperature of 150 °C, desolvation temperature of 150 °C, cone gas (N_2) flow rate of 150 L/h and desolvation gas (N_2) flow of 550 L/h, whereas in the analyser, collision gas (Ar) pressure was 0.5 bar. MS/MS experiments were performed by fragmentation of the deprotonated $[M-H]^-$ or protonated $[M-H]^+$ molecule, depending on each compound, which was selected as the precursor ion. MRM transitions as well as the cone voltage and collision energy values of the target analytes were automatically optimised by the direct infusion of individual standards of each oestrogenic compound at 1 mg/L in a 50/50 (v/v) mixture of MeOH/H_2O.

Table III.3.- Gradient programme used for the UHPLC-MS/MS separation of Section IV.5.

Time (min)	% A	% B	Flow (mL/min)	Curve
0	10	90	0.5	-
0.5	60	40	0.5	6
8	100	0	0.5	6
10	100	0	0.4	6
12	10	90	0.5	6

* Curve 6 correlates with a linear gradient.

III.5.4.- UHPLC-MS/MS analysis (Section IV.5, analysis of kefir and cheese samples, and Section IV.6)

Chromatographic separation was carried at 40 °C following the gradient indicated in Table III.4. MeOH was considered as mobile phase A and Milli-Q water as mobile phase B for myco- and phytoestrogens, while for natural and synthetic oestrogens MeOH/ACN 50/50 (v/v) was considered as mobile phase A and 2 mM ammonium hydroxide as mobile phase B. The MS system was operated in MRM mode with a capillary voltage of 2.6 kV, a source temperature of 150 °C, a desolvation temperature of 150 °C, a cone gas (N_2) flow rate of 150 L/h, a desolvation gas (N_2) flow of 550 L/h and a collision gas (Ar) pressure of 0.5 bar. MS/MS experiments were performed by fragmentation of the deprotonated $[M-H]^-$ or protonated $[M-H]^+$ molecule, depending on each compound, and obtaining two product ions. MRM transitions as well as the cone voltage and collision energy values of the target analytes were automatically optimised by the direct infusion of individual standards of each oestrogen at 2 mg/L in a mixture of A/B 50/50 (v/v) for each group of compounds.

Table III.4.- Gradient programme used for the UHPLC-MS/MS separation of Sections IV.5 and IV.6.

Time (min)	% A	% B	Flow (mL/min)	Curve
0	10	90	0.3	-
0.5	40	60	0.3	6
8	99.9	0.1	0.4	6
10	99.9	0.1	0.4	6
12	10	90	0.3	6
14	10	90	0.3	6

* Curve 6 correlates with a linear gradient.

III.6.- Synthesis of the [PPIm][PF_6] IL

1,3-dipenthylimidazolium hexafluorophosphate ([PPIm][PF_6]) synthesis involves two steps as shown in Figure III.1: the synthesis of the reaction intermediate 1,3-dipenthylimidazolium bromide ([PPIm][Br]) and the incorporation of the PF_6^- anion. Initially, a suspension of NaH (3.25 g) in THF (71.5 mL) was magnetically stirred at 500 r.p.m. Then, a solution of 7.15 g of imidazole in 71.5 mL of THF at 0 °C was added drop by drop to this mixture under an argon atmosphere and the reaction was stirred at room temperature for 2 h. After the dropwise addition of 26.8 mL of 1-bromopentane at room temperature, the mixture was refluxed for 7 h, filtered through celite and concentrated using a rotavapor. The residue was dissolved in DCM and purified by filtration using a 4 cm i.d. classical chromatographic column filled with sea sand, silica gel, celite and vegetal carbon. The concentrated residue was washed with n-hexane and dried under vacuum. The obtained yield of [PPIm][Br] was 88 %. Afterwards, 15 g of $NaPF_6$ were added to a solution of 20 g of [PPIm][Br] in 400 mL of water and the reaction was magnetically stirred for 30 min. Then, the IL layer was separated and dissolved in 40 mL of DCM. The solution was washed two times with 50 mL of water and evaporated under vacuum. The residue was dissolved in DCM, purified, and washed in the same way as for [PPIm][Br]. Finally, the synthesised [PPIm][PF_6] was dried under vacuum obtaining a yield of 94 %.

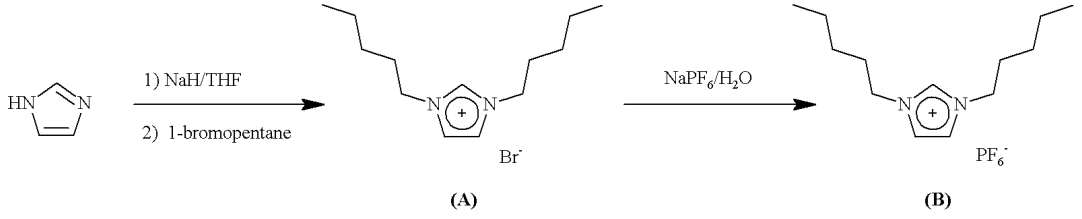


Figure III.1.- Scheme of the IL synthesis. (A) [PPIm][Br] structure; (B) [PPIm][PF_6] structure.

III.7.- Synthesis of core-shell Fe_3O_4@polydopamine m-NPs

This procedure was developed in two steps as shown in Figure III.2. Firstly, 5.41 g of $FeCl_3 \cdot 6H_2O$ and 2.78 g of $FeSO_4$ nH_2O (2:1 molar ratio) were dissolved in 200 mL of 0.5 M HCl by magnetic stirring for m-NPs preparation. Then, the solution was dropwise added to a 1.25 M NaOH solution (300 mL) under vigorous stirring (850 r.p.m.) at room temperature. Once the addition was finished, the black precipitated (Fe_3O_4) was stirred for 30 min more and

the NPs dispersion, which had a pH value of approximately 13.3, was neutralised with HCl 25 % (w/w). Secondly, the polydopamine (pDA) coating was generated by dispersing the previously prepared m-NPs at a concentration of 2.75 g/L in a 15 mM DA solution of phosphate buffered saline (PBS) at pH 8.3 and the polymerisation process was maintained for 6 h under magnetic stirring (850 r.p.m.) at room temperature. Afterwards, the synthesised Fe_3O_4@pDA m-NPs were washed with ACN/H_2O 50/50 (v/v) six times to remove the non-reacted DA. Then, they were washed once more with ACN, centrifuged at 4400 r.p.m. during 15 min and dried at 40 °C and 180 mbar. Fe_3O_4 m-NPs were coated and used even after 4 weeks of their synthesis without observing a decrease in the extraction efficiency. Regarding pDA coated m-NPs, they were used during one week after their synthesis.

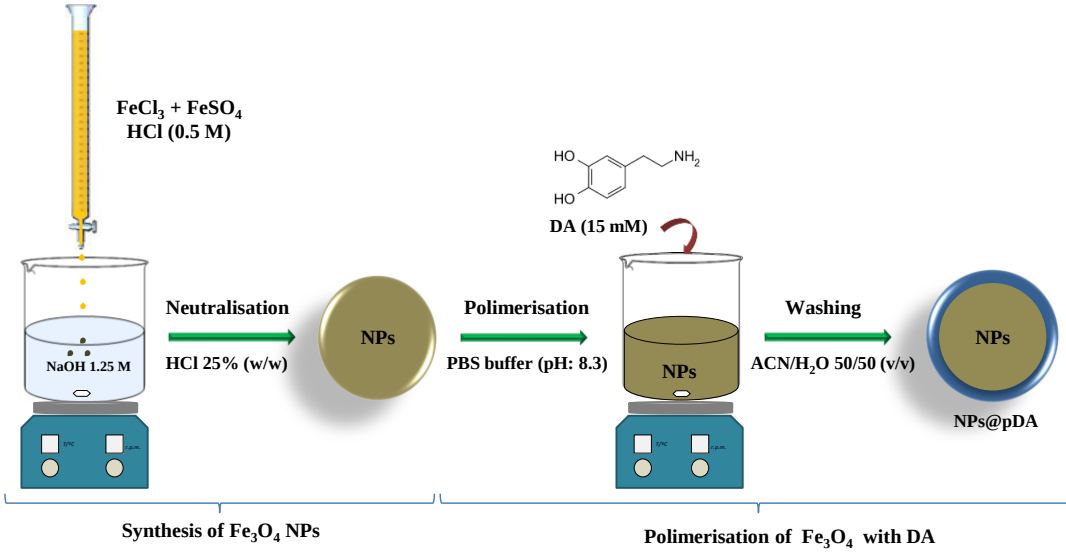


Figure III.2.- Scheme of Fe_3O_4@pDA synthesis.

III.8.- Sample pretreatment procedures

III.8.1.- Milk and dairy products extraction by HF-LPME (Section IV.1)

Milk samples deproteinisation. Three millilitres of spiked or non-spiked milk were introduced in a 50 mL PP centrifuge tube. Then, 6 mL of ACN and 150 µL of acetic acid were added to the matrix and it was vortex-shaken (2800 r.p.m.) for 1 min to produce protein precipitation. The mixture was maintained in the darkness for 15 min and centrifuged at 4400 r.p.m. for 15 min. The supernatant was evaporated at 40 °C and 180 mbar, the residue

(approximately 3 mL) was then dissolved in 7 mL of Milli-Q water to a total volume of 10 mL, and 1 g of NaCl was added. The resulting solution was filtered through a 0.45 μm Minisart SRP 15 PTFE filter into a 20 mL vial (See Figure III.3).

Yogurt samples deproteinisation. Three grams of spiked or non-spiked yogurt were introduced in a 50 mL PP centrifuge tube. Then, 9 mL of ACN and 150 μL of acetic acid were added and the sample was vortex-shaken during 3 min. After that, settling, evaporation, dissolution and filtration were carried out in the same way as described for milk samples.

Probiotic product deproteinisation. Three millilitres of spiked or non-spiked probiotic product were introduced in a 50 mL PP centrifuge tube. Then, 9 mL of ACN and 175 μL of acetic acid were added to the sample and vortex shaking was carried out for 3 min. The rest of steps were developed in the same way as for the other two samples.

Cheese samples deproteinisation. Three grams of spiked or non-spiked cheese were introduced in a 50 mL PP centrifuge tube and 9 mL of ACN and 150 μL of acetic acid were added. The sample was homogenised using a T10 Ultra-Turrax during 3 min at a speed of 3 (approximately 11500 r.p.m.). After settling in the darkness for 15 min, the mixture was centrifuged 15 min at 4400 r.p.m. The supernatant was transferred to another 50 mL centrifuge tube and 4 mL of n-hexane were added. Manual shaking was carried out for 30 s to extract the fat. Then, the tube was centrifuged again for 5 min at 4400 r.p.m. and the upper layer of n-hexane was discarded, whereas the ACN layer was evaporated at 40 °C and 180 mbar. Finally, the dry residue was dissolved in Milli-Q water like the previous matrices. In all cases, the samples were spiked 24 h before their analysis.

HF-LPME procedure. The aqueous solution obtained from the previous pretreatments (10 mL of a 10 % (w/v) NaCl aqueous sample extract) was adjusted to pH 6.0 with 8 M NaOH. A non-treated PP fibre piece of 2.0 cm was inserted into the needle tip of a 25 μL syringe that was previously filled with 1-octanol. Afterwards, the solvent was slowly introduced in the HF and when the membrane was completely impregnated, the remaining volume of 1-octanol of the syringe was introduced into the lumen of the fibre. Then, it was immediately immersed in the aqueous solution extract (See Figure III.3). Extraction was developed for 60 min at room temperature with magnetic stirring at 1250 r.p.m. After that time, and with the aim of inducing analyte back extraction, the fibre attached to the syringe was pulled out of the sample, introduced in a HPLC sample vial containing 500 μL of ACN and submitted to ultrasounds for

7 min in an ultrasonic bath. Following, and once the fibre was discarded, the liquid phase was evaporated under a gentle steam of nitrogen and reconstituted with 100 µL of the initial composition of the mobile phase (1 mM formic acid in ACN/1 mM formic acid aqueous solution 15/85 (v/v)). Finally, 20 µL of this mixture was injected in the HPLC-DAD/FD system for its analysis.

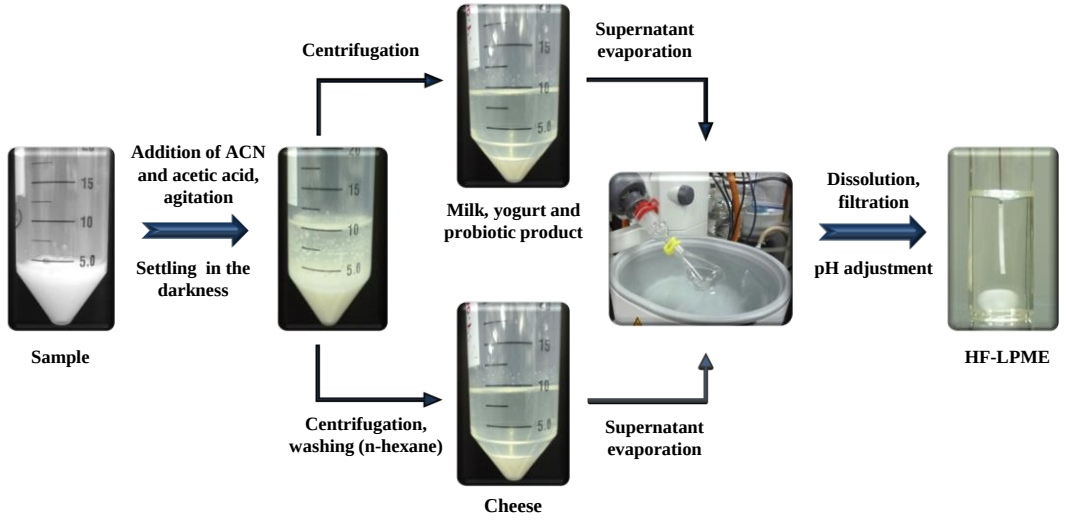


Figure III.3.- Scheme of milk and dairy products deproteinisation and extraction by HF-LPME.

III.8.2.- Water samples extraction by IL-DLLME (Section IV.2)

IL-DLLME procedure. Ten millilitres of spiked or non-spiked water samples, previously filtered with a Chromafil® Xtra PET-20/25 filter, were adjusted to pH 8 with a 0.1 M NaOH solution and introduced in a 15 mL PP centrifuge tube. A mixture of 60 mg of [PPIm][PF_6] as extraction solvent and 500 µL of ACN as dispersant was rapidly injected into the aqueous solution producing a cloudy dispersion that was vortex-shaken for 1 min to assist the extraction process. Following, the mixture was centrifuged at 4400 r.p.m. for 10 min and the fine droplets formed during the dispersion were settled at the bottom of the tube. Then, 40 µL of the IL containing the target analytes were collected with a micropipette and transferred into a HPLC vial. Finally, it was dissolved in 300 µL of 1 mM formic acid in ACN and 20 µL were injected in the HPLC system.

III.8.3.- Water and milk samples extraction by m-NPs µ-dSPE (Sections IV.3 and IV.6)

Milk samples deproteinisation. One and a half millilitres of spiked or non-spiked cow, goat, sheep and human breast milk were introduced into a 50 mL PP centrifuge tube. Afterwards, 3 mL of ACN and 75 µL of acetic acid were added and the mixture was vortex-shaken for 1 min in order to produce protein precipitation. The mixture was maintained in the darkness for 15 min and centrifuged at 4400 r.p.m. for 15 min. Then, the supernatant was evaporated at 40 °C and 180 mbar. The obtained residue (approximately 1.5 mL) was dissolved until a total volume of 25 mL and the pH was adjusted to 7 with an 8 M NaOH solution. Finally, the sample was filtered through a 0.45 µm Chromafil® Xtra PET-45/25 filter.

m-NPs µ-dSPE procedure. Twenty five millilitres of spiked or non-spiked water samples were adjusted to pH 7 and introduced in a flask containing 60 mg of the sorbent while the solution obtained from milk deproteinisation was directly filtered on a 50 mL PP tube containing 80 mg of the sorbent. After manual agitation for 30 s, a permanent magnet was located at the bottom of the flask for 10 min in order to settle the sorbent as shown in Figure III.4. Afterwards, the sample was discarded retaining the sorbent in the extraction recipient with the help of the magnet. NPs with the retained analytes were dried with a nitrogen flow. Analytes were then eluted by the addition of 6 or 8 mL of MeOH for water and milk samples, respectively, with a slight agitation for 30 s and a magnetic deposition period of 5 min. Finally, the elution solvent containing the analytes was separated and evaporated at 40 °C and 220 mbar and the residue was reconstituted in 500 µL of the initial composition of the mobile phase, filtered, an injected in the LC system. Core-shell m-NPs were used once, trying to avoid an excessive solvent consumption for their washing and possible carry over effects.

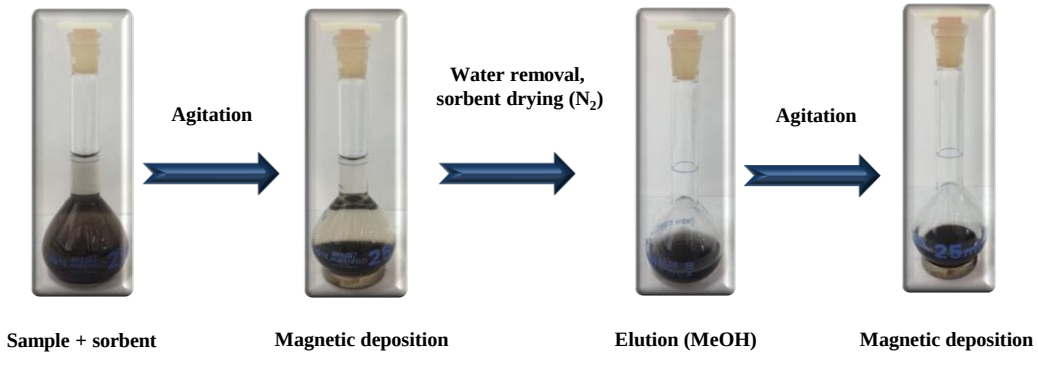


Figure III.4.- Scheme of the m-NPs µ-dSPE procedure used in Sections IV.3 and IV.6.

III.8.4.- Water samples and infant milk formula extraction by MWCNTs µ-dSPE (Section IV.4)

Infant milk formula extraction. Infant formula was previously prepared as indicated by the manufacturer in the packaging (1.4 g of powder milk were dissolved in 10 mL of water at 40 °C). Then, 3 mL of the sample were introduced into a 50 mL PP centrifuge tube and 150 µL of acetic acid and 6 mL of ACN were added. The sample was vortex-shaken for 1 min in order to produce protein precipitation. The mixture was kept in the darkness for 15 min and centrifuged at 4400 r.p.m. for 15 min. The supernatant was collected and evaporated at 40 °C and 180 mbar. The residue (approximately 2.5 mL) was then dissolved up to 25 mL with Milli-Q water, the pH was adjusted to 3 with a 0.1 M HCl solution and the mixture was filtered through a Chromafil® Xtra PET-45/25 filter.

MWCNTs µ-dSPE procedure. Fifty millilitres of spiked or non-spiked water samples (pH previously adjusted to 3 with HCl 0.1 M and filtered with a Chromafil® Xtra PET-45/25 filter) or 25 mL of the extract obtained from infant milk deproteinisation, were introduced in a flask containing 80 mg of MWCNTs as shown in Figure III.5. After agitation for 1 min, the dispersed MWCNTs solution was passed through a SPE glass tube that contained two PTFE frits, using a Visiprep™ DL-SPE vacuum system. Afterwards, another frit was located onto the sorbent and vacuum was applied for 30 min with the aim of drying the stationary phase. Then, the retained analytes were eluted with 30 mL of MeOH/acetone 50/50 (v/v) and the solvent was evaporated to dryness at 220 mbar and 40 °C. Finally, the residue was reconstituted in 500 µL of the initial mobile phase and filtered using a Chromafil® Xtra PET-20/15 filter.

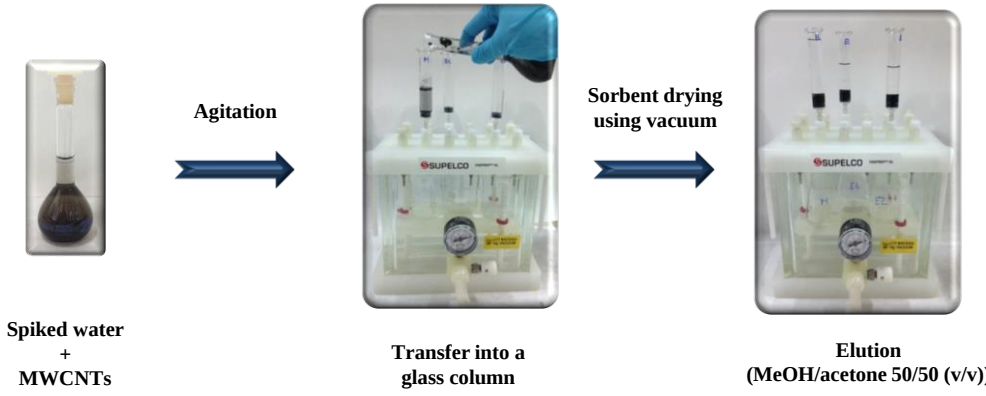


Figure III.5.- Scheme of the MWCNTs µ-dSPE procedure used in Section IV.4.

III.8.5.- Milk and dairy products extraction using the QuEChERS method (Section IV.5)

Dry weight content of milk and yogurt samples. Dry weight content (dw, %) was determined according to standardised methods, ČSN ISO 6731:2011 and ČSN ISO 13580:2007, for milk and yogurt samples, respectively. Briefly, 3 mL of milk were introduced in a metallic capsule, previously dried in a heater at 102 °C for 1 h, and weighed in an analytical balance. After that, the capsule with milk was located in a water bath at 60 °C during 30 min and then introduced in an oven for 2 h at 100 °C. Following, it was introduced in a desiccator until it reached room temperature and was weighed in an analytical balance. Afterwards, the capsule was again introduced in the oven under the same conditions and the process was repeated until a difference lower than 0.5 g between measurements was obtained. For yogurt samples the methodology was similar with the exception that, in this case, 5 g of yogurt were used and 2 g of sand were also introduced in the capsule in order to favour the yogurt drying since it is a dense sample. Drying time was also different maintaining the samples for 4 h into the oven.

QuEChERS extraction of milk and yogurt samples. Fifteen millilitres of spiked or non-spiked milk were transferred into a 50 mL PP centrifuge tube. In the case of yogurt, 10 g of sample were weighed into a 50 mL PP centrifuge tube and 5 mL of Milli-Q water were added and mixed by shaking for 1 min. Then, 15 mL of ACN were added to both types of samples and they were manually shaken during 1 min for the isolation of the target compounds. Afterwards, 6 g of anhydrous $MgSO_4$ and 1.5 g of NaCl were added and the tube was immediately shaken again for 1 min and centrifuged for 5 min at 10000 r.p.m. and 5 °C (See Figure III.6). Then, the upper organic layer of the supernatant was transferred to a new centrifuge tube containing 180 mg of C_{18} sorbent and 1.8 g of anhydrous $MgSO_4$, which was shaken again for 1 min and centrifuged for 5 min at 10000 r.p.m. and 20 °C. Subsequently, 8 mL of the purified extract were evaporated to dryness at 40 °C and 160 mbar and the residue was dissolved in 250 µL of MeOH. The reconstituted extract was filtered through a 0.22 µm nylon centrifuge tube filter (2 min, 5000 r.p.m., 20 °C) and 5 µL were injected in the UHPLC-MS/MS system.

QuEChERS extraction of cheese and kefir samples. All samples were initially homogenised using a T10 basic Ultra-Turrax for 3 min at a speed of approximately 11500 r.p.m. Then, 10 g of each matrix were weighed into a 50 mL PP centrifuge tube and 5 mL of Milli-Q water were added and mixed by hand shaking for 1 min. After that, 15 mL of ACN

were added and the mixture was again hand 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 more. 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 under the same previous conditions (see Figure III.6). Afterwards, 8 mL of the supernatant were collected and evaporated at 40 °C and 180 mbar. Finally, the residue was reconstituted in 500 µL of the initial mobile phase, filtered using a Chromafil® Xtra PET-20/15 filter and 5 µL were injected in the UHPLC-MS/MS system. In the case of whole cheese, 500 mg of C_{18} were necessary to remove the fat of the matrix during the clean up step.

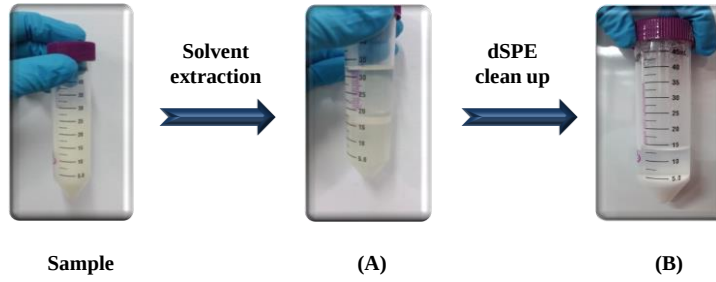


Figure III.6.- Appearance of the milk or dairy product samples after the different steps of the QuEChERS method. (A) Sample after the extraction step. (B) Sample after the clean up step.

CHAPTER IV
RESULTS AND DISCUSSION

IV.- RESULTS AND DISCUSSION

IV.1.- Hollow-fibre liquid-phase microextraction for the determination of natural and synthetic oestrogens in milk and dairy products

In this section, a new methodology was developed for the determination of nine oestrogenic compounds, four natural (E_3, 17α-E_2, 17α-E_2, and E_1) and four synthetic (EE_2, DES, DS and HEX) oestrogens as well as one metabolite (2-OHE_2) in different milk, cheese, yogurt and probiotic samples based on an initial deproteinisation followed by a HF-LPME using 1-octanol as extraction solvent. Separation, determination and quantification were achieved by HPLC-DAD/FD. Deproteinisation conditions, as well as parameters affecting the extraction efficiency in HF-LPME (pH and ionic strength, extraction time, stirring speed, temperature and back extraction conditions) were investigated and optimised. Calibration, precision and accuracy studies were carried out to validate the methodology for its application in each sample.

IV.1.1.- Background

As it was indicated in Section I.5, among the techniques used in the separation, determination and quantification of oestrogens, LC and their different approaches constitute the most adequate chromatographic systems for this purpose. However, a previous sample treatment is usually necessary. In the case of complex matrices such as milk and dairy products, protein precipitation followed by LLE (Hartmann et al., 1998), SPE (Shi et al., 2011; Yuan et al., 2012) or MSPD (Su et al., 2011) are the procedures conventionally applied. However, new alternative methodologies based on the miniaturisation of the extraction process have recently attracted much interest in order to reduce solvent consumption and to simplify the procedure.

HF-LPME was introduced in 1999 as an alternative to solve the drop stability problems observed in the application of SDME (Pedersen-Bjergaard and Rasmussen, 1999). This approach provides a great number of advantages including its easy handling, high preconcentration factors and low cost and low solvent consumption as well as a high capacity to provide very clean extracts (Pedersen-Bjergaard and Rasmussen, 1999). However, and despite such advantages, the technique has been scarcely applied for the analysis of oestrogenic compounds. Until the development of this work, HF-LPME had only been applied for the analysis of some oestrogens in water samples (Basheer et al., 2005; Li et al., 2011, Liu et al.,

2008) and their use for the extraction of these types of analytes from milk or dairy products had not been carried out. However, modified HFs have been used in two occasions to extract stilbenes from milk samples (Liu et al., 2010; Yang et al., 2012) using a more complicated approach similar to SPME. In the first case, Liu et al. (Liu et al., 2010) applied a MIP-coated HF to determine DES, DS and HEX in milk after deproteinisation of the sample with ACN containing HCl. After centrifugation and evaporation, the residue was reconstituted with MeOH in which the MIP-HF was immersed. In the second case (Yang et al., 2012), a MWCNTs/silica composite-reinforced HF was prepared and after impregnation with 1-octanol, DES was extracted from milk samples with different contents of fats, carbohydrates and proteins. As can be observed, both procedures used modified HFs, however, a LPME approach was not applied in any of them.

With respect to the analysis of oestrogenic compounds in milk and dairy products, and despite the fact that the association of these compounds with the development of several hormonal disorders and that the use of some of them as veterinary drugs in farm animals has been forbidden by the regulatory authorities of the EU, there exists an important lack of studies concerning the presence of these hormones in animal origin products such as milk or dairy samples. In fact, and although such analytes have been evaluated in some matrices of interest (Kinsell et al., 2009), this work constitutes the first methodology developed for the analysis of synthetic stilbenes (also for the analysis of the metabolite 2-OHE_2) in dairy products different from milk. Besides, it is also the first time that an analytical methodology has been developed to evaluate the presence of oestrogens in probiotic products and that HF-LPME is used for the extraction of these compounds from dairy products.

IV.1.2.- Specific objectives

In view of the foregoing, the following specific objectives have been established for this work:

- The development of a new analytical methodology based on a HF-LPME approach to carry out the determination of a group of nine oestrogenic compounds including four natural oestrogens (E_3, 17α-E_2, 17β-E_2 and E_1), four synthetic oestrogens (DES, DS, HEX and EE_2) and the metabolite 2-OHE_2 in whole, semi-skimmed and skimmed cow milk samples as well as skimmed and whole natural yogurt, white cheese and a probiotic product.
- The development of the separation and determination of the target analytes by HPLC using

DAD and FD in series as well as the obtaining of optimum separation and detection conditions.

- The evaluation of the influence of the parameters affecting the extraction efficiency of the HF-LPME procedure (i.e. pH and ionic strength of the donor phase, extraction time, stirring speed, temperature and back extraction conditions) to achieve the best extraction efficiency.
- The validation of the whole methodology in terms of calibration, precision and accuracy as well as the obtaining of the LODs and LOQs of the method.
- The study of the applicability of the methodology for the analysis of the different samples of interest.

IV.1.3.- Separation by HPLC-DAD/FD

Initially, chromatographic separation was carried out in a C_{18} 150 mm × 3.9 mm × 4 μm Nova-Pak column, using a DAD system working at 215 nm. Different ACN/H_2O mixtures were used as mobile phases, containing small amounts of ammonium hydroxide or formic acid, since previous works have described the use of basic (Farke et al., 2011) or acidic (Malekinejad et al., 2006) media for the separation of oestrogens by HPLC. Isocratic and gradient elutions at room temperature were studied at different ACN/H_2O ratios for both additives. The best separation was obtained using the gradient elution described in Section III.5.1 without any additive and maintaining the analytes in their neutral form since, as can be seen in Table I.1, pK_a values of the target analytes are above 9. However, peak resolution between DES and DS was not suitable. For this reason, and taking into account that the column employed in this case allows developing separations at 50-55 °C under non-extreme pH conditions, the temperature was raised up to 40 °C observing then an improvement in the resolution of DES and DS and a good separation of the rest of analytes. With the aim of improving the sensitivity of the method and since several of the selected oestrogens show native fluorescence (E_3, 17α-E_2, 17β-E_2, EE_2 and HEX), the next step consisted in coupling in series both DAD and FD systems in order to improve instrumental sensitivity. Therefore, because the fluorescence signal may also be influenced by the mobile phase composition, the addition of small percentages of an acid or a base was again considered. At the same time, a screening study was developed to determine the maximum absorption, excitation and emission wavelength for each compound, carrying out different scans. As a result, 215 nm was found as optimum absorption wavelength for E_1 and 230 nm for DES and DS, while in FD 280 nm was found as the best excitation wavelength for

all analytes and as emission wavelength, 320 nm was the optimum for 2-OHE_2 and 310 nm for the rest of compounds. Regarding mobile phase additives, it could be observed that, in general terms, the fluorescence of the analytes was not increased, however, in the case of 2-OHE_2 the addition of formic acid clearly improved it. As a consequence, the addition of 1 mM of formic acid to the mobile phase was found adequate. Figure IV.1 shows the separation of the nine compounds under the optimum separation and detection conditions.

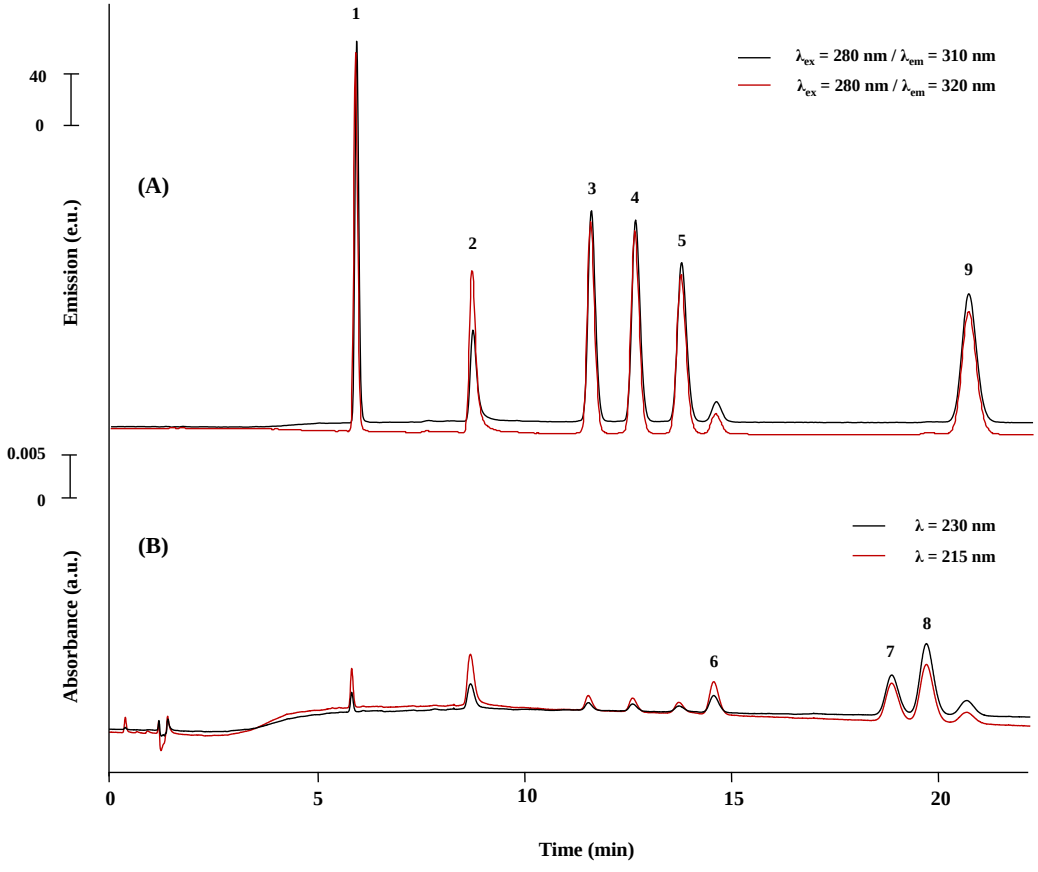


Figure IV.1.- HPLC-DAD/FD chromatograms of the target analytes at their maximum excitation/emission (A) and absorption (B) wavelengths. Flow rate: 1 mL/min. Injection volume: 20 µL. Sample dissolved in ACN/H_2O 15/85 (v/v) containing 1 mM formic acid. Analyte identification and concentration: (1) E_3 (0.75 mg/L), (2) 2-OHE_2 (4.30 mg/L), (3) 17β-E_2 (0.75 mg/L), (4) 17α-E_2 (0.75 mg/L), (5) EE_2 (0.75 mg/L), (6) E_1 (2.20 mg/L), (7) DES (2.20 mg/L), (8) DS (2.20 mg/L) and (9) HEX (0.75 mg/L). Gradient described in Section III.5.1.

In order to study the repeatability of the separation, a study consisting of three consecutive injections (n = 3) of a mixture of the analytes at two levels of concentration in three different days (n = 9) was carried out. Good repeatability for the retention time and peak areas was observed in the same day and between days, with relative standard deviations (RSDs) lower than 0.2 and 2.0 %, and 0.7 and 3.7 %, respectively, as shown in Table IV.1.

After that, instrumental calibration curves based on the peak areas were obtained for each oestrogenic compound injecting seven different concentration levels (n = 7) in triplicate (see Table IV.2), obtaining determination coefficients (R^2) higher than 0.9964 for all of them. Instrumental LODs and LOQs were also calculated as the concentration which provided a signal-to-noise ratio (S/N) of 3 and 10, respectively. These values ranged between 3.2 and 80 μg/L (LODs) and 11 and 267 μg/L (LOQs) for the analytes detected by FD, and between 176 and 507 μg/L (LODs) and 587 μg/L and 1.7 mg/L (LOQs) for those detected by DAD. These results evidence the higher sensitivity that is usually achieved with FD, compared with DAD.

Table IV.1.- Instrumental repeatability (expressed as % RSD) for the HPLC-DAD/FD separation of the selected compounds.

Analyte	**Intraday precision[a] (*n* = 3)**		**Interday precision[a] (*n* = 9)**	
	Retention time	Area	Retention time	Area
E_3 **	0.1	0.6	0.7	3.3
2-OHE_2 **	0.1	1.3	0.5	1.6
17β-E_2 **	0.1	0.5	0.5	2.5
17α-E_2 **	0.1	0.6	0.5	2.5
EE_2 **	0.1	0.4	0.5	2.6
E_1 *	0.1	1.2	0.5	1.6
DES*	0.1	2.0	0.4	2.6
DS*	0.2	1.1	0.4	1.9
HEX**	0.2	0.2	0.4	3.7

* Determined by DAD. ** Determined by FD. [a] Concentration of 0.75 mg/L for all analytes. except for E_1, DES and DS (2.20 mg/L) and 2-OHE_2 (3.20 mg/L).

IV.1.4.- Optimisation of the HF-LPME procedure

As it was previously indicated, HF-LPME is an interesting alternative for the extraction of oestrogens in a simple, fast and efficient way. However, its application in complex matrices like milk or dairy products requires a previous deproteinisation step, which is

normally carried out with an organic solvent and/or the addition of an acid. Then, after evaporation of the organic solvent, an aqueous extract is obtained, which, once diluted, can act as the donor phase in the LPME step. With the aim of not introducing matrix effects and having a better vision of the influence of each factor in the extraction process, the optimisation of the influencing factors (pH, ionic strength, extraction time and temperature, stirring speed and back extraction parameters) was developed with Milli-Q water prior to the application of the methodology to the samples of study.

Table IV.2.- Calibration data for the HPLC-DAD/FD separation of the selected compounds.

Analyte	Calibration data (n = 7)				LOD[a)] (µg/L)	LOQ[b)] (µg/L)
	Range of concentration tested (mg/L)	Slope	Intercept	R^2		
E_3**	0.01-0.43	$4.58 \cdot 10^4 \pm 1.85 \cdot 10^4$	$5.13 \cdot 10^5 \pm 4.46 \cdot 10^5$	0.9989	3.2	11
2-OHE_2**	0.27-10.7	$2.94 \cdot 10^3 \pm 0.21 \cdot 10^3$	$-8.83 \cdot 10^5 \pm 11.85 \cdot 10^5$	0.9966	80	267
17β-E_2**	0.02-0.34	$4.55 \cdot 10^4 \pm 0.30 \cdot 10^4$	$6.26 \cdot 10^5 \pm 5.48 \cdot 10^5$	0.9972	6.7	22
17α-E_2**	0.03-0.25	$4.30 \cdot 10^4 \pm 0.36 \cdot 10^4$	$4.21 \cdot 10^5 \pm 5.07 \cdot 10^5$	0.9973	7.6	25
EE_2**	0.03-0.90	$4.03 \cdot 10^4 \pm 0.25 \cdot 10^4$	$-4.80 \cdot 10^5 \pm 10.86 \cdot 10^5$	0.9978	9.1	31
E_1*	1.70-25.4	$2.28 \cdot 10 \pm 0.22 \cdot 10$	$2.05 \cdot 10^4 \pm 3.06 \cdot 10^4$	0.9964	507	1689
DES*	0.99-29.7	$5.92 \cdot 10 \pm 0.14 \cdot 10$	$-1.48 \cdot 10^4 \pm 2.20 \cdot 10^4$	0.9997	295	982
DS*	0.60-17.9	$9.04 \cdot 10 \pm 0.14 \cdot 10$	$-1.85 \cdot 10^4 \pm 2.90 \cdot 10^4$	0.9992	176	587
HEX**	0.05-1.40	$5.52 \cdot 10^4 \pm 0.21 \cdot 10^4$	$2.85 \cdot 10^5 \pm 15.01 \cdot 10^5$	0.9991	14	46

* Determined by DAD. ** Determined by FD. R^2: Determination coefficient. [a)] Calculated as the concentration associated with a S/N of 3. [b)] Calculated as the concentration associated with a S/N of 10.

Preliminary experiments were carried out using a two-phase HF-LPME procedure impregnating it in 1-octanol since this solvent has provided good results in two-phase HF-LPME of some of the selected analytes in water samples, such as E_1, EE_2, DES (Basheer et al., 2005) or 17β-E_2 (Basheer et al., 2005; Sarafraz-Yazdi and Amiri, 2010). In fact, it is widely known that 1-octanol constitutes an excellent solvent for this type of applications, due to its low volatility, low solubility in water, higher affinity for the target analytes and its non-polar character which favours its retention in the pores of the HF (Pedersen-Bjergaard and Rasmussen, 2008).

The fibre was cut into pieces of 2 cm and impregnated as described in Section III.8.1. All the experiments during the optimisation of the HF-LPME procedure were developed in

duplicate, using 10 mL of spiked Milli-Q water as donor phase containing the target analytes at a concentration of 20 μg/L for 17β-E_2, 17α-E_2, E_3, EE_2 and HEX, 50 μg/L for E_1, DES and DS, and 100 μg/L for 2-OHE_2. A new fibre was used for each experiment.

IV.1.4.1.- Extraction time selection

The mass transference from the donor to the acceptor phase is a process that depends on the extraction time until the equilibrium is achieved. However, since HF-LPME is a non-equilibrium technique, not excessively long extractions are required, as long as a good sensitivity and precision are also reached. In this case, the influence of the extraction time was studied up to 120 min, maintaining the rest of the conditions as follows: 10 mL of Milli-Q water at pH 6, 2 cm of fibre impregnated in 1-octanol and a stirring speed of 1000 r.p.m. at 25 ºC. The recovery of the analytes at the end of the extraction was carried out by retraction of the extractant solvent in the syringe. Figure IV.2 shows the peak areas obtained at each extraction time for the analytes determined by FD (Figure IV.2A) and by DAD (Figure IV.2B). As can be seen, the area generally increases with time, even after 120 min of extraction, but for some analytes a slight reduction of the extraction efficiency is observed after 90 min. This fact suggests a partial back extraction of these analytes into the donor phase. Based on that, and also taking into account the increase of the standard deviation at high extraction times, 60 min was taken as a compromise.

IV.1.4.2.- Evaluation of the pH of the aqueous phase

The effect of the pH of the donor phase on the extraction efficiency was studied under the described conditions applying an extraction time of 60 min. Since pK_a values of the target compounds are between 9.7 and 10.7, the pH was varied between 5 and 10 to cover the range in which they are in their neutral form and, consequently, migrate to the organic phase through the hydrophobic SLM. As can be seen in Figure IV.3, at pH values higher than 6-7, a considerable decrease in the extraction efficiency was obtained for the majority of the oestrogens. This is still more pronounced above pH 9, which can be attributed to the fact that the target analytes are in their anionic forms at these pH values, and their affinity for the aqueous phase is higher than that for the organic solvent. Based on these results, a pH value of 6 was selected for further experiments.

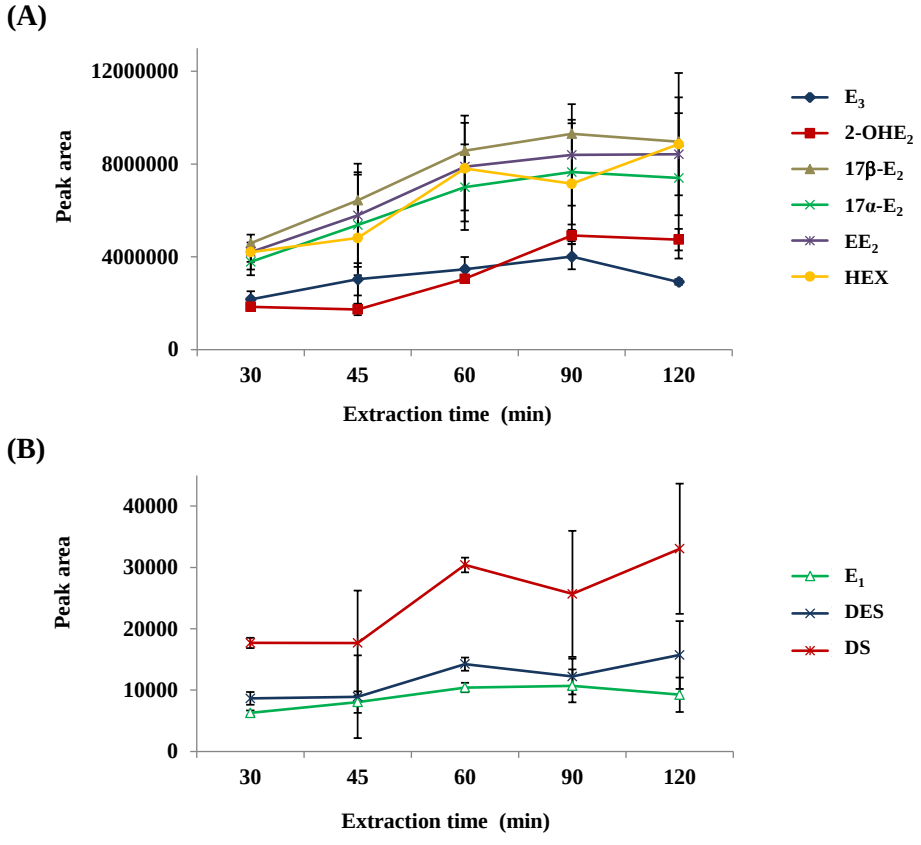


Figure IV.2.- Effect of the extraction time on the peak area of the target analytes after the HF-LPME procedure (2.0 cm fibre, 1-octanol as extraction solvent). Extraction conditions: 10 mL of spiked Milli-Q water at pH 6, stirring at 1000 r.p.m. and 25 °C. Two extractions (n = 2) were carried out in each case. Concentration of the analytes: 20 µg/L of 17β-E_2, 17α-E_2, E_3, EE_2 and HEX, 50 µg/L of E_1, DES and DS, and 100 µg/L of 2-OHE_2.

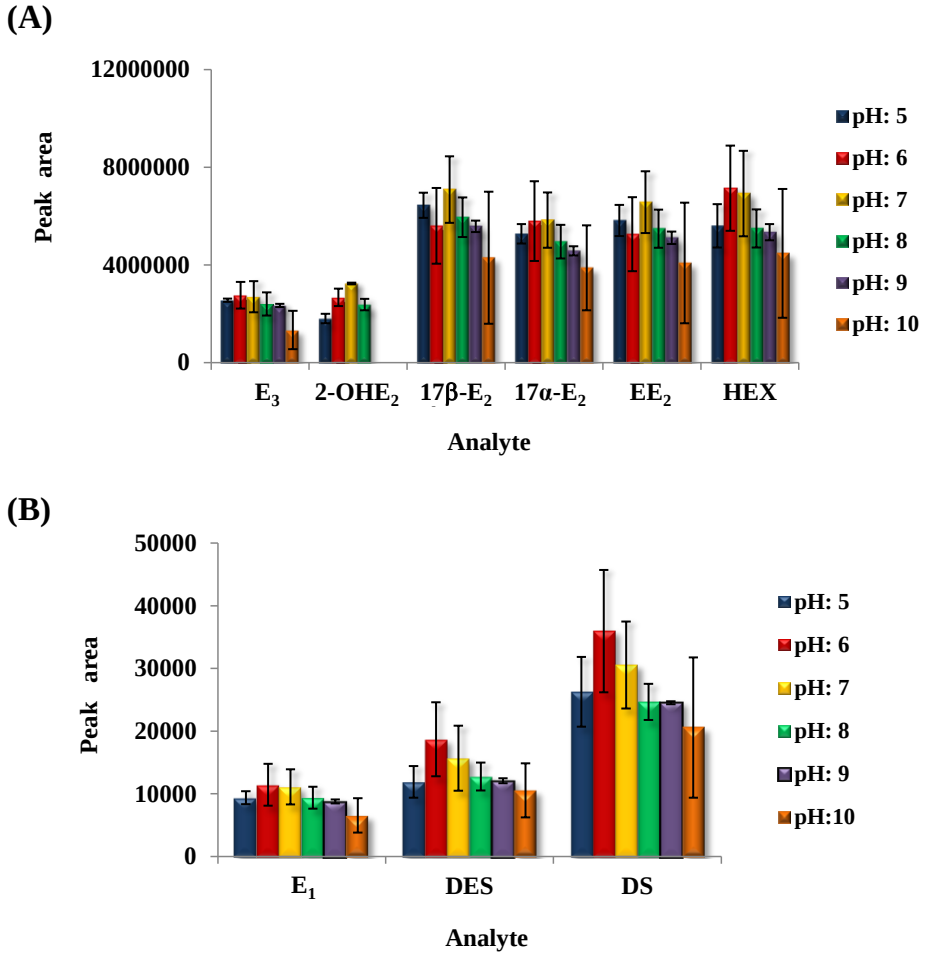


Figure IV.3.- Effect of the sample pH on the peak area of the target analytes after the HF-LPME procedure. Extraction conditions: 10 mL of spiked Milli-Q water, 60 min of extraction at 1000 r.p.m. and 25 °C. Two extractions (n = 2) were carried out in each case. Concentration of the analytes: 20 µg/L of 17β-E_2, 17α-E_2, E_3, EE_2 and HEX, 50 µg/L of E_1, DES and DS, and 100 µg/L of 2-OHE_2.

IV.1.4.3.- Study of the ionic strength of the aqueous phase

In general terms, an increase of the ionic strength of the donor phase usually favours the extraction capacity of the organic solvent. This phenomenon is known as salting-out effect and for LPME techniques is commonly evaluated by the addition of NaCl to the aqueous donor phase. In this regard, the percentage of NaCl was varied between 0 and 25 % (w/v), maintaining the rest of the conditions as previously obtained. As can be seen in Figure IV.4, the

addition of a 10 % (w/v) of NaCl to the donor phase improves the extraction of the analytes, in some cases in even 2-3 times, a fact also observed by other authors who have extracted oestrogens from water samples (Basheer et al., 2005; Li et al., 2011). However, the efficiency starts to decrease above this value. This fact can be associated with the possible changes that can be produced in the physicochemical conditions of the diffusion layer when the ionic strength is increased, which can difficult the transport of the analytes through it towards the organic solvent of the SLM (Lambropoulou and Albanis, 2007). For this reason, the addition of a 10 % (w/v) of NaCl to the donor phase was considered.

IV.1.4.4.- Effect of the stirring rate

The stirring speed is critical to assist the mass transference by the physical movement of the analytes. In the present work, it was studied between 250 and 1500 r.p.m. A progressive increase of the extraction efficiency up to 1500 r.p.m. (the highest available speed of the stirrer) was observed. However, it was experimentally demonstrated that a high stirring speed near to this limit produced a random rotation of the stir bar in the sample vial precluding the adequate reproducibility of the results. For this reason, a value of 1250 r.p.m. was taken as the speed which allowed a good extraction of the analytes with suitable standard deviations.

IV.1.4.5.- Influence of the extraction temperature

Despite the fact that most HF-LPME applications present in the literature have been developed without temperature control (Asensio-Ramos et al., 2011b), it has been reported that this parameter may affect considerably the extraction efficiency of the procedure. Indeed, it is known that it can produce two antagonistic effects on the extraction efficiency of HF-LPME. On the one hand, an increment of the temperature favours the mass transference of the analytes towards the acceptor phase, but, in the other, it diminishes the K_{ow} values of the analytes generating a lower affinity of them for the organic acceptor solvent (Xiong and Hu, 2008). To study the influence of both effects, the temperature was varied between 25 (room temperature) and 60 °C under the previously optimised conditions. At 25 °C, a better efficiency as well as an enhanced repeatability between extractions was obtained. In addition, at high temperatures the metabolite 2-OHE_2 was not extracted at all. Consequently, the extractions were carried out at room temperature in the subsequent studies.

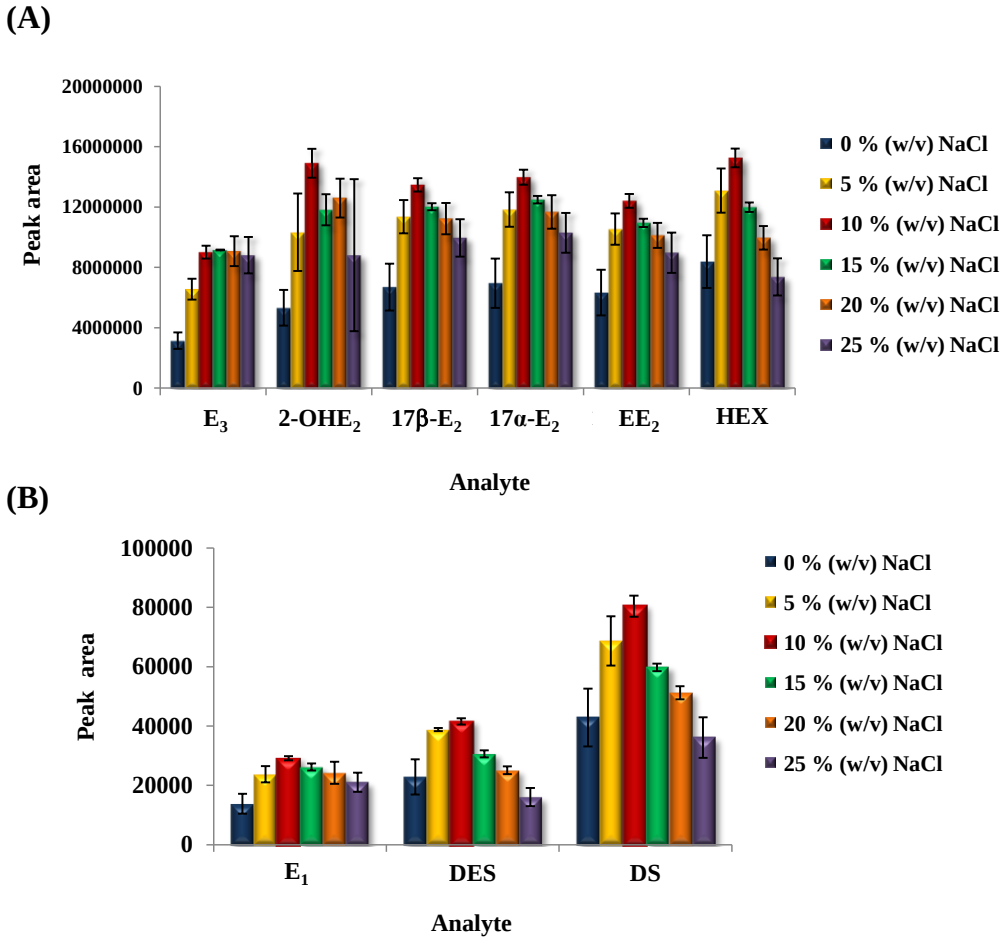


Figure IV.4.- Effect of the ionic strength expressed as NaCl percentage (w/v) on the peak areas of the target analytes after the HF-LPME procedure. Extraction conditions: 10 mL of spiked Milli-Q water at pH 6, 60 min of extraction at 1000 r.p.m. and 25 °C. Two extractions (n = 2) were carried out in each case. Concentration of the analytes: 20 µg/L of 17β-E_2, 17α-E_2, E_3, EE_2 and HEX, 50 µg/L of E_1, DES and DS, and 100 µg/L of 2-OHE_2.

IV.1.4.6.- Effect of the back extraction solvent

In some manuscripts published in the literature it has been suggested that the back extraction of the analytes from the fibre with a second solvent may improve the recovery values (Basheer et al., 2005; Yang et al., 2012). In this sense, some experiences were developed in order to evaluate the back extraction efficiency of different solvents. Due to the

dimensions of the injection vial and the length of the fibre, a volume of 500 μL of solvent was necessary to completely cover the HF during the back extraction. Several solvents with different polarities and properties were tested, including: ACN, acetone, DCM, MeOH and cyclohexane, maintaining the rest of the conditions as previously optimised. In all cases, the back extraction process was carried out for 5 min with the assistance of ultrasounds. After that, the solvents were evaporated under a stream of nitrogen, the dry extract was reconstituted in 100 μL of the initial composition of the mobile phase and 20 μL were injected in the chromatographic system. As can be seen in Figure IV.5, for some analytes the peak areas were duplicated with respect to the normal retraction of 1-octanol contained in the lumen of the fibre. In general, ACN and acetone provided the best results; however, taking into account the repeatability between extractions, ACN was finally selected. Afterwards, a brief study of the sonication time was developed to observe if longer times provided better efficiency. Times between 5 and 12 min were evaluated, observing that 7 min provided the maximum efficiency, so this value was chosen for the application of the methodology.

IV.1.5.- Validation of the HF-LPME-HPLC-DAD/FD method in Milli-Q water

With the aim of validating the proposed method, a linearity study was carried out, by the obtaining of the calibration curves as well as the evaluation of the LODs and LOQs of the method. Precision and accuracy studies were also carried out at two levels of concentration.

For the calibration study, aliquots of 10 mL of Milli-Q water spiked at six different levels of concentration (n = 6) were submitted to the already optimised conditions (pH 6, 10 % (w/v) of NaCl, 2 cm of HF impregnated in 1-octanol, extraction at room temperature for 60 min at 1250 r.p.m. and back extraction in 500 μL of ACN for 7 min with the help of ultrasounds) to obtain the calibration curves of the whole method. Table IV.3 shows that R^2 values were higher than 0.9961 for all compounds, except for 2-OHE_2, for which it was 0.9901. LODs of the method were between 0.12 and 6.0 μg/L for those determined by FD and between 2.8 and 6.0 μg/L for those determined by DAD, which were experimentally checked extracting Milli-Q water samples spiked at these concentrations and calculating the S/N.

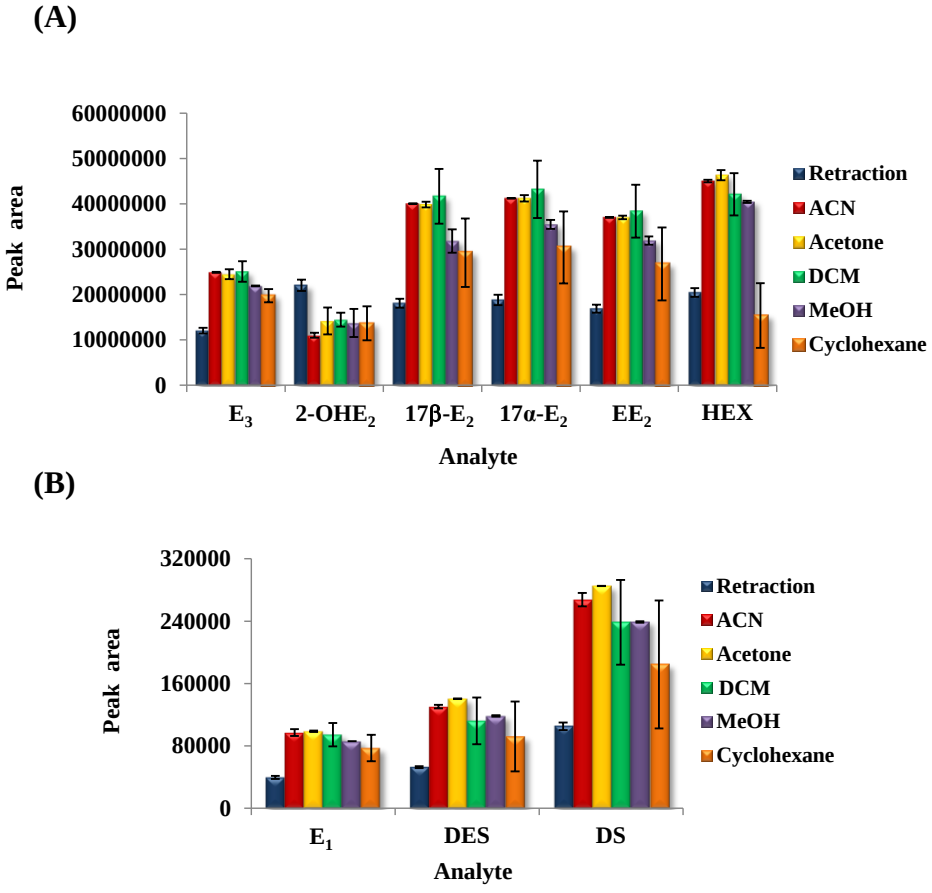


Figure IV.5.- Effect of the type of the back extraction solvent on the peak area of the target analytes after the HF-LPME procedure. Extraction conditions: 10 mL of spiked Milli-Q water at pH 6 containing 10 % (w/v) of NaCl and 60 min of extraction at 1250 r.p.m. and 25 °C. Back extraction conditions: 500 µL of solvent and assisted by 5 min of ultrasounds. Two extractions (n = 2) were carried out in each case. Concentration of the analytes: 20 µg/L of 17β-E_2, 17α-E_2, E_3, EE_2 and HEX, 50 µg/L of E_1, DES and DS, and 100 µg/L of 2-OHE_2.

For precision and accuracy studies, Milli-Q water samples were spiked at two concentration levels and the optimised method was applied five times at each level (n = 5). Table IV.4 shows the results of this study, in which a statistical comparison between the spiked and the found concentrations was carried out using the Student's *t* test. As can be seen, experimental *t* values were equal or lower than the tabulated one (2.78 for n - 1 = 4, P = 0.05),

thus the null hypothesis can be accepted because there are not significant differences between the real and the found concentrations. Besides, good accuracy was obtained with values ranged between 86 and 118 %, showing that the method is highly repeatable and accurate.

Table IV.3.- Calibration data of the selected compounds in Milli-Q water after the application of the HF-LPME-HPLC-DAD/FD method.

Analyte	Calibration data (n = 6)				LOD[a)] (µg/L)	LOQ[b)] (µg/L)
	Range of concentration tested (mg/L)	Slope	Intercept	R^2		
E_3**	0.40 - 12.1	$1.17 \cdot 10^6 \pm 5.68 \cdot 10^4$	$7.46 \cdot 10^4 \pm 3.37 \cdot 10^5$	0.9993	0.12	0.40
2-OHE_2**	80.2 - 601	$9.87 \cdot 10^4 \pm 1.81 \cdot 10^4$	$-9.95 \cdot 10^6 \pm 6.27 \cdot 10^6$	0.9901	6.0	20
17β-E_2**	0.60 - 18.1	$2.12 \cdot 10^6 \pm 6.82 \cdot 10^4$	$-2.01 \cdot 10^5 \pm 6.08 \cdot 10^5$	0.9997	0.18	0.60
17α-E_2**	0.70 - 20.6	$1.50 \cdot 10^6 \pm 1.72 \cdot 10^5$	$4.02 \cdot 10^5 \pm 1.94 \cdot 10^6$	0.9961	0.21	0.69
EE_2**	0.86 - 25.9	$1.65 \cdot 10^6 \pm 1.18 \cdot 10^5$	$-1.01 \cdot 10^6 \pm 1.50 \cdot 10^6$	0.9985	0.26	0.86
E_1*	20.0 - 1000	$1.32 \cdot 10^3 \pm 1.20 \cdot 10^2$	$-2.15 \cdot 10^4 \pm 6.60 \cdot 10^4$	0.9976	6.0	20
DES*	15.2 - 304	$2.13 \cdot 10^3 \pm 9.48 \cdot 10$	$-1.05 \cdot 10^4 \pm 1.56 \cdot 10^4$	0.9994	4.6	15
DS*	9.39 - 282	$5.15 \cdot 10^3 \pm 4.31 \cdot 10^2$	$-4.33 \cdot 10^4 \pm 5.97 \cdot 10^4$	0.9979	2.8	9.4
HEX**	1.20 - 24.1	$1.67 \cdot 10^6 \pm 7.24 \cdot 10^4$	$6.26 \cdot 10^4 \pm 9.47 \cdot 10^5$	0.9994	0.36	1.2

* Determined by DAD. ** Determined by FD. R^2: Determination coefficient. a) Calculated as the concentration associated with a S/N of 3. b) Calculated as the concentration associated with a S/N of 10.

IV.1.6.- Optimisation of the deproteinisation process in milk and dairy product samples

Once the developed method was validated in Milli-Q water, its suitability for the extraction of oestrogens in milk and dairy products was also evaluated. As it has been previously indicated, the application of HF-LPME to these types of samples requires a previous deproteinisation step in order to reduce matrix effects and also to avoid the damage of the LC column since proteins may irreversibly link to the stationary phase.

Firstly, the procedure was optimised for milk samples. Based on previous studies in which the determination of oestrogens in this matrix was carried out (Farlow et al., 2009; Kyul et al., 2011; Tso and Aga, 2010), the use of weak acids together with organic solvents and the modification of the ionic strength were considered. With this aim, spiked samples of whole cow milk were used, since it was the most complex of the three selected milk products. In every experiment, two replicates and a non-spiked sample were analysed in parallel to ensure the absence of possible chromatographic interferences and residues of the target analytes in the samples.

Table IV.4.- Results of the precision and accuracy study of the HF-LPME-HPLC-DAD/FD method for the selected compounds in Milli-Q water.

Analyte	Spiked level (µg/L)	Found[a)] (µg/L)	Accuracy	$t^{b)}$
E_3**	2.01	1.95 ± 0.22	97	0.11
	7.23	6.21 ± 0.25	86	0.65
2-OHE_2**	100	117 ± 43	118	2.52
	361	324 ± 37	90	0.90
17β-E_2**	3.02	2.87 ± 0.69	95	0.18
	10.9	11.1 ± 0.7	102	1.14
17α-E_2**	3.43	3.62 ± 0.79	106	0.16
	12.4	12.8 ± 0.8	103	1.01
17α-EE_2**	4.32	4.23 ± 0.03	98	0.08
	15.5	15.3 ± 0.0	98	0.54
E_1*	100	115 ± 8	116	0.53
	360	356 ± 8	98	0.72
DES*	76.0	81.7 ± 21.9	105	0.50
	274	288 ± 31	105	1.28
DS*	47.0	48.6 ± 13.0	99	0.29
	169	167 ± 137	99	0.40
HEX**	6.02	5.67 ± 0.39	108	0.20
	21.7	23.4 ± 0.6	108	1.76

* Determined by DAD. ** Determined by FD. [a)] Average value ± standard deviation of five determinations (95 % confidence value). [b)] t_{tab} = 2.78, α = 0.05.

Initially, and since the optimised HF-LPME procedure required the addition of 10 % (w/v) of NaCl, an increase of the ionic strength was applied adding 1.0 g of NaCl to 2 mL of milk. After vortex agitation for 1 min and centrifugation for 30 min at 4400 r.p.m., the supernatant was collected and diluted with Milli-Q water up to 10 mL, filtered through a 0.45 µm filter and the pH was adjusted to 6.0. However, due to an incomplete precipitation, the addition of acetic acid was evaluated. With this purpose, 100 µL of acetic acid were added to 2 mL of milk and after vortex shaking (1 min) the sample was centrifuged for 30 min at 4400 r.p.m. Then 8.8 mL of Milli-Q water were added to the supernatant to reach a final volume of 10 mL. One gram of NaCl was then incorporated; the pH was adjusted to 6 using a solution 8 M of NaOH and the sample was filtered prior to the HF-LPME procedure. In this case, the precipitation of the proteins was adequate and a clean extract was obtained. However, the extraction efficiency was considerably low. The variation of the conditions did not improve the results, not even using ultrasounds to assist the precipitation step, a fact that can be attributed to the high lipophilic character of the oestrogens.

In order to solve this problem and, taking into account the good results obtained in previous publications with the addition of organic solvents (Msagati and Nindi, 2006b; Shao et al., 2005), different experiences were developed using MeOH or ACN in a proportion between 1/2 and 1/3 (v/v) milk/solvent and changing the volume (50-200 μL) and type of acid (acetic or formic). The mixture was vortex stirred for 1 min and maintained 15 min in the darkness to favour proteins precipitation. Fifteen minutes of centrifugation at 4400 r.p.m. were enough to produce phase separation. Then, the organic solvent was evaporated in a rotavapor at 40 °C and to the obtained aqueous residue, Milli-Q water was added up to 10 mL as well as 1.0 g of NaCl. The pH was then adjusted to 6, the sample was filtered and HF-LPME was carried out under the optimised conditions. In this case, the extraction efficiency was much higher, especially in the case of ACN since it can improve the deproteinisation and the extraction of the analytes from the aqueous matrix.

The best results were obtained using 100 μL of acetic acid and 4 mL of ACN, except for the metabolite, 2-OHE_2, for which MeOH resulted to be slightly better. However, phase separation was poorer with MeOH, so ACN was finally selected maintaining a proportion of 1/2 (v/v) milk/ACN because it provides the best performance as it has been previously suggested in the literature (Tso and Aga, 2010). Regarding acid addition, the use of formic acid resulted in a diminution of peak areas for all analytes, especially again for 2-OHE_2, an effect that was also observed when using volumes of acetic acid lower than 100 μL. Following, an increase of the sample volume was considered using 3 and 4 mL of milk with the same proportion of acid and solvent with the objective of increasing the LODs of the method. When 4 mL of sample were used, several interferences that hindered the identification and quantification of the analytes were found. However, when 3 mL were extracted with 6 mL of ACN and 150 μL of acetic acid, no interferences were observed as can be seen in Figure IV.6 in which the blanks of the different types of milk samples analysed as well as a spiked cow whole milk are shown. Consequently, these conditions were chosen as the optimum ones for the deproteinisation step.

Because of the high complexity of dairy products, the direct application of the developed procedure to the analysis of milk samples did not show satisfactory results for the extraction of the target analytes. However, it was observed that slight modifications clearly improved the results. To evaluate such modification, each type of matrix was studied independently by carrying out the experiments in duplicate and by performing extractions of blank samples in parallel to verify the absence of interfering peaks.

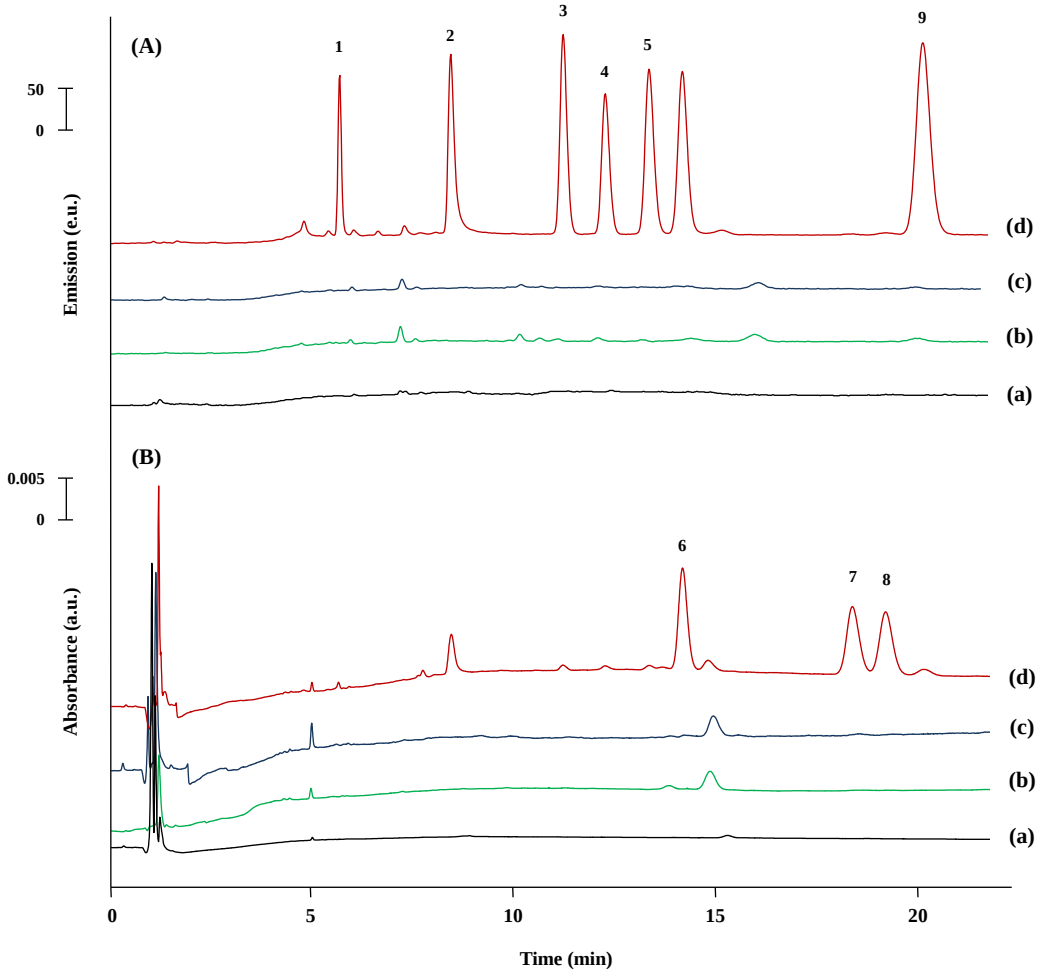


Figure IV.6.- HPLC-DAD/FD chromatograms corresponding to a blank sample of whole (a), semi-skimmed (b) and skimmed (c) cow milk and a spiked whole cow milk sample (d) after the application of the optimised method. Detection wavelengths: (A) FD at λ_{ex} = 280 nm, λ_{em} = 310 nm and (B) DAD at λ = 215 nm. Analyte identification and concentration in milk: (1) E_3 (0.02 mg/L), (2) 2-OHE_2 (0.97 mg/L), (3) 17β-E_2 (0.03 mg/L), (4) 17α-E_2 (0.03 mg/L), (5) EE_2 (0.06 mg/L), (6) E_1 (1.47 mg/L), (7) DES (3.00 mg/L), (8) DS (1.48 mg/L) and (9) HEX (0.25 mg/L).

In the case of yogurt samples, the application of the methodology developed for milk samples provided a decrease of the extraction efficiencies and a troublesome filtration after the deproteinisation, due to the presence of suspended solids, especially for skimmed yogurt. These results pointed to an incomplete precipitation of the proteins since, while milk samples

contained around 3 g protein/100 g, the selected skimmed yogurt had around 4 g/100 g (See Section III.4). Taking this aspect into account, the sample/ACN ratio was modified from 3 g of yogurt/6 mL of ACN up to 3 g yogurt/12 mL ACN always adding 150 μL of acetic acid. With this change, the dielectric constant of the medium was lower and promoted the precipitation of the proteins and, at the same time, improved the extraction of the analytes. In addition, and due to the more viscous consistency of this sample, higher vortex times (from 1 min up to 4 min) were considered. In this way, the contact between the sample, the analytes and the precipitation/extraction solution increased. Finally, a 1/3 (w/v) yogurt/ACN ratio, the addition of 150 μL of acetic acid and the vortex assistance of the extraction for 3 min, followed by the HF-LPME procedure provided similar extraction efficiencies as for milk samples. As can be seen in Figure IV.7 no interferences that precluded the correct analysis of the analytes were found.

The methodology was also applied to the analysis of a commercial liquid probiotic dairy fermented product to which active live cultures (*Lactobacillus casei*) were added. As it happened in the analysis of yogurt samples, the precipitation step previously applied for milk samples was not suitable to produce an appropriate extract. In this case, a 1/3 v/v probiotic product/ACN ratio and the addition of 150 μL of acetic acid followed by 3 min vortex also provided the best results, but still the extraction efficiency was not good enough and precipitation of the proteins was not repeatable. To solve this problem, the volume of acetic acid was increased up to 200 μL. Results showed that the use of 175 μL of acid provided a cleaner extract after deproteination and also improved the sensitivity, being similar to that obtained for yogurt and milk samples. Figure IV.8 shows the chromatograms of a spiked and blank sample obtained in this case with the optimised conditions.

For cheese analysis, the application of the previously developed methodology was not effective at all. In this case, the amounts of fats and proteins were considerably higher than for the other matrices (14 g and 10 g/100 g of cheese, respectively) and, consequently, the addition of higher amounts of ACN was necessary. Initially, 3 g of chopped cheese were treated in the same way as yogurt samples (9 mL of ACN, 150 μL of acetic acid) but a homogenisation at high speed for 3 min with an Ultra-Turrax laboratory homogeniser was necessary due to the solid character of the sample. After centrifugation, fat droplets were visible in the supernatant and the walls of the tube. Besides, the filter was blocked when the extract was filtered, extraction efficiency was very low and 2-OHE_2 was not recovered at all. Even with the use of

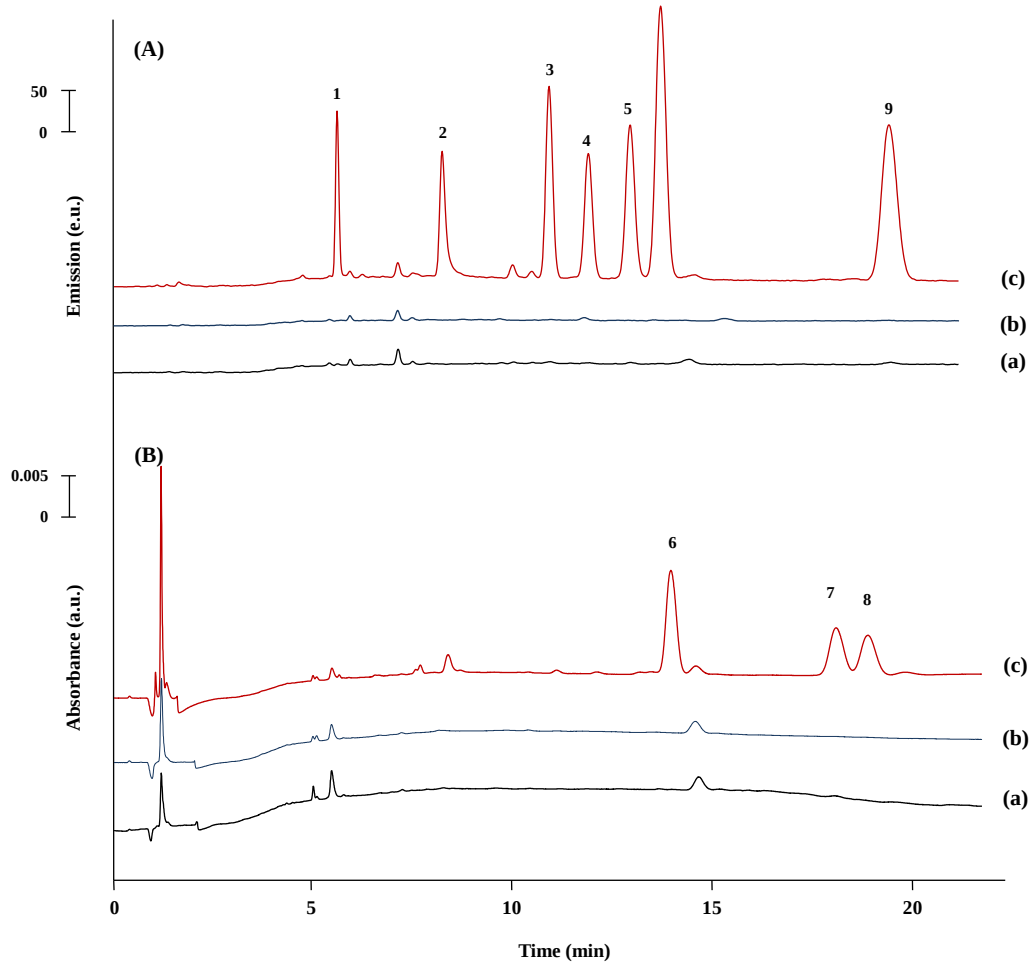


Figure IV.7.- HPLC-DAD/FD chromatograms of a blank sample of whole yogurt (a), a blank sample of skimmed yogurt (b) and spiked whole yogurt (c) after the application of the method. Detection wavelengths: (A) FD at λ_{ex} = 280 nm, λ_{em} = 310 nm and (B) DAD at λ = 215 nm. Analyte identification and concentration in the yogurt: (1) E_3 (0.02 mg/kg), (2) 2-OHE_2 (0.31 mg/kg), (3) 17β-E_2 (0.03 mg/kg), (4) 17α-E_2 (0.03 mg/kg), (5) EE_2 (0.05 mg/kg), (6) E_1 (2.37 mg/kg), (7) DES (4.87 mg/kg), (8) DS (2.00 mg/kg) and (9) HEX (0.29 mg/kg).

higher amounts of ACN or acetic acid, results did not improve and fat was still observed. For this reason, n-hexane was used as a lipid remover, considering the good results obtained in the literature when fatty matrices were analysed (Giménez et al., 2013; Zeng et al., 2005). The

solvent was added to the ACN supernatant layer obtained after the precipitation step. After suitable agitation and centrifugation, the upper lipid phase was removed, while the rest of the extract was evaporated as the other samples. It was found that 4 mL of n-hexane were necessary and sufficient to produce a suitable defatting of the supernatant, obtaining better LODs (similar to the other dairy products) and cleaner chromatograms (See Figure IV.9).

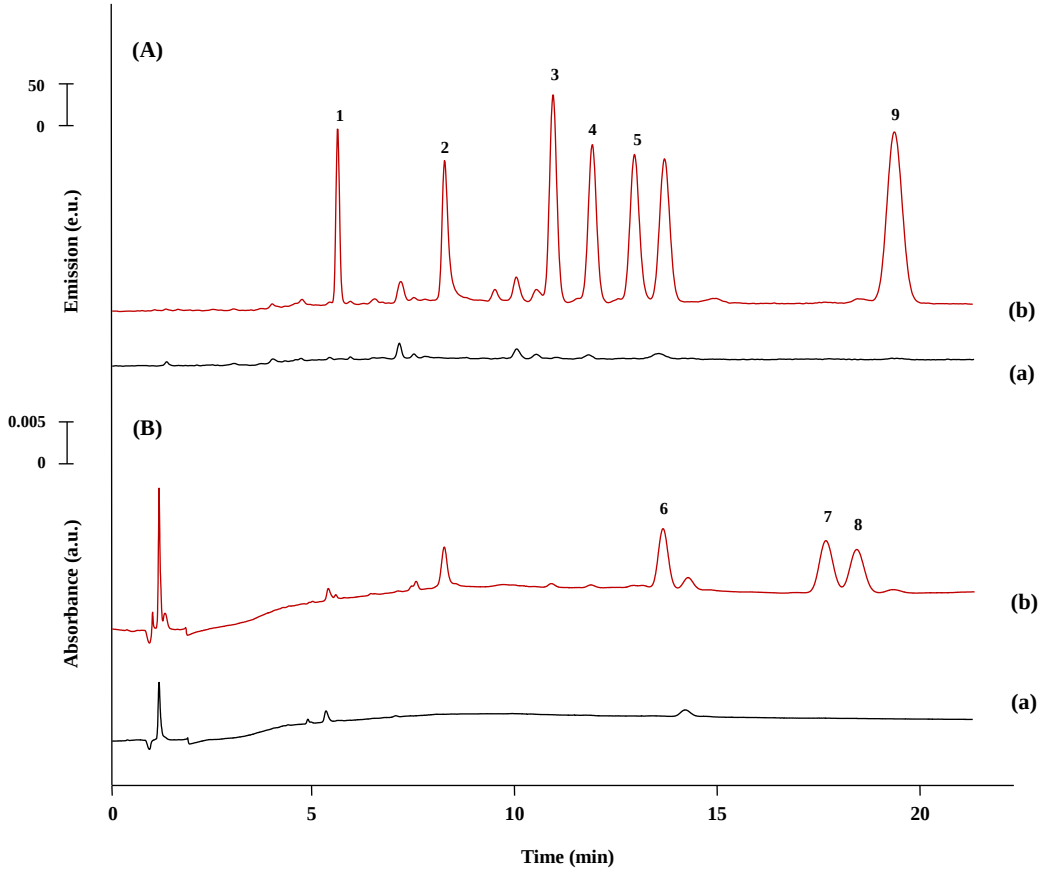


Figure IV.8.- HPLC-DAD/FD chromatograms of a blank sample (a) and a spiked sample (b) of the probiotic product after the application of the method. Detection wavelengths: (A) FD at λ_{ex} = 280 nm, λ_{em} = 310 nm and (B) DAD at λ = 215 nm. Analyte identification and concentration in the probiotic product: (1) E_3 (0.02 mg/kg), (2) 2-OHE_2 (0.48 mg/kg), (3) 17β-E_2 (0.02 mg/kg), (4) 17α-E_2 (0.03 mg/kg), (5) EE_2 (0.05 mg/kg), (6) E_1 (0.96 mg/kg), (7) DES (4.28 mg/kg), (8) DS (1.75 mg/kg) and (9) HEX (0.28 mg/kg).

The use of higher volumes diminished the extraction efficiency. In contrast, and despite our efforts, 2-OHE_2 could not be extracted from cheese even after changing the deproteinisation/defatting conditions.

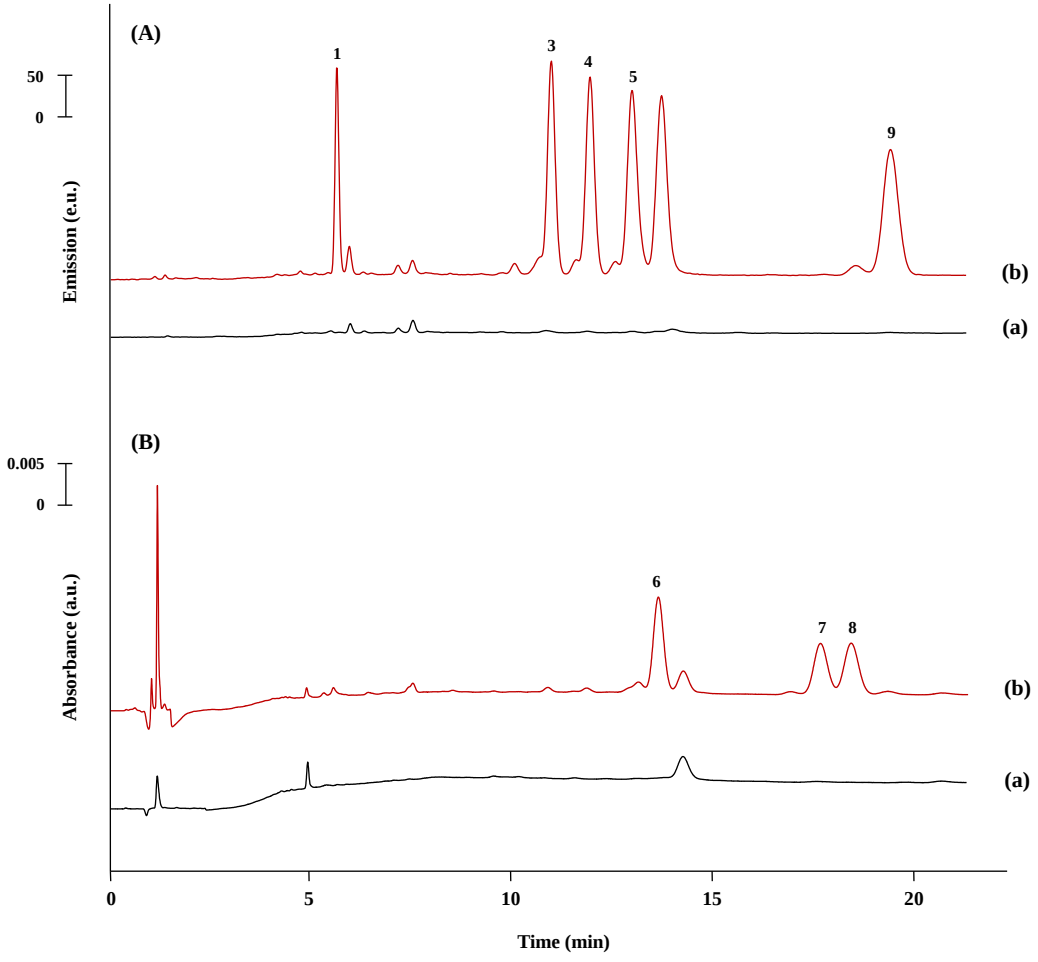


Figure IV.9.- HPLC-DAD/FD chromatograms of a blank sample (a) and a spiked sample (b) of white cheese after the application of the method. Detection wavelengths: (A) FD at λ_{ex} = 280 nm, λ_{em} = 310 nm and (B) DAD at λ = 215 nm. Analyte identification and concentration in cheese: (1) E_3 (0.02 mg/kg), (3) 17β-E_2 (0.03 mg/ kg), (4) 17α-E_2 (0.04 mg/ kg), (5) EE_2 (0.06 mg/ kg), (6) E_1 (1.43 mg/ kg), (7) DES (3.17 mg/ kg), (8) DS (1.91 mg/ kg) and (9) HEX (0.19 mg/ kg).

IV.1.7.- Validation of the HF-LPME-HPLC-DAD/FD method in milk and dairy products

After developing the optimisation of the whole method, it was further validated in terms of linearity, LODs, LOQs, precision and accuracy.

For linearity studies, calibration curves of the method were obtained spiking each matrix at six different concentration levels (n = 6). Table IV.5 shows the results of this study in the different samples, observing very good linearity with R^2 higher than 0.9902 in all cases. The LODs and LOQs were calculated as the concentration of the analyte which provided a S/N of 3 and 10, respectively, and were experimentally checked. LODs were in the range 0.28-107 μg/L for milk, between 0.29 and 73 μg/kg for yogurt, in the range 0.23-64 μg/L for the probiotic product, and between 0.35 and 48 μg/kg for cheese (in this last case, 2-OHE_2 was left out of the study since it was not extracted at all). As it was expected, in general, LODs were higher for those analytes detected by DAD due to the great sensitivity provided by FD.

Afterwards, a precision and accuracy study was developed spiking each matrix with the selected oestrogens at two different concentration levels and performing the extraction in quintuplicate (n = 5). The comparison between the found concentrations, calculated using the previous calibration curves, and the spiked concentrations was developed with a Student's *t*-test, by comparing the experimental *t* value with the tabulated one for n = 5 (t_4 = 2.78, P = 0.05). As shown in Table IV.6, all experimental *t*-values were lower than t_4, with good accuracy percentages, between 81 and 118 %.

IV.1.8.- Comparison with other methodologies applied for the analysis of the selected oestrogens in milk and dairy products

As indicated in Section I.4.1, despite the known harmful effects that these types of compounds may produce in the consumers, regulatory authorities have not established MRLs for oestrogens in milk or dairy products. That is why a comparison of the data obtained with other methodologies seems to be very useful to evaluate the suitability of the developed methodology in the control and evaluation of oestrogenic compounds in the selected matrices.

In general, and regarding the analysis of milk samples, the LODs obtained in this work are of the same order of magnitude than those achieved by other authors with the use of LC coupled to UV or FD systems (Ding et al., 2011; Liu et al., 2010; Markopoulou and Koundourellis, 2006; Yan et al., 2009; Yang et al., 2012; Zhong et al., 2012). In fact, in some

Table IV.5.- Calibration data of the selected compounds in the different milk and dairy samples after the HF-LPME-HPLC-DAD/FD method.

Analyte	Sample	Calibration data (n = 6)				LOD_{method} [a)] (µg/kg)[#]	LOQ_{method} [b)] (µg/kg)[#]
		Range of concentration tested (µg/kg)[#]	Slope	Intercept	R^2		
E_3[**]	WM	1.1-27	$3.14 \cdot 10^5 \pm 1.02 \cdot 10^4$	$-3.84 \cdot 10^4 \pm 1.43 \cdot 10^5$	0.9995	0.32	1.6
	SSM	0.95-28	$2.98 \cdot 10^5 \pm 3.26 \cdot 10^4$	$7.58 \cdot 10^4 \pm 4.57 \cdot 10^5$	0.9965	0.28	0.94
	SM	1.2-62	$2.49 \cdot 10^5 \pm 9.32 \cdot 10^3$	$2.36 \cdot 10^5 \pm 2.83 \cdot 10^5$	0.9993	0.37	1.2
	SY	1.3-66	$2.15 \cdot 10^5 \pm 0.20 \cdot 10^5$	$7.00 \cdot 10^5 \pm 6.69 \cdot 10^5$	0.9952	0.40	1.3
	WY	1.0-28	$2.72 \cdot 10^5 \pm 0.45 \cdot 10^5$	$6.56 \cdot 10^3 \pm 63.76 \cdot 10^3$	0.9920	0.29	0.96
	PrP	0.8-14	$3.58 \cdot 10^5 \pm 0.22 \cdot 10^5$	$-1.48 \cdot 10^5 \pm 3.86 \cdot 10^5$	0.9981	0.23	0.77
	WC	1.2-58	$2.75 \cdot 10^5 \pm 0.22 \cdot 10^5$	$3.96 \cdot 10^5 \pm 5.39 \cdot 10^5$	0.9954	0.35	1.2
$2\text{-}OHE_2$[**]	WM	236-2062	$6.66 \cdot 10^3 \pm 4.82 \cdot 10^2$	$-3.85 \cdot 10^5 \pm 6.14 \cdot 10^5$	0.9973	35	117
	SSM	80-1021	$1.23 \cdot 10^4 \pm 2.35 \cdot 10^3$	$-8.99 \cdot 10^5 \pm 1.28 \cdot 10^6$	0.9961	8.7	29
	SM	54-1625	$3.83 \cdot 10^4 \pm 2.88 \cdot 10^3$	$-3.52 \cdot 10^6 \pm 2.45 \cdot 10^6$	0.9971	16	54
	SY	148-2473	$9.74 \cdot 10^3 \pm 2.63 \cdot 10^3$	$-4.96 \cdot 10^5 \pm 38.67 \cdot 10^5$	0.9920	15	49
	WY	16-780	$2.82 \cdot 10^4 \pm 0.23 \cdot 10^4$	$-8.28 \cdot 10^5 \pm 9.98 \cdot 10^5$	0.9970	4.7	16
	PrP	24-433	$1.95 \cdot 10^4 \pm 0.18 \cdot 10^4$	$-7.52 \cdot 10^5 \pm 10.38 \cdot 10^5$	0.9954	7.2	24
	WC	-	-	-	-	-	-
$17\beta\text{-}E_2$[**]	WM	2.5-63	$2.97 \cdot 10^5 \pm 1.49 \cdot 10^4$	$-1.05 \cdot 10^5 \pm 4.72 \cdot 10^5$	0.9987	0.75	2.5
	SSM	1.7-51	$3.95 \cdot 10^5 \pm 3.98 \cdot 10^4$	$4.50 \cdot 10^5 \pm 1.14 \cdot 10^6$	0.9970	0.50	1.7
	SM	1.8-36	$4.84 \cdot 10^5 \pm 3.25 \cdot 10^4$	$1.61 \cdot 10^5 \pm 6.41 \cdot 10^5$	0.9987	0.54	1.8
	SY	4.1-68	$3.02 \cdot 10^5 \pm 0.46 \cdot 10^5$	$3.20 \cdot 10^6 \pm 1.68 \cdot 10^6$	0.9932	0.40	1.3
	WY	1.3-37	$3.65 \cdot 10^5 \pm 0.45 \cdot 10^5$	$7.39 \cdot 10^5 \pm 8.33 \cdot 10^5$	0.9956	0.38	1.3
	PrP	1.1-19	$4.48 \cdot 10^5 \pm 0.41 \cdot 10^5$	$1.00 \cdot 10^6 \pm 1.02 \cdot 10^6$	0.9956	0.32	1.1
	WC	1.6-81	$3.58 \cdot 10^5 \pm 0.39 \cdot 10^5$	$1.12 \cdot 10^6 \pm 1.59 \cdot 10^6$	0.9936	0.48	1.6
$17\alpha\text{-}E_2$[**]	WM	2.7-67	$2.73 \cdot 10^5 \pm 1.42 \cdot 10^4$	$4.29 \cdot 10^3 \pm 4.83 \cdot 10^5$	0.9986	0.81	2.7
	SSM	5.6-24	$2.02 \cdot 10^5 \pm 6.17 \cdot 10^4$	$1.12 \cdot 10^6 \pm 1.41 \cdot 10^6$	0.9970	0.55	1.8
	SM	1.9-38	$2.85 \cdot 10^5 \pm 4.65 \cdot 10^4$	$3.13 \cdot 10^5 \pm 9.69 \cdot 10^5$	0.9922	0.57	1.9
	SY	4.9-48	$2.67 \cdot 10^5 \pm 0.14 \cdot 10^5$	$2.71 \cdot 10^6 \pm 0.42 \cdot 10^6$	0.9992	0.48	1.6
	WY	1.6-48	$1.65 \cdot 10^5 \pm 0.28 \cdot 10^5$	$7.82 \cdot 10^5 \pm 7.46 \cdot 10^5$	0.9918	0.48	1.6
	PrP	1.4-41	$4.06 \cdot 10^5 \pm 0.44 \cdot 10^5$	$3.06 \cdot 10^5 \pm 9.35 \cdot 10^5$	0.9940	0.40	1.4
	WC	2.0-40	$3.54 \cdot 10^5 \pm 0.41 \cdot 10^5$	$4.33 \cdot 10^5 \pm 9.25 \cdot 10^5$	0.9931	0.58	1.9
EE_2[**]	WM	5.7-142	$1.78 \cdot 10^5 \pm 4.09 \cdot 10^3$	$-5.15 \cdot 10^4 \pm 2.65 \cdot 10^5$	0.9997	1.7	5.7
	SSM	3.4-44	$1.41 \cdot 10^5 \pm 2.36 \cdot 10^4$	$1.23 \cdot 10^6 \pm 2.09 \cdot 10^6$	0.9918	1.0	3.4
	SM	3.1-94	$2.67 \cdot 10^5 \pm 2.81 \cdot 10^4$	$3.47 \cdot 10^5 \pm 1.39 \cdot 10^6$	0.9942	0.94	3.1
	SY	2.3-55	$3.65 \cdot 10^5 \pm 0.38 \cdot 10^5$	$1.10 \cdot 10^6 \pm 1.21 \cdot 10^6$	0.9943	0.68	2.3
	WY	2.7-134	$1.78 \cdot 10^5 \pm 0.08 \cdot 10^5$	$1.08 \cdot 10^6 \pm 0.51 \cdot 10^6$	0.9990	1.8	2.7
	PrP	2.4-71	$2.25 \cdot 10^5 \pm 0.14 \cdot 10^5$	$1.52 \cdot 10^5 \pm 5.36 \cdot 10^5$	0.9979	0.71	2.4
	WC	2.9-144	$1.99 \cdot 10^5 \pm 0.21 \cdot 10^5$	$7.61 \cdot 10^5 \pm 14.96 \cdot 10^5$	0.9944	0.86	2.9

Table IV.5.- (Continued).

Analyte	Sample	Calibration data (n = 6)				LOD_{method} [a] (µg/kg)[#]	LOQ_{method} [b] (µg/kg)[#]
		Range of concentration tested (µg/kg)[#]	Slope	Intercept	R^2		
E_1[*]	WM	44-1094	$2.72 \cdot 10^2 \pm 1.90 \cdot 10$	$-6.98 \cdot 10^3 \pm 1.02 \cdot 10^4$	0.9975	13	43
	SSM	100-1304	$2.41 \cdot 10^2 \pm 4.19 \cdot 10$	$-4.03 \cdot 10^3 \pm 3.95 \cdot 10^4$	0.9910	30	100
	SM	82-1633	$2.86 \cdot 10^2 \pm 4.42 \cdot 10$	$1.17 \cdot 10^4 \pm 4.09 \cdot 10^4$	0.9923	24	81
	SY	89-2128	$2.02 \cdot 10^2 \pm 0.10 \cdot 10^2$	$1.08 \cdot 10^4 \pm 1.25 \cdot 10^4$	0.9989	26	88
	WY	119-3560	$1.41 \cdot 10^2 \pm 0.17 \cdot 10^2$	$3.34 \cdot 10^4 \pm 3.59 \cdot 10^4$	0.9954	36	118
	PrP	48-864	$2.96 \cdot 10^2 \pm 0.31 \cdot 10^2$	$1.49 \cdot 10^3 \pm 23.06 \cdot 10^3$	0.9942	14	48
	WC	71-1427	$2.99 \cdot 10^2 \pm 0.36 \cdot 10^2$	$6.97 \cdot 10^3 \pm 29.21 \cdot 10^3$	0.9926	21	71
DES[*]	WM	271-6767	$3.20 \cdot 10 \pm 6.11 \cdot 10^{-1}$	$7.08 \cdot 10^3 \pm 1.92 \cdot 10^3$	0.9999	81	269
	SSM	358-5375	$5.17 \cdot 10 \pm 8.82$	$6.26 \cdot 10^3 \pm 5.54 \cdot 10^4$	0.9914	107	358
	SM	167-3340	$1.53 \cdot 10^2 \pm 9.50$	$-1.78 \cdot 10^4 \pm 1.72 \cdot 10^4$	0.9989	50	167
	SY	209-5021	$2.26 \cdot 10^2 \pm 0.08 \cdot 10^2$	$2.04 \cdot 10^3 \pm 22.86 \cdot 10^3$	0.9997	63	209
	WY	244-12177	$4.13 \cdot 10 \pm 0.38 \cdot 10$	$1.68 \cdot 10^4 \pm 2.42 \cdot 10^4$	0.9975	73	243
	PrP	214-3849	$7.75 \cdot 10 \pm 1.13 \cdot 10$	$2.24 \cdot 10^3 \pm 29.88 \cdot 10^3$	0.9940	64	214
	WC	158-7920	$6.84 \cdot 10 \pm 0.32 \cdot 10$	$1.26 \cdot 10^4 \pm 1.34 \cdot 10^4$	0.9994	47	158
DS[*]	WM	314-7844	$7.67 \cdot 10 \pm 7.26$	$-9.66 \cdot 10^3 \pm 2.65 \cdot 10^4$	0.9974	94	313
	SSM	199-557	$1.32 \cdot 10^2 \pm 2.5 \cdot 10$	$-1.64 \cdot 10^4 \pm 8.22 \cdot 10^4$	0.9902	60	199
	SM	82.1-1643	$3.12 \cdot 10^2 \pm 1.93 \cdot 10$	$-1.78 \cdot 10^4 \pm 1.72 \cdot 10^4$	0.9989	25	82
	SY	237-5696	$4.17 \cdot 10^2 \pm 0.21 \cdot 10^2$	$-2.36 \cdot 10^4 \pm 7.32 \cdot 10^4$	0.9992	71	237
	WY	100-5000	$1.16 \cdot 10^2 \pm 0.13 \cdot 10^2$	$1.13 \cdot 10^4 \pm 3.41 \cdot 10^4$	0.9963	30	99
	PrP	87-1572	$1.99 \cdot 10^2 \pm 0.24 \cdot 10^2$	$2.58 \cdot 10^3 \pm 25.55 \cdot 10^3$	0.9959	26	88
	WC	95-4767	$1.52 \cdot 10^2 \pm 0.14 \cdot 10^2$	$1.90 \cdot 10^4 \pm 3.39 \cdot 10^4$	0.9955	29	95
HEX[**]	WM	44-1100	$3.65 \cdot 10^4 \pm 3.56 \cdot 10^3$	$-4.88 \cdot 10^5 \pm 1.82 \cdot 10^6$	0.9972	13	44
	SSM	20-55	$5.61 \cdot 10 \pm 9.97 \cdot 10^3$	$-7.08 \cdot 10^5 \pm 3.02 \cdot 10^6$	0.9913	5.9	20
	SM	15-281	$1.25 \cdot 10^5 \pm 6.91 \cdot 10^3$	$-7.65 \cdot 10^5 \pm 1.96 \cdot 10^6$	0.9991	4.2	14
	SY	7.1-171	$1.92 \cdot 10^5 \pm 0.04 \cdot 10^5$	$1.18 \cdot 10^5 \pm 4.17 \cdot 10^5$	0.9999	2.1	7.1
	WY	14-715	$4.33 \cdot 10^4 \pm 0.33 \cdot 10^4$	$1.12 \cdot 10^6 \pm 1.23 \cdot 10^6$	0.9983	4.3	14
	PrP	14-251	$7.18 \cdot 10^4 \pm 1.07 \cdot 10^4$	$2.71 \cdot 10^5 \pm 18.52 \cdot 10^5$	0.9935	4.2	14
	WC	9.6-477	$5.66 \cdot 10^4 \pm 0.07 \cdot 10^5$	$1.27 \cdot 10^6 \pm 16.61 \cdot 10^6$	0.9932	2.8	9.4

WM: Whole milk; SSM: Semi-skimmed milk; SM: Skimmed milk; SY: Skimmed natural yogurt; WY: Whole natural yogurt; PrP: Probiotic product; WC: White cheese. R^2: Determination coefficient. [*] Determined by DAD. [**] Determined by FD. [#] For milk samples and the liquid probiotic product, the results are expressed in µg/L. [a] Calculated as the concentration associated with a S/N of 3. [b] Calculated as the concentration associated with a S/N of 10.

Table IV.6.- Results of the precision and accuracy study of the HF-LPME-HPLC-DAD/FD method for the selected compounds in the different milk and dairy samples.

Analyte	Sample	Spiked level (µg/kg)[#]	Found[a)] (µg/kg)[#]	Accuracy	$t^{b)}$	Analyte	Sample	Spiked level (µg/kg)[#]	Found[a)] (µg/kg)[#]	Accuracy	$t^{b)}$
E_3**	WM	2.78	2.45 ± 0.43	88	2.68	E_1*	WM	109	105 ± 34	96	0.24
		16.6	14.8 ± 0.5	89	0.58			657	720 ± 10	110	1.94
	SSM	3.22	2.59 ± 1.25	90	2.73		SSM	341	336 ± 18	99	0.06
		13.3	12.8 ± 1.1	98	0.36			1404	1450 ± 193	103	0.53
	SM	4.97	5.13 ± 1.12	103	1.09		SM	327	371 ± 100	113	2.54
		22.3	22.9 ± 1.0	103	1.53			1470	1289 ± 116	88	2.47
	SY	5.28	4.32 ± 3.19	82	2.63		SY	355	374 ± 51	105	0.11
		27.7	29.5 ± 2.9	106	1.36			1862	1790 ± 61	96	0.39
	WY	3.83	3.40 ± 1.81	89	0.50		WY	475	536 ± 174	113	1.32
		23.0	20.9 ± 2.1	91	1.98			2848	2770 ± 180	97	0.69
	PrP	3.04	2.41 ± 1.06	81	1.14		PrP	192	180 ± 72	94	1.97
		12.9	13.0 ± 1.0	101	0.21			816	787 ± 28	96	1.09
	WC	4.62	3.89 ± 2.22	84	2.63		WC	285	270 ± 80	95	0.54
		20.8	22.0 ± 2.0	106	2.36			1284	1295 ± 94	101	0.57
2-OHE$_2$**	WM	295	317 ± 78	107	0.25	DES*	WM	677	710 ± 51	105	0.25
		1767	1653 ± 423	94	0.07			4060	3818 ± 857	94	0.22
	SSM	98.2	94.1 ± 14.6	96	0.33		SSM	1218	1068 ± 128	88	0.48
		404	363 ± 52	90	0.60			5017	5701 ± 850	114	0.42
	SM	217	190 ± 58	88	2.72		SM	668	689 ± 80	103	0.37
		975	870 ± 52	89	1.52			3006	2808 ± 102	93	0.70
	SY	198	190 ± 26	96	0.06		SY	837	1051 ± 75	93	2.68
		1039	1206 ± 179	116	1.87			4394	4297 ± 104	98	0.09
	WY	62.4	59.1 ± 14.6	95	2.40		WY	974	1149 ± 487	118	2.44
		375	346 ± 38	92	0.65			5845	5724 ± 367	93	0.17
	PrP	96.1	82.5 ± 52.7	86	2.09		PrP	855	873 ± 286	102	0.31
		408	389 ± 47	95	1.88			3635	3534 ± 128	97	0.28
	WC	-	-	-	-		WC	634	666 ± 160	105	0.13
		-	-	-	-			2851	3012 ± 161	106	0.57
17β-E$_2$**	WM	6.27	6.87 ± 1.46	110	0.46	DS*	WM	784	823 ± 295	105	0.16
		37.6	44.4 ± 2.5	118	0.75			4706	4575 ± 251	97	0.10
	SSM	5.72	5.61 ± 1.53	98	0.36		SSM	677	685 ± 186	101	0.06
		23.6	26.7 ± 1.3	113	2.38			2786	3129 ± 345	112	0.41
	SM	7.26	8.04 ± 0.93	111	2.18		SM	329	368 ± 40	112	1.41
		32.7	31.5 ± 1.2	97	0.74			1479	1542 ± 53	104	0.42
	SY	5.43	5.63 ± 4.44	104	0.06		SY	949	1073 ± 134	113	1.31
		28.5	33.7 ± 4.1	118	0.47			4984	4750 ± 176	95	0.18

Table IV.6.- (Continued).

Analyte	Sample	Spiked level (µg/kg)[#]	Found[a)] (µg/kg)[#]	Accuracy	$t^{b)}$	Analyte	Sample	Spiked level (µg/kg)[#]	Found[a)] (µg/kg)[#]	Accuracy	$t^{b)}$
17β-E_2[**]	WY	5.04	4.52 ± 1.77	90	1.32	DS[*]	WY	400	452 ± 96	113	0.38
		30.3	31.4 ± 2.3	104	1.05			2400	2753 ± 300	114	0.39
	PrP	4.27	3.89 ± 1.05	91	2.01		PrP	349	353 ± 96	101	0.17
		18.1	16.7 ± 0.9	92	0.31			1484	1472 ± 43	99	0.08
	WC	6.47	5.53 ± 2.49	85	1.31		WC	381	344 ± 36	96	0.10
		29.1	28.2 ± 4.0	97	0.11			1716	1898 ± 218	111	1.98
	WM	6.73	6.46 ± 1.64	96	1.25		WM	110	121 ± 42	110	0.31
		40.4	38.1 ± 1.6	94	0.24			660	781 ± 52	118	0.54
	SSM	6.30	6.62 ± 0.94	89	1.35		SSM	66.6	69.0 ± 16.6	104	0.16
		25.9	24.8 ± 9.7	96	0.14			274	323 ± 42	118	2.38
	SM	7.68	6.65 ± 2.40	87	0.32		SM	56.3	62.8 ± 6.1	112	1.26
		34.5	36.4± 4.3	105	0.20			253	267 ± 8	105	0.54
17α-E_2[**]	SY	6.47	5.76 ± 1.18	89	0.23	HEX[**]	SY	28.5	27.9 ± 1.6	98	0.08
		34.0	30.6 ± 0.7	90	1.40			149	166 ± 2	111	1.18
	WY	6.34	6.57 ± 3.32	104	2.08		WY	57.2	58.4 ± 23.0	102	1.06
		38.0	36.3 ± 4.4	95	0.17			343	394 ± 26	115	0.45
	PrP	5.41	4.58 ± 2.11	85	2.01		PrP	55.9	55.6 ± 19.3	99	0.09
		23.0	22.1 ± 0.8	96	1.08			237	226 ± 8	95	0.50
	WC	7.92	6.90 ± 2.21	87	1.51		WC	38.3	39.7 ± 11.8	92	0.18
		35.6	37.2 ± 2.6	104	1.61			172	187 ± 9	106	0.49
	WM	14.2	13.3 ± 1.5	93	0.84						
		85.4	78.9 ± 0.2	92	0.30						
	SSM	11.7	10.6 ± 0.2	91	0.55						
		48.0	58.4 ± 0.2	118	0.70						
	SM	12.6	13.1 ± 4.7	105	0.71						
		56.6	56.7 ± 2.0	100	0.04						
EE_2[**]	SY	9.09	7.98 ± 0.79	88	2.78						
		47.7	51.2 ± 3.4	107	2.69						
	WY	10.8	10.1 ± 2.8	94	0.87						
		64.5	70.2 ± 2.7	109	1.56						
	PrP	9.48	8.90 ± 2.20	94	1.37						
		40.3	39.3 ± 0.9	98	0.55						
	WC	11.5	9.8 ± 5.3	85	1.45						
		51.9	54.1 ± 6.4	104	0.15						

WM: Whole milk; SSM: Semi-skimmed milk; SM: Skimmed milk; SY: Skimmed natural yogurt; WY: Whole natural yogurt; PrP: Probiotic product; WC: White cheese
[*] Determined by DAD. [**] Determined by FD. [#] For milk samples and the probiotic product, the results are expressed in µg/L. [a)] Average value ± standard deviation of five determinations (95% confidence value). [b)] $t_4 = 2.78$, $\alpha = 0.05$.

cases they are even lower. This is the case of the determination of EE_2 in human breast milk by Markopoulou and Koundourellis (Markopoulou and Koundourellis, 2006) who achieved a LOD of 1.8 μg/L using a SPE-HPLC-UV method, while in this work it ranged between 0.94 and 1.71 μg/L. In the same way, Ding et al. (Ding et al., 2011) obtained LODs of 1.21 μg/L and 2.35 μg/L for 17β-E_2 and EE_2, respectively, using a CNTs-SPE-HPLC-FD methodology, values that were higher than the ones here obtained (0.50-0.75 μg/L for 17β-E_2 and 0.94-1.71 μg/L for EE_2). Regarding the metabolite 2-OHE_2, it has only been previously analysed in milk in one work (Farlow et al., 2009), but nothing was specifically mentioned about the LOD achieved, so comparison cannot be carried out in this case. It is worth mentioning that in general, the LODs obtained in this work are slightly higher than others previously published for these analytes with the use of LC-MS or LC-MS/MS (Farke et al., 2011; Gao et al., 2011, Malekinejad et al., 2006; Msagati and Nindi, 2006a, 2006b; Serrano et al., 2001), something reasonable, taking into account the better sensitivity of MS versus UV or FD which also suggests that the LODs could be improved by the use of a MS detector.

With respect to the rest of the analysed samples, at the time of the development of the present work, only few articles had reported the analysis of oestrogens and, among them, only four natural oestrogens (i.e. E_1, 17α-E_2, 17β-E_2 and E_3) and EE_2 were determined in such cases. One of these works was carried out by Hartmann et al. (Hartmann et al., 1998), in which E_1, 17α-E_2, 17β-E_2 and E_3 together with other eight naturally-occurring steroid hormones were analysed in yogurt and cheese among other types of samples using a LLE-column chromatography-GC-MS method. Authors only indicated that their LODs were around 0.01-0.3 μg/kg but no data about the content of each analyte and samples was specified. Such values were similar to the ones obtained in our work for E_3, 17α-E_2 and 17β-E_2 (0.29-0.58 μg/kg for yogurt and cheese and 0.23-0.40 μg/L for the probiotic product). In the case of E_1, the value achieved in our work was even slightly higher (21.3-35.5 μg/kg and 14.3 μg/L). In another work previously published, Shi et al. (Shi et al., 2011) carried out the determination of 17β-E_2 in yogurt using a MIP followed by HPLC-UV. The LODs achieved, 0.12-0.46 nmol/kg (approximately 0.03-0.13 μg/kg), were slightly lower than the ones obtained in our work due to the great specificity of the MIP.

IV.1.9.- Conclusions

From the results obtained in this section the following conclusions can be drawn:

- In this work, a methodology based on a HF-LPME procedure followed by the determination using a HPLC-DAD/FD system has been proposed for the first time for the extraction and analysis of nine different oestrogens compounds (E_3, 17α-E_2, 17α-E_2, E_1, EE_2, DES, DS, HEX and 2-OHE_2) in whole, semi-skimmed and skimmed cow milk, whole and skimmed natural yogurt, white cheese and a probiotic product.

- The chromatographic separation of the nine analytes was successfully optimised and validated obtaining LODs in the range 3.2-507 μg/L for all analytes. RSDs for retention times and peak areas were lower than 0.2 and 2.0 %, and 0.7 and 3.7 % in the same day and between days respectively, which clearly demonstrates the repeatability of the HPLC-DAD/FD system.

- The HF-LPME procedure was optimised using a step by step approach in which Milli-Q water was selected with the aim of not introducing matrix effects and having a better vision of the influence of each factor in the extraction process. The best results were achieved using a PP fibre of 2 cm of length and 1-octanol as acceptor phase for the extraction of 10 mL of an aqueous extract (donor phase) at pH 6 containing 10 % (w/v) of NaCl. The extraction was performed for 60 min with a stirring agitation of 1250 r.p.m. at room temperature. It was experimentally demonstrated that the best extraction efficiencies were obtained when the analytes were back extracted from the fibre using 500 μL of ACN with the assistance of ultrasounds during 7 min.

- Prior to the application of the HF-LPME procedure to milk and dairy products, an initial deproteinisation was optimised and applied in order to avoid matrix effects and the damage of the LC column since proteins may irreversibly link to the stationary phase. The deproteinisation step consisted of the addition of ACN containing acetic acid followed by agitation, settling in the darkenss for 15 min and centrifugation to favour the separation of the phases. The methodology was validated in terms of linearity and the LODs obtained were in the μg/L and μg/kg range. A precision and accuracy study was also carried out at two levels of concentration and in quintuplicate for each matrix. The methodology resulted to be simple, effective and selective, with a low consumption of organic solvents and with a relatively low cost. It provided a high preconcentration of the analytes and a good

sensitivity with LODs similar to those previously reported in the literature using the same detection systems.

- To the best of our knowledge, this work constitues the first application of HF-LPME for the extraction of these compounds from dairy products. It is also the first published methodology in which stilbenes and the metabolite 2-OHE_2 have been analysed in dairy products different than milk and the first publication in which the determination of oestrogens in probiotic products has been carried out.

IV.2.- Determination of oestrogens in water samples using the ionic liquid 1,3-dipentylimidazolium hexafluorophosphate as extraction solvent in dispersive liquid-liquid microextraction

In this section, a group of three natural (E_3, 17β-E_2 and 17α-E_2) and four synthetic (EE_2, DES, DS and HEX) oestrogenic compounds as well as one mycotoxin with oestrogenic activity (ZEN) has been determined in water samples after a DLLME procedure using a symmetric dialkyl-substituted IL, 1,3-dipenthylimidazolium hexafluorophosphate ([PPIm][PF_6]), as extraction solvent. The methodology was applied for the analysis of the selected analytes in Milli-Q, mineral and wastewater. Separation, determination, and quantification were developed by HPLC-DAD and FD systems connected in series. Factors influencing the IL-DLLME procedure (sample pH, type and volume of the dispersant solvent, amount of IL, ionic strength and assistance of vortex agitation) were investigated and optimised by means of a step by step approach. Once optimum extraction conditions were established, the calibration curves of the whole method (IL-DLLME-HPLC-DAD/FD) were obtained and a precision and an accuracy study were developed.

IV.2.1.- Background

Water quality is an important concern for the EU. In fact, diverse directives have been published with the aim of controlling the presence of hazardous pollutants that can be a health risk for consumers in this type of samples. In this regard, oestrogenic compounds are clear candidates to be evaluated. In fact, by the end of 2011 both EE_2, which is commonly used as contraceptive, and 17β-E_2, which is usually employed in human hormone replacement therapy after menopause, were included in the list of the 33 principal pollutants that should be controlled in environmental water by the Proposal Directive COM (2011) 876, together with other 13 pharmaceutical compounds.

In addition to these two analytes, other natural oestrogens such as E_1 and E_3 are also particularly present in the effluents coming from wastewater and sludge treatment plants, principally as a result of the excretion of farming animals (Hamid and Eskicioglu, 2012). Therefore, other oestrogenic compounds such as ZEN, that may be present in cereals, as well as synthetic stilbenes like DES, DS and HEX, used in some occasions as growth-promoters, can also appear in other types of water as a result of their release from animal wastes into different environmental compartments (run-off and groundwater, soils, sediments, etc.) (Hoerger et al.,

2009; Kolok and Sellin, 2008; Maragos, 2012).

As indicated in Section I.6.3.2., DLLME, introduced by Rezaee et al. (Rezaee et al., 2006) in 2006, was the last of the three LPME techniques to be proposed. Besides the inherent advantages of this group of techniques, DLLME offers a more rapid achievement of equilibrium conditions, which increases the velocity of the extraction. Apart from that, it should be highlighted that since the introduction of DLLME, diverse modifications have been applied with the aim of increasing the extraction capacity as well as the simplicity of the technique. In this regard, the use of non-conventional solvents and, specifically, the use of ILs, have been one of the main points of actuation. The particular characteristics of these types of salts, which are constituted by an organic cation and either an organic or inorganic anion with melting points below 100 °C, has expanded the range of possibilities since its first applications in DLLME in 2008 (Fan et al., 2008; Zhou et al., 2008). Among the different known ILs, the family of hexafluorophosphates of non-symmetric 1-alkyl-3-alkylimidazolium has been one of the most applied in diverse kinds of applications, including DLLME, since they present similar features to conventional solvents such as good extraction capacity for many organic analytes, low solubility in water, higher density than water and high solubility in the most common dispersion solvents (e.g. ACN, MeOH, acetone). In this sense, 1-hexyl-3-methylimidazolium hexafluorophosphate ([HMIm][PF_6]) has been the IL most used by far in DLLME (Asensio-Ramos et al., 2011a; Mao et al., 2009; Ravelo-Pérez, et al., 2009; Zhou et al., 2008), followed by others like 1-octyl-3-methylimidazolium hexafluorophosphate ([OMIm][PF_6]) (Pena et al., 2009) or 1-butyl-3-methylimidazolium hexafluorophosphate ([BMIm][PF_6]) (Fan et al., 2008). On the contrary, and concerning the use of symmetrical 1,3-dialkylimidazolium hexafluorophosphate ILs, only dibutyl ([BBIm][PF_6]) (He et al., 2010) and [PPIm][PF_6] (Asensio-Ramos et al., 2012) have been employed one occasion each in DLLME for the analysis of pesticides, after their synthesis since they are not commercially available.

At the time of the development of the present PhD Thesis, only few articles had reported the application of DLLME to the extraction of oestrogenic compounds (Antep and Merdivan, 2012; Arroyo-Manzanares et al., 2013a, 2013b; Chang and Huang, 2010; Hadjmohammadi and Ghoreishi, 2011; Lima et al., 2013; Liu et al., 2011). In most of them, a low number of analytes were extracted using toxic chlorinated solvents. In this sense, tetrachloromethane was used for the extraction of E_1, E_2 and DES from tap, river and well water (Hadjmohammadi and Ghoreishi, 2011), chlorobenzene to extract E_2 and EE_2 from tap,

surface and wastewater (Lima et al., 2013) and chloroform for the extraction of ZEN from beer (Antep and Merdivan, 2012), milk thistle (Arroyo-Manzanares et al., 2013a) and seed (Arroyo-Manzanares et al., 2013b). In addition, 1-undecanol has also been used in two cases for the determination of E_1, E_2, E_3 and EE_2 (Chang and Huang, 2010) as well as E_2, E_3 and EE_2 (Liu et al., 2011) in different types of water samples using a method based on a SFO-DLLME approach. However, the application of IL-DLLME with this purpose has only been reported once before the development of this work for the extraction of E_1, E_2, E_3, EE_2 and DES from river, waste and seawater using acetone as dispersion solvent and [HMIm][PF_6] as extractant (Wu et al., 2012). In this sense, there is still an important field of study, especially in the area of symmetric IL-DLLME which application has not been fully evaluated although it could provide great benefits in the development and application of the technique.

IV.2.2.- Specific objectives

In view of the foregoing, the following specific objectives have been established for this work:

- The development of a new analytical methodology based on a DLLME approach to carry out the extraction of a group of eight oestrogenic compounds including three natural oestrogens (E_3, 17β-E_2 and 17α-E_2), four synthetic oestrogens (EE_2, DES, DS and HEX) and one mycotoxin (ZEN) from Milli-Q, mineral and wastewater samples.
- The development of the separation and determination of the target analytes by HPLC using both DAD and FD systems in series as well as to obtain the optimum separation and detection conditions.
- The synthesis and evaluation of the potential of the symmetric dialkyl-substituted IL [PPIm][PF_6] as extraction solvent in DLLME.
- The study of the influence of the parameters affecting the extraction efficiency in DLLME (i.e. pH of the sample, type and amount of dispersant solvent, IL amount, ionic strength of the donor phase and assistance of vortex agitation) to achieve the best extraction efficiency for all analytes.
- The validation of the whole methodology in terms of calibration, precision and accuracy as well as the obtaining of the LODs and LOQs of the method.

- The study of the applicability of the methodology to the analysis of different samples of interest.

IV.2.3.- HPLC-DAD/FD method

With the aim of carrying out the correct separation and determination of the selected group of compounds, separation conditions discussed in the previous section were selected for this group of analytes obtaining excellent results in terms of selectivity and resolution, also for ZEN, which was incorporated in this study and detected by DAD at 230 nm since it does not have native fluorescence. Figure IV.10 shows the chromatograms of the separation.

A part from that, and taking into account the low solubility of ILs in water media which can constitute a problem in the analytical process, preliminary tests to establish the best injection conditions were done in order to know the limitations of [PPIm][PF_6] in this respect. Results showed that 150 μL of ACN were enough to achieve the complete solubilisation of 20 μL of the IL, obtaining good peak shapes with adequate resolution. The same ratio should be maintained if a higher or lower amount of the IL is collected after the DLLME procedure.

IV.2.4.- IL-DLLME optimisation

In order to achieve the best extraction efficiency for all analytes, a step by step optimisation of the IL-DLLME procedure studying the influence of the most critical parameters was carried out. All experiments were done in duplicate following the procedure described in Section III.8.2, using 10 mL of Milli-Q as donor phase to avoid the possible influence of the matrices during the optimisation procedure. The spiking concentrations of the studied analytes were: 10 μg/L for 17β-E_2, 17α-E_2, E_3, EE_2 and HEX, 30 μg/L for DES and DS and 14 μg/L for ZEN.

The IL used was synthesised and purified at the laboratory, following the procedure described in Section III.6, and subsequently characterised using nuclear magnetic resonance (NMR). Further information about the synthesis and characterisation of the material can be found in (Asensio-Ramos et al., 2012).

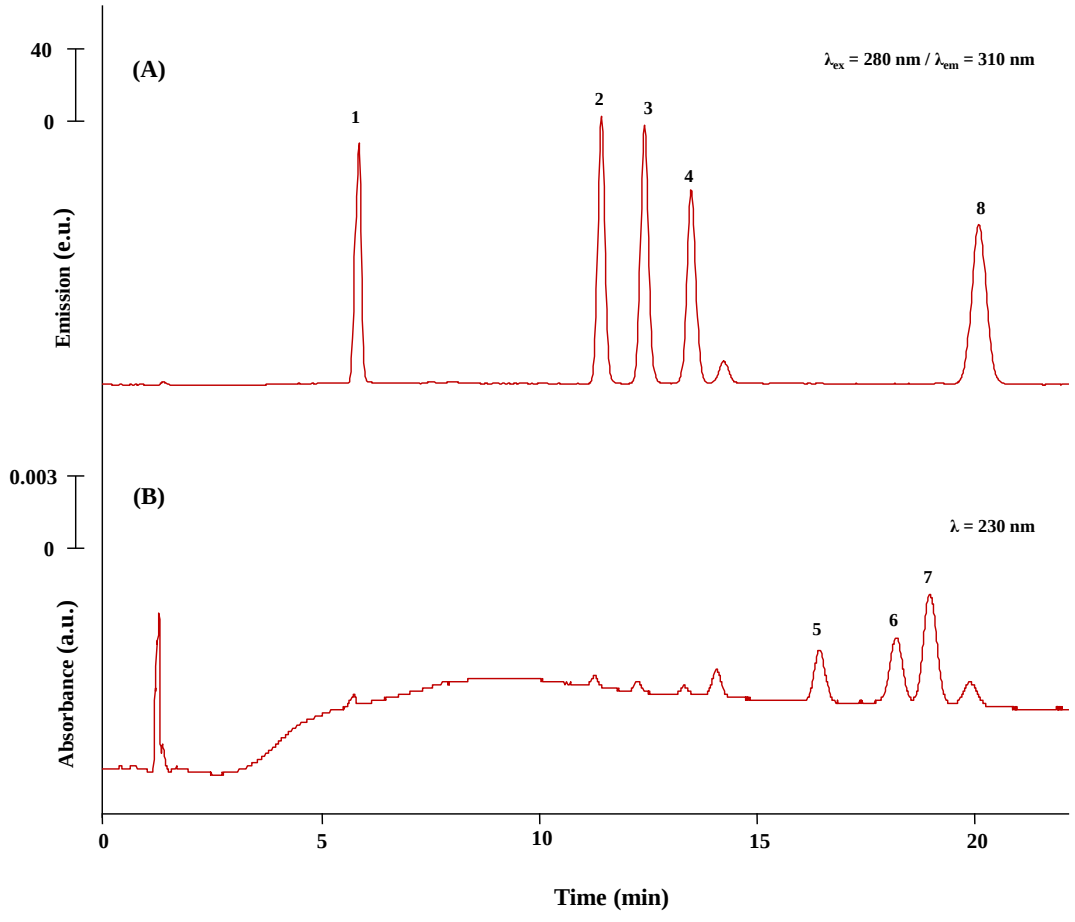


Figure IV.10.- HPLC-DAD/FD chromatograms of the target analytes at their maximum excitation/emission (A) and absorption (B) wavelengths. Flow rate: 1 mL/min. Injection volume: 20 µL. Sample dissolved in ACN containing 1 mM formic acid. Analyte identification and concentration: (1) E_3 (0.30 mg/L), (2) 17β-E_2 (0.30 mg/L), (3) 17α-E_2 (0.30 mg/L), (4) EE_2 (0.30 mg/L), (5) ZEN (0.50 mg/L), (6) DES (0.90 mg/L), (7) DS (0.90 mg/L) and (8) HEX (0.30 mg/L). Gradient described in Section III.5.1.

IV.2.4.1.- Influence of the pH of the donor phase

One of the critical aspects to take into consideration to develop an adequate extraction is the form in which the analytes are present in the donor phase. For this reason, and based on the fact that this group of analytes have pK_a values in the range 7.58-10.27 as can be seen in Tables I.1 and I.2, the pH of the aqueous phase was modified between 3 and 10. As starting

point, and taking into account the experience of the previous application of this technique for the extraction of pesticides from soils (Asensio-Ramos et al., 2012), 60 mg of [PPIm][PF_6] and 500 µL of ACN as dispersant solvent were used and 1 min of vortex agitation was applied to assist the extraction process. As can be observed in Figure IV.11, the best recovery was obtained when pH 8 was established with values in the range 78-92 % for all the selected analytes, except for E_3 that was 40 %. However, when the pH was higher than 9, a considerable decrease in the extraction efficiency was found, which can be attributed to the fact that at these values of pH the analytes are under their anionic forms and, as consequence, their affinity for the aqueous phase is higher than for the organic solvent, decreasing the extraction efficiency. Taking these results into account, pH 8 was maintained for further experiments.

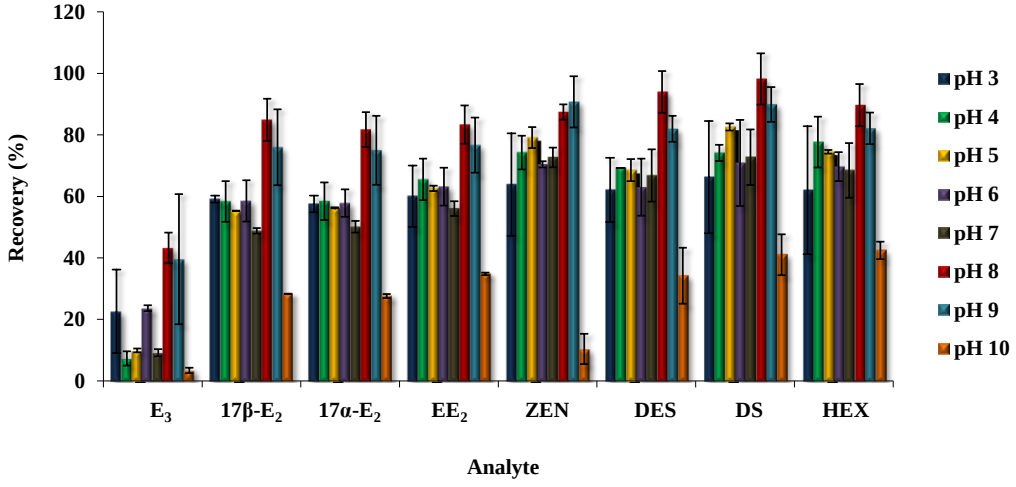


Figure IV.11.- Effect of the pH of the donor phase on the recovery of the target analytes after the DLLME procedure. Extraction conditions: 10 mL of spiked Milli-Q water, 0 % (w/v) of NaCl, 60 mg of [PPIm][PF_6], 500 µL of ACN as dispersant solvent and vortex agitation for 1 min. Two extractions (n = 2) were carried out in each case. Concentration of the analytes: 10 µg/L of 17β-E_2, 17α-E_2, E_3, EE_2 and HEX, 30 µg/L of DES and DS and 14 µg/L of ZEN.

IV.2.4.2.- Selection of the dispersant solvent

The dispersant solvent plays an important role in the extraction process favouring the dispersion of the extractant drops in the donor phase. For this reason, and as it has been previously mentioned, the dispersant solvent should be miscible with both the extraction

solvent and the aqueous phase. Since MeOH has also been used as dispersant with the IL applied in this work (Asensio-Ramos et al., 2012) a comparative study between MeOH and ACN was carried out using 60 mg of [PPIm][PF_6] and 500 μL of each solvent. In this occasion, the use of ACN provided the best results in terms of extraction efficiency of the analytes with recovery values higher than those obtained with MeOH, which were around 50-60 %, except for E_3 that was 18 %, so this solvent was used in subsequent studies.

IV.2.4.3.- Study of the IL amount

In principle, an increase of the amount of IL could enhance recovery values; however, and owing to the high absorption of imidazolium ILs in the UV-Vis region, when a DAD system is used, peak overlapping of the analytes of interest is a common problem that makes more difficult compounds detection (Fan et al., 2008; Liu et al., 2009; Ravelo-Pérez et al., 2009; Zhou et al., 2008). For this reason, an exhaustive study of the amount of IL used in this work was developed, modifying the quantity between 30 and 80 mg. The best recovery was found for 60 mg, as can be observed in Figure IV.12. Lower amounts were not enough to achieve the adequate extraction of the analytes while for higher amounts, both peak overlapping as well as peak deformation were observed together with a decrease in the efficiency of the extraction. In fact, some analytes, such as ZEN, DES and DS, could not been determined when 80 mg of the IL were applied. That is why, 60 mg of [PPIm][PF_6] was chosen to continue with the optimisation procedure. With this amount, 40 μL of the IL were collected at the end of the extraction and, in accordance with the previous results obtained, 300 μL of ACN were used for dissolving the IL prior to the HPLC injection in order to maintain the adequate IL-organic solvent ratio for the correct determination of the analytes.

IV.2.4.4.- Evaluation of the volume of the dispersant solvent

The volume of the dispersant solvent used is critical for the correct formation of the cloudy dispersion. For this reason, volumes between 300 and 700 μL were tested. As can be seen in Figure IV.13, the recovery values were above 78 % for all the selected analytes, except for E_3 that was around 40 %, using 500 μL of ACN. Lower dispersion solvent volumes were not enough to achieve an adequate dispersion while higher volumes resulted in a poorer extraction as a result of the solution of the analytes in the dispersant solvent instead of in the extractant. As a consequence, this volume was chosen as the optimum value for subsequent studies.

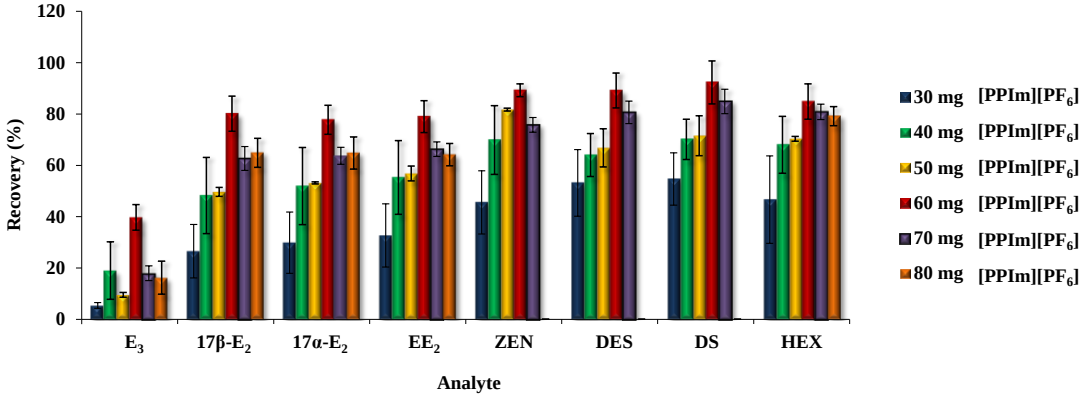


Figure IV.12.- Effect of the [PPIm][PF_6] amount on the recovery of the target analytes after the DLLME procedure. Extraction conditions: 10 mL of spiked Milli-Q water, 0 % (w/v) of NaCl, pH 8, 500 µL of ACN as dispersant solvent and vortex agitation for 1 min. Two extractions (n = 2) were carried out in each case. Concentration of the analytes: 10 µg/L of 17β-E_2, 17α-E_2, E_3, EE_2 and HEX, 30 µg/L of DES and DS and 14 µg/L of ZEN.

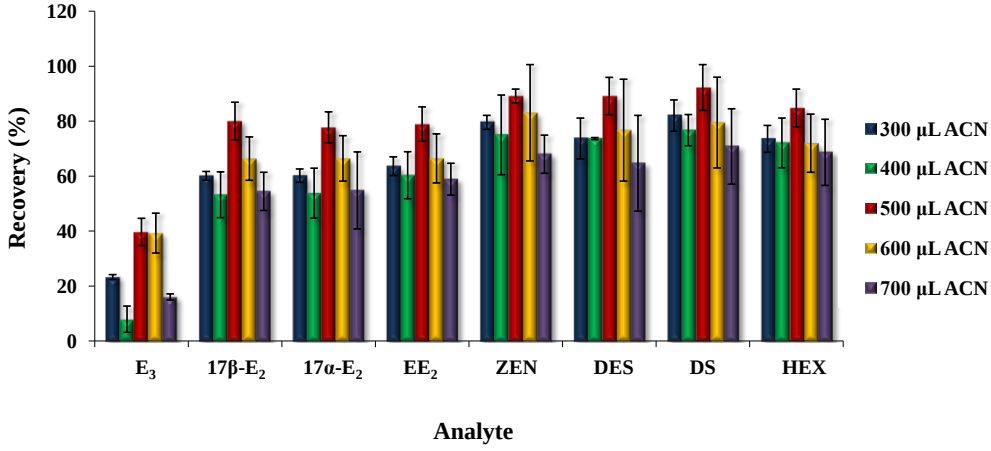


Figure IV.13.- Effect of the dispersant volume on the recovery of the target analytes after the DLLME procedure. Extraction conditions: 10 mL of spiked Milli-Q water, 0 % (w/v) of NaCl, pH 8, 60 mg of [PPIm][PF_6] as extractant solvent and vortex agitation for 1 min. Two extractions (n = 2) were carried out in each case. Concentration of the analytes: 10 µg/L of 17β-E_2, 17α-E_2, E_3, EE_2, and HEX, 30 µg/L of DES and DS and 14 µg/L of ZEN.

IV.2.4.5.- Influence of the ionic strength of the aqueous phase

As it is widely known, the salting-out effect is usually studied during the optimisation of all LPME procedures in order to improve the extraction efficiency since the increase of the ionic strength of the donor phase commonly decreases the solubility of the extractant in the donor phase and therefore, favours analytes transference (He et al., 2010). With this aim, an increase of the ionic strength of the aqueous phase was carried out by adding different proportions of NaCl, i.e. 0, 5, 10, 15 and 20 % (w/v), and maintaining the rest of the conditions as previously described. Contrary to what it might have been expected, no important differences were found between extractions. Indeed, the highest recovery values were obtained in the absence of salt which could be attributed to the fact that an increase in the chloride concentration may produce the change of the IL anion and generate a halide-based IL with a higher solubility in the aqueous phase, which produces a decrease of its extraction capability (Pena et al., 2009). Consequently, no salt was added in further experiments.

IV.2.4.6.- Study of the effect of vortex assisted agitation

The use of vortex assistance is usually applied in DLLME in order to favour the dispersion and, consequently, to improve the extraction efficiency of the procedure (Zhang et al., 2012). Based on that, vortex agitation time was evaluated between 0 and 3 min. Results showed the importance of this step in the efficiency of the extraction. As can be seen in Figure IV.14, when no vortex assistance was applied, poor recovery was obtained (below 58 %) as well as a worse repeatability between extractions. However, vortex times of 1 min considerably improved the results, while higher times did not provide a clear improvement (recovery values even decreased for the first four compounds). Then, 1 min of vortex shaking was selected as optimum.

IV.2.5.- Validation of the methodology

Once the DLLME was optimised, the whole method was validated in three different samples (Milli-Q, mineral and wastewater) in terms of linearity, precision and accuracy. The LODs and LOQs of the method were also determined in each case.

First of all, spiked and non-spiked samples were extracted by the optimised DLLME procedure and injected in the analytical system. Figure IV.15 shows the chromatograms of each sample in which it can be observed that, although some non-identified peaks appeared,

especially in the case of wastewater with FD, no interferences that precluded the correct integration of the peaks of interest were found. Besides, and as it was expected due to the high absorbance of the IL in the UV-Vis range, a wide signal associated with such absorption could also be observed with the diode array detector but it did not interfer in the adequate determination of the oestrogens.

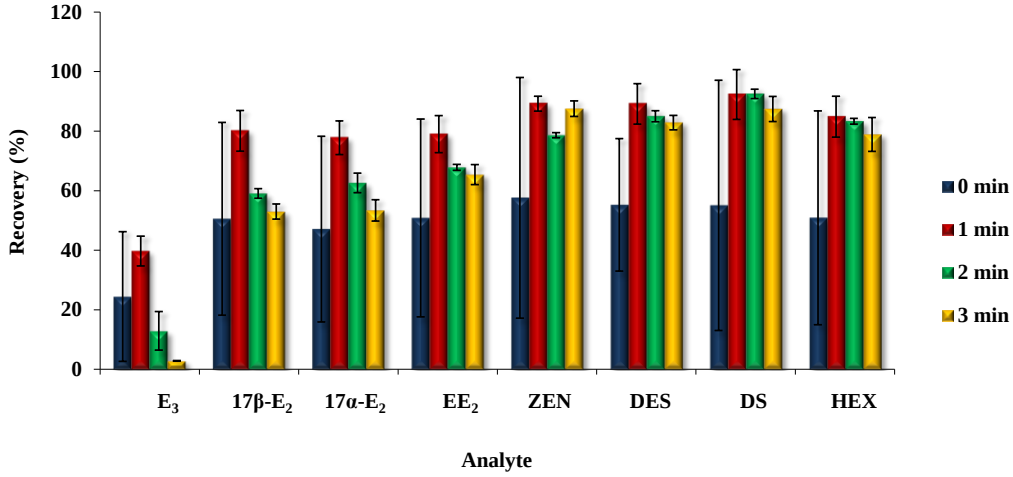


Figure IV.14.- Effect of vortex shaking time on the recovery of the target analytes after the DLLME procedure. Extraction conditions: 10 mL of spiked Milli-Q water sample at pH 8, 0 % (w/v) of NaCl, 500 µL of ACN as dispersant and 60 mg of [PPIm][PF_6]. Two extractions (n = 2) were carried out in each case. Concentration of the analytes: 10 µg/L of 17β-E_2, 17α-E_2, E_3, EE_2 and HEX, 30 µg/L of DES and DS and 14 µg/L of ZEN.

Linearity studies were carried out by the preparation of the calibration curves in each matrix. With this purpose, five aliquots of each water sample free of oestrogens were spiked with the target compounds at five different concentration levels, were submitted to the DLLME procedure and then injected in the chromatographic system in duplicate. As can be seen in Table IV.7, in which calibration data is summarised, R^2 values were above 0.9902 in all cases, with good linearity and sensitivity. The LODs and LOQs were calculated as the concentrations associated with a S/N of 3 and 10, respectively, and also checked experimentally. LODs values were in the range 14-37 µg/L for determinations by DAD and in the range 0.30-1.8 µg/L for the analytes determined by FD. As can be concluded from these results, despite the low limits obtained (in the µg/L range) it is obvious that FD provided the highest sensitivity and that the combination of this extraction technique with other more sensitive detection systems such as

MS could considerably improve these limits.

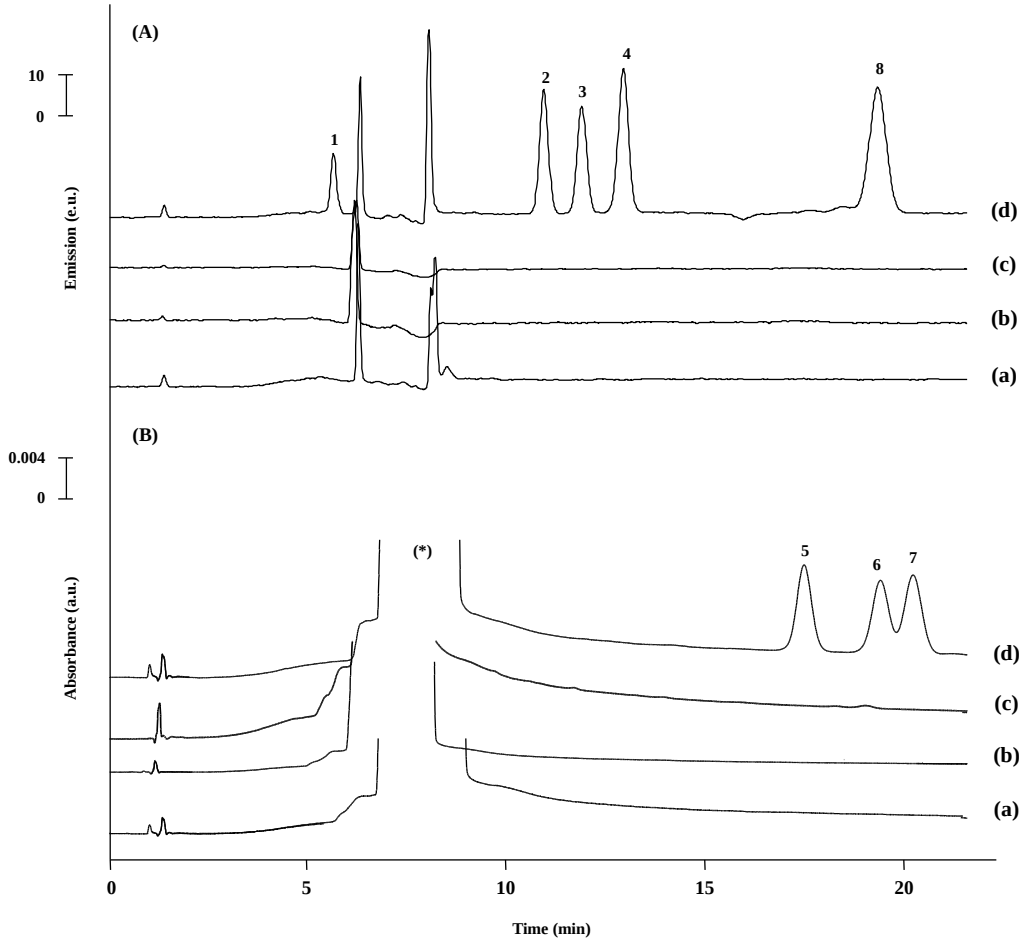


Figure IV.15.- HPLC-DAD/FD chromatograms of a blank sample of wastewater (a), mineral (b) and Milli-Q (c) water and a spiked wastewater sample (d) after the application of the optimised method at (A) λ_{ex} = 280 nm, λ_{em} = 310 nm and (B) λ= 230 nm. Analyte identification and concentration in the water samples: (1) E_3 (0.02 mg/L), (2) 17β-E_2 (0.01 mg/L), (3) 17α-E_2 (0.01 mg/L), (4) EE_2 (0.01 mg/L), (5) ZEN (0.35 mg/L), (6) DES (0.52 mg/L), (7) DS (0.31 mg/L), (8) HEX (0.01 mg/L), and (*) interference due to the absorption of [PPIm][PF_6].

For precision and accuracy studies, each water sample was spiked at two concentration levels in quintuplicate and submitted to the whole optimised method. Table IV.8 summarises the statistical comparison (Student's *t*-test) of the concentration found and the spiked one as

Table IV.7.- Calibration data of the selected compounds in the water samples after the IL-DLLME-HPLC-DAD/FD method.

Analyte	Type of water sample	Calibration data (n = 5)				LOD_{method} [a] (µg/L)	LOQ_{method} [b] (µg/L)
		Range of concentration tested (µg/L)	Slope	Intercept	R^2		
E_3**	Milli-Q	1.5-15	$1.40 \cdot 10^5 \pm 0.17 \cdot 10^5$	$1.82 \cdot 10^5 \pm 1.35 \cdot 10^5$	0.9984	0.45	1.5
	Mineral	6.1-61	$1.37 \cdot 10^5 \pm 0.24 \cdot 10^5$	$-5.92 \cdot 10^5 \pm 8.31 \cdot 10^5$	0.9906	1.8	6.1
	Wastewater	2.6-11	$1.16 \cdot 10^5 \pm 0.21 \cdot 10^5$	$-1.34 \cdot 10^4 \pm 27.70 \cdot 10^4$	0.9902	0.79	2.7
17β-E_2**	Milli-Q	1.0-17	$6.15 \cdot 10^5 \pm 0.26 \cdot 10^5$	$2.91 \cdot 10^5 \pm 2.04 \cdot 10^5$	0.9988	0.30	1.0
	Mineral	1.7-29	$6.98 \cdot 10^5 \pm 0.53 \cdot 10^5$	$5.79 \cdot 10^5 \pm 6.71 \cdot 10^5$	0.9957	0.52	1.7
	Wastewater	1.2-12	$5.40 \cdot 10^5 \pm 0.51 \cdot 10^5$	$1.39 \cdot 10^5 \pm 3.22 \cdot 10^5$	0.9954	0.36	1.2
17α-E_2**	Milli-Q	1.0-17	$6.16 \cdot 10^5 \pm 0.27 \cdot 10^5$	$5.29 \cdot 10^4 \pm 22.26 \cdot 10^4$	0.9986	0.31	1.0
	Mineral	1.7-29	$6.42 \cdot 10^5 \pm 0.48 \cdot 10^5$	$5.01 \cdot 10^5 \pm 6.09 \cdot 10^5$	0.9957	0.52	1.7
	Wastewater	1.2-12	$5.21 \cdot 10^5 \pm 0.55 \cdot 10^5$	$8.76 \cdot 10^4 \pm 33.92 \cdot 10^5$	0.9932	0.35	1.2
EE_2**	Milli-Q	1.2-21	$6.46 \cdot 10^5 \pm 0.14 \cdot 10^5$	$1.70 \cdot 10^4 \pm 13.41 \cdot 10^4$	0.9997	0.37	1.3
	Mineral	1.8-12	$7.42 \cdot 10^5 \pm 0.54 \cdot 10^5$	$5.29 \cdot 10^5 \pm 7.30 \cdot 10^5$	0.9961	0.55	1.8
	Wastewater	1.3-13	$6.70 \cdot 10^5 \pm 0.60 \cdot 10^5$	$1.92 \cdot 10^5 \pm 4.10 \cdot 10^5$	0.9959	0.39	1.3
ZEN*	Milli-Q	72-1193	$2.37 \cdot 10^3 \pm 0.06 \cdot 10^3$	$5.28 \cdot 10^3 \pm 34.46 \cdot 10^3$	0.9996	22	72
	Mineral	68-1138	$2.49 \cdot 10^3 \pm 0.17 \cdot 10^3$	$4.42 \cdot 10^4 \pm 8.44 \cdot 10^4$	0.9966	21	68
	Wastewater	53-318	$2.27 \cdot 10^3 \pm 0.19 \cdot 10^3$	$2.45 \cdot 10^3 \pm 55.91 \cdot 10^3$	0.9960	16	53
DES*	Milli-Q	123-2059	$1.47 \cdot 10^3 \pm 0.09 \cdot 10^3$	$3.92 \cdot 10^4 \pm 9.19 \cdot 10^4$	0.9972	37	123
	Mineral	138-1732	$1.58 \cdot 10^3 \pm 0.10 \cdot 10^3$	$3.59 \cdot 10^4 \pm 8.01 \cdot 10^4$	0.9960	31	68
	Wastewater	78-523	$1.37 \cdot 10^3 \pm 0.11 \cdot 10^3$	$2.41 \cdot 10^4 \pm 4.60 \cdot 10^4$	0.9964	24	78
DS*	Milli-Q	73-436	$2.85 \cdot 10^3 \pm 0.09 \cdot 10^3$	$-1.12 \cdot 10^4 \pm 5.18 \cdot 10^4$	0.9993	22	73
	Mineral	61-1020	$2.75 \cdot 10^3 \pm 0.16 \cdot 10^3$	$2.78 \cdot 10^4 \pm 752 \cdot 10^4$	0.9967	18	61
	Wastewater	46-462	$2.67 \cdot 10^3 \pm 0.21 \cdot 10^3$	$-4.64 \cdot 10^3 \pm 54.66 \cdot 10^3$	0.9970	14	46
HEX**	Milli-Q	1.5-24	$1.09 \cdot 10^6 \pm 0.03 \cdot 10^6$	$7.79 \cdot 10^4 \pm 36.02 \cdot 10^4$	0.9994	0.42	1.4
	Mineral	1.9-32	$9.90 \cdot 10^5 \pm 0.68 \cdot 10^5$	$6.24 \cdot 10^5 \pm 9.95 \cdot 10^5$	0.9953	0.57	1.9
	Wastewater	1.4-14	$8.14 \cdot 10^5 \pm 0.47 \cdot 10^5$	$1.82 \cdot 10^5 \pm 3.59 \cdot 10^5$	0.9983	0.41	1.4

* Determined by DAD. ** Determined by FD. R^2: Determination coefficient. [a] Calculated as the concentration associated with a S/N of 3.

[b] Calculated as the concentration associated with a S/N of 10.

Table IV.8.- Results of the precision and accuracy study of the IL-DLLME-HPLC-DAD/FD method for the selected compounds in the different water samples.

Analyte	Type of water sample	Spiked level (µg/L)	Found[a] (µg/L)	Accuracy	t[b]	Analyte	Type of water sample	Spiked level (µg/L)	Found[a] (µg/L)	Accuracy	t[b]
E_3**	Milli-Q	2.52	2.86 ± 0.55	113	1.62	ZEN*	Milli-Q	119	108 ± 15	91	2.55
		14.1	16.0 ± 2.6	113	1.20			668	688 ± 15	103	0.39
	Mineral	10.1	11.7 ± 3.7	116	0.19		Mineral	114	102 ± 35	89	1.52
		56.5	50.0 ± 3.8	88	1.97			637	655 ± 36	103	1.13
	Wastewater	4.41	3.78 ± 1.89	86	0.98		Wastewater	88.2	91.9 ± 20.6	105	0.91
		24.7	21.8 ± 2.1	88	0.60			494	523 ± 27	106	0.79
17β-E_2**	Milli-Q	1.70	1.72 ± 0.36	101	0.34	DES*	Milli-Q	206	199 ± 67	96	0.64
		9.51	9.77 ± 0.41	103	0.09			1153	1173 ± 66	102	0.22
	Mineral	2.86	2.64 ± 1.00	97	1.00		Mineral	173	151 ± 51	87	2.52
		16.0	16.2 ± 1.0	101	0.33			970	972 ± 52	100	0.08
	Wastewater	1.99	1.86 ± 0.53	94	1.14		Wastewater	131	136 ± 28	104	0.82
		11.1	12.1 ± 0.7	105	0.91			732	797 ± 37	109	0.99
17α-E_2**	Milli-Q	1.70	1.60 ± 0.38	94	2.05	DS*	Milli-Q	121	119 ± 20	98	0.55
		9.51	10.90 ± 0.20	115	1.91			678	659 ± 19	97	0.41
	Mineral	2.86	2.62 ± 0.99	92	0.74		Mineral	102	92.3 ± 29.4	90	2.20
		16.0	16.2 ± 1.0	101	0.37			571	589 ± 28	103	1.41
	Wastewater	1.93	1.79 ± 0.56	94	0.92		Wastewater	77	87.5 ± 16.9	114	2.75
		10.8	11.8 ± 0.8	105	0.81			431	457 ± 22	106	0.71
EE_2**	Milli-Q	2.06	2.03 ± 0.23	98	0.53	HEX**	Milli-Q	2.43	2.28 ± 0.36	94	2.74
		11.6	12.1 ± 0.2	105	1.91			13.6	13.0 ± 0.3	95	0.69
	Mineral	3.07	2.65 ± 1.03	86	1.80		Mineral	3.15	2.67 ± 1.09	85	2.63
		17.2	17.6 ± 1.0	102	0.83			17.7	18.0 ± 1.0	102	0.74
	Wastewater	2.16	2.00 ± 0.52	95	0.84		Wastewater	2.28	2.38 ± 0.37	104	0.66
		12.1	13.4 ± 0.7	105	0.62			12.8	11.7 ± 0.4	92	0.35

* Determined by DAD. ** Determined by FD. [a] Average value ± standard deviation of five determinations (95% confidence value). [b] $t_4 = 2.78$, $\alpha = 0.05$.

well as the accuracy percentages obtained for each analyte and sample. As can be observed, experimental *t*-values ($t_{exp} \leq 2.74$ for Milli-Q water, $t_{exp} \leq 2.63$ for mineral water and $t_{exp} \leq 2.75$ for wastewater), were lower than the tabulated one (2.78, at 95 % confidence level, P = 0.05) for all the studied analytes. Therefore, the null hypothesis could be accepted concluding that there were no significant differences between the real and the found concentration. Acceptable relative recovery values were also obtained in all cases with accuracy percentages above 85 % for the three types of water. All these results revealed that the developed IL-DLLME-HPLC-DAD/FD method is repeatable, sensitive and selective and, as a result, suitable for the analysis of the eight selected oestrogens in Milli-Q, mineral and wastewater samples.

IV.2.6.- Conclusions

From the results obtained in this section the following conclusions could be drawn:

- In this work a methodology based on an IL-DLLME procedure followed by HPLC-DAD/FD has been proposed for the extraction and analysis of eight different oestrogenic compounds (E_3, 17α-E_2, 17β-E_2, EE_2, DES, DS, HEX and ZEN) in Milli-Q, mineral and wastewater.
- The DLLME procedure was optimised using a step by step approach with Milli-Q water. The best results were achieved using 60 mg of [PPIm][PF_6] as extractant and 500 µL of ACN as dispersant solvents together with vortex agitation for 1 min for the extraction of 10 mL of water at pH 8 without modification of the ionic strength. The use of MeOH as disperant solvent clearly decreased the extraction recovery, while the addition of NaCl could produce the change of the IL anion and generate a halide-based IL with a higher solubility in the aqueous phase, producing a decrease of its extraction capability. Vortex agitation was also found necessary to effectively improve the dispersion.
- The methodology was validated in terms of linearity by the preparation of the calibration curves of the method for each matrix obtaining LODs in the range of µg/L. A precision and accuracy study was also carried out at two levels of concentration and in quintuplicate. Results obtained clearly demonstrated the linearity of the method, in spite of the water sample selected, as well as its excellent precision and accuracy as well as low LODs and LOQs, in the µg/L range.
- In spite of the UV-Vis absorption of the IL, which could be clearly observed in HPLC-DAD

chromatograms, it was possible to perfectly identify and quantify the target analytes.

- The methodology, which resulted to be simple, effective, sensitive, selective and environmentally friendly, constitutes the first published work in which [PPIm][PF_6] is used as extraction solvent in DLLME for the determination of oestrogenic compounds and the second work in the literature in which this IL is used for the DLLME of organic pollutants. Furthermore, this work is the second application of IL-DLLME to the analysis of oestrogens in water samples and one of the first applications of DLLME to the determination of this type of analytes.
- Taking into account the excellent results obtained, this IL could be used to extract other types of compounds (after a suitable optimisation of the extraction conditions) also from different matrices and the methodology could be applied for routine analysis. Besides, the combination of the extraction procedure with other sensitive detection systems such as MS could be studied in order to decrease even more the LODs of the methodology, but taking special care in not introducing the IL in the system since it has an extremely low volatility.

IV.3.- Core-shell poly(dopamine) magnetic nanoparticles as sorbents in micro-dispersive solid-phase extraction for the determination of oestrogenic compounds in water samples prior to high-performance liquid chromatography-mass spectrometry analysis

In this section, a new sorbent consisting of core-shell Fe_3O_4@pDA m-NPs was prepared, characterised and applied for the m-μ-dSPE of twelve oestrogenic compounds of interest (i.e. 17α-E_2, 17β-E_2, E_1, HEX, EE_2, DES, DS, ZEN, α-ZAL, β-ZAL, α-ZEL and β-ZEL) from different water samples. Separation, determination and quantification were carried out by HPLC coupled to IT-MS with an ESI. NPs@pDA were synthesised by a chemical coprecipitation procedure and characterised by different surface characterisation techniques (XRD, XPS, thermogravimetric analysis (TGA), transmission electron microscopy (TEM), scanning electron microscopy (SEM), infrared (IR) and Raman spectroscopy, vibrating sample magnetometry, microelectrophoresis and adsorption/desorption isotherms). Parameters affecting the extraction efficiency of m-μ-dSPE (i.e. polymerisation time, pH and volume of the sample, extraction and elution conditions) were studied and optimised. Finally, the developed methodology was validated in Milli-Q, mineral, tap and wastewater samples using 2-MeOE_2 as IS.

IV.3.1.- Background

Nanotechnology has recently become an important tool for Analytical Chemistry due to the great number of advantages that the use of nanomaterials provides in this field. Among the different materials developed, NPs and, particularly, m-NPs, have been one of the most largely applied since they can be easily isolated from the matrix using an external magnet which considerably simplifies the procedure and diminishes the time consumed during the extraction (González-Sálamo et al., 2016b). Nowadays, there exist diverse types of m-NPs commonly used in different areas of Analytical Chemistry but, without any doubt, the iron oxides magnetite (Fe_3O_4) and maghemite (γ-Fe_2O_3) have been the ones most widely applied due to their good biocompatibility, particular physical and chemical properties and high potential of application (Martín et al., 2014a, 2014b; Shi et al., 2014). However, and as it was described in the Introduction Section of this PhD Thesis, NPs are usually grafted or coated with inorganic or organic layers in order to obtain a wide variability of materials and to increase their stability since they tend to form agglomerates and suffer oxidation easily. Therefore, such coatings not only offer a protective function but also provide them further functionalisation

and, as a result, the establishment of specific interactions with the targets analytes.

DA, which is a neurotransmitter mimic of the adhesive proteins, presents interesting characteristics for the formation of coating layers on both organic and inorganic surfaces since it is able to self-polymerise in aqueous phase under weak alkaline conditions (Jia et al., 2013). The structure of the resulting polymer is not completely known but it is thought that it is generated by the reaction between the primary amino groups and the quinone groups of the oxidised DA through the formation of Schiff bases and/or Michael addition adducts (Jia et al., 2013; Martín et al., 2014b). The result of this reaction is a high biocompatible coating with high dispersion capacity in water and active groups on their surface that allow the introduction of further modifications (Jia et al., 2013). Besides, the polymer also presents amino and catechol groups that allow the establishment of hydrogen bonds, as well as aromatic systems which can establish π-π staking interactions with the target analytes. Such interaction is particularly important for many organic pollutants (Qiao et al., 2014; Wang et al., 2013).

Nevertheless, and despite the advantages that the combination of m-NPs and pDA provides for their application as sorbents in dSPE, the number of publications until the development of this work was reduced. Indeed, it had only been applied for the determination of six polycyclic aromatic hydrocarbons (PAHs) in environmental water samples (Wang et al., 2013), the alkaloid berberine in a Chinese medical plant (Shi et al., 2014), four aflatoxins in red wine (McCullum et al., 2014) and seven antibiotics, three perfluorinated compounds and benzo(a)pyrene in lake and tap water (Ma et al., 2013). However, their application for the extraction of oestrogenic compounds had not been previously reported.

IV.3.2.- Specific objectives

In view of the foregoing, the following specific goals have been established for this work:

- The synthesis and characterisation of core-shell Fe_3O_4@pDA NPs and their evaluation as potential sorbents in m-μ-dSPE for the extraction of oestrogenic compounds from water samples.
- The optimisation of the separation and determination conditions of twelve oestrogenic compounds including three natural (17α-E_2, 17β-E_2 and E_1) and four synthetic (HEX, EE_2, DES and DS) oestrogens as well as five mycoestrogens (ZEN, α-ZAL, β-ZAL, α-ZEL and

β-ZEL) of interest by HPLC using IT-MS as detection system.

- The optimisation of m-μ-dSPE conditions such as pH and volume of the sample, extraction and elution conditions as well as Fe_3O_4@pDA NPs polymerisation time following a step by step approach, for the successful extraction of the selected analytes from mineral, tap and wastewater samples.
- The validation of the whole methodology in terms of recovery and calibration as well as the determination of the LODs and LOQs of the method for each sample.

IV.3.3.- Core-shell Fe_3O_4@pDA synthesis and characterisation

Core-shell Fe_3O_4@pDA NPs were synthesised by co-precipitation of Fe^{2+}/Fe^{3+} ions in alkali media, as indicated in Section III.7. The resulting black precipitate was then neutralised and m-NPs were coated with a thin film of pDA through the spontaneous oxygen-mediated self-polymerisation of DA in an aqueous solution (pH 8.3). The polymerisation process was carried out for 3, 6, 9 and 12 hours, in order to determine which of the coatings provided a higher retention of the selected analytes.

Following, several characterisation methods were employed in order to identify the correct phase and composition of the m-NPs and to investigate the polymerisation of DA onto their surface. Firstly, the XRD pattern of m-NPs was obtained. Figure IV.16 shows the XRD spectrum in which the main peaks were assigned to the Bragg reflections corresponding to the (111), (220), (311), (222), (400), (422), (551) and (440) diffraction line of magnetite (Fe_3O_4) (Joint Committee on Powder Diffraction Standards (JCPDS) card 75-0449). These results confirmed the polycrystalline nature of m-NPs with a cubic spinel structure (Martín et al., 2014a, 2014b). The presence of very broad peaks indicated the ultra-fine nature and small crystallite size of the NPs. Thus, the crystallite size was calculated using the Debye-Scherrer equation using the full-width at half diffraction (FWHM) value of the (331) XRD line and presented a value of 11 nm. Finally, the lattice parameter (a) and interplanar spacing (dhkl) were determined by using Bragg's law and were found to be 8.405 and 2.534 Å, respectively, which are very close to the reported values for pure magnetite (8.394 and 2.531 Å, respectively) (Martín et al., 2014a).

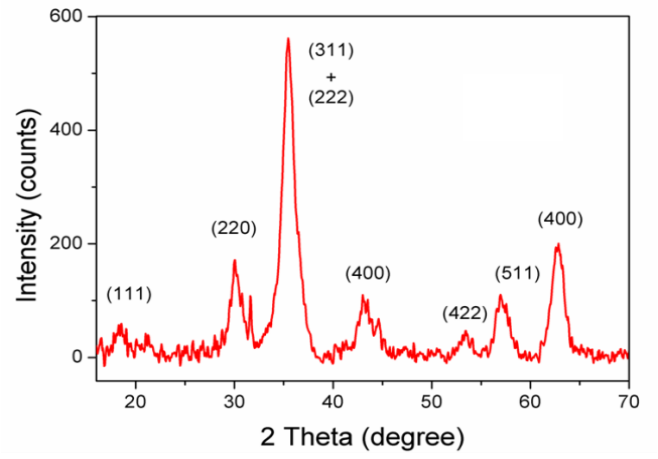


Figure IV.16.- XRD spectrum image of m-NPs synthesised by the co-precipitation method.

To study the morphological properties of m-NPs, SEM and TEM images were also obtained. Figure IV.17A shows a typical SEM image where m-NPs form amorphous aggregates due to electrostatic and dipolar interactions as well as van der Waals forces. TEM images also showed a very small size of the m-NPs in good agreement with XRD data reported above. In addition, a high-resolution TEM image shown in Figure IV.17B confirmed that m-NPs were well crystallised with an interplanar distance of 0.48 nm, which is consistent with the lattice spacing of the (111) spinel planes of the Fe_3O_4 crystal structure (Martín et al., 2014b).

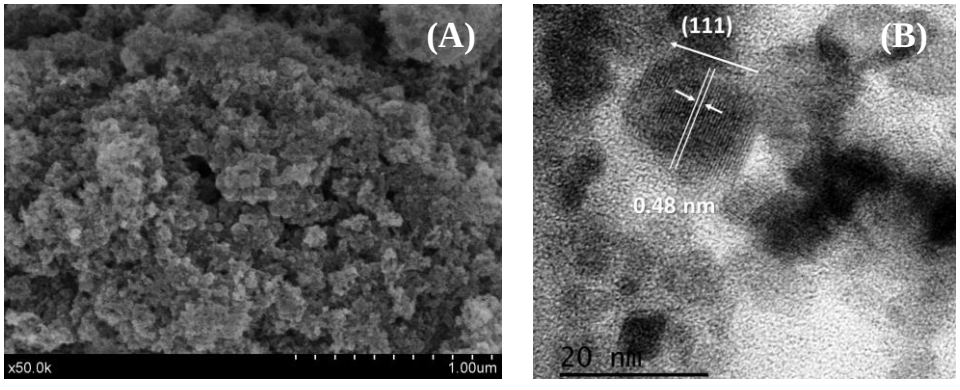


Figure IV.17.- (A) SEM and (B) high-resolution TEM images of the laboratory prepared m-NPs.

The specific surface area and the porosity of the m-NPs were measured in order to demonstrate their potential use as sorbents in m-μ-dSPE. Thus, standard nitrogen adsorption-desorption tests were performed. Hysteresis loop between the adsorption and desorption isotherms was found, which is characteristic of porous materials with capillary condensation. This aspect confirms the formation of porous structures in the magnetic aggregates. According to the International Union of Pure and Applied Chemistry (IUPAC) classification, the isotherms can be considered as a type-IV curve, which typically illustrates the presence of mesopores (pore diameter: 2-50 nm). Estimation of the Brunauer-Emmett-Teller (BET) specific surface area and the Barrett-Joyner-Halenda pore-size distribution demonstrated that m-NPs have a high specific surface area of up to 146.3 m^2/g and cluster-like aggregates pores with an average size of 27 nm.

In addition, the core-shell structure of m-NPs@pDA was studied using different techniques. First, a similar XRD pattern was observed for m-NPs@pDA using different polymerisation times, suggesting that the crystalline structure of the m-NP was not affected by the coating. The correct polymerisation of pDA onto the m-NPs was confirmed with XPS, IR, Raman spectroscopy and high-resolution TEM. Fourier transform (FT)-IR spectrum of m-NPs@pDA with 9 h of deposition time exhibited in Figure IV.18A shows a main absorption band at 580 cm^{-1} assigned to the Fe-O stretching modes of magnetite (Martín et al., 2014b). m-NPs@pDA showed additional bands in the range of 1000-1700 cm^{-1} which may be related to the aromatic rings of pDA (1614 cm^{-1}) and the amide I, amide II and C-N stretching bands (1639, 1535, and 1230 cm^{-1}, respectively) of the structure. Finally, a broad and strong band in the 3000 to 3400 cm^{-1} region, was assigned to the overlapping of the O-H of water adsorbed in pDA coating and N-H of the polymeric film (Wang et al., 2013; Zhu et al., 2011).

Taking into account that the XRD pattern of hematite (γ-Fe_2O_3; JCPDS card 39-1346) is quite similar, Raman spectrometry was used to confirm the correct phase. Raman spectra of m-NPs and m-NPs@pDA (Figure IV.18B) showed the most intense Fe_3O_4 band (A1g mode) at 683 cm^{-1} which could be clearly identified (Chourpa, et al., 2005). Furthermore, m-NPs@pDA presented two overlapping peaks at 1400 cm^{-1} (associated with the stretching of catechol) and 1600 cm^{-1} (associated with the deformation of catechol), confirming the core-shell configuration (Ryu et al., 2010). Finally, the evolution of the intensity of these last two peaks over time, confirmed the increase of the film thickness with the polymerisation time.

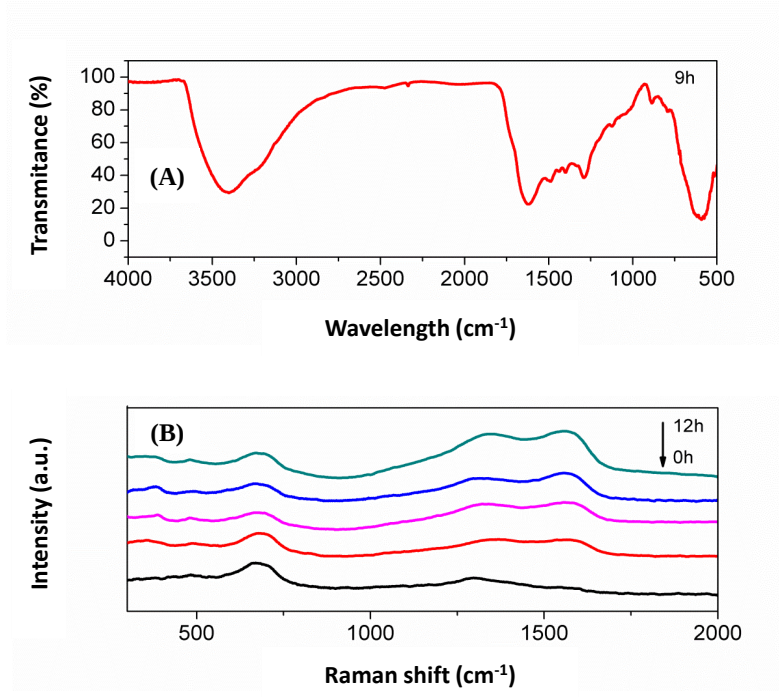


Figure IV.18.- (A) FT-IR transmittance spectrum of m-NPs@pDA with a polymerisation time of 9 hours; (B) Raman spectra of m-NPs (0 h) and m-NPs@pDA with different polymerisation times (0, 3, 6, 9 and 12 h).

In order to obtain a better understanding of the formation of the film, the TGA of m-NPs and m-NPs@pDA with different polymerisation times were developed. As it is shown in Figure IV.19, all NPs showed a weight loss of 5-8 % in the 100-200 °C range related to the removal of physically adsorbed water. In the next range (200-700 °C), m-NPs presented a weight loss of 4 % (bonded water contribution) meanwhile m-NPs@pDA had greater weight losses due to the thermal oxidation of the polymeric film. The polymeric shell content evolution over time (inset of Figure IV.19) showed a higher polymerisation rate during the first hours and a progressive decrease with time, as it has been previously reported by other authors (Lee et al., 2007), with a variation of the polymeric content in the range 10 and 25 % for the different polymerisation times.

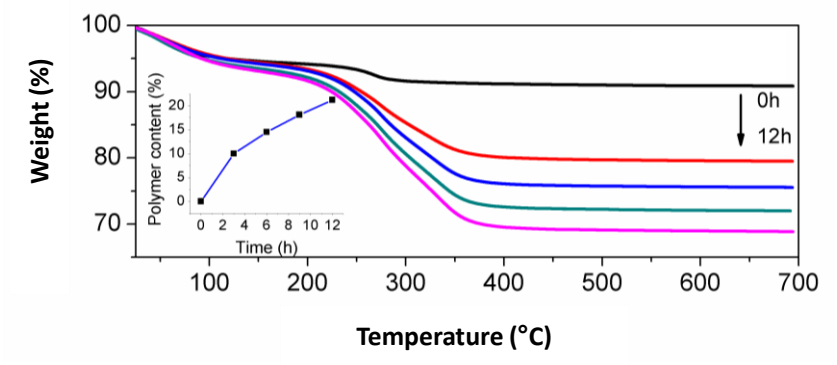


Figure IV.19.- TGA of m-NPs (0 h) and m-NPs@pDA with different polymerisation times (0, 3, 6, 9 and 12 h).

Afterwards, XPS analysis for m-NPs@pDA confirmed the polymerisation of pDA onto the m-NP surface (Martín et al., 2014b) and the thickness coating (about 2-3 nm) was determined by high-resolution TEM. Besides, the determination of the electrostatic potential (Z potential, Pz) or the charge near the surface of the NPs, which was carried out at different pH values by microelectrophoresis (Figure IV.20), revealed that the isoelectric point (IP) presented a value of 4.3 which is also in good agreement with data reported previously (Ball, 2010; Liu et al., 2014).

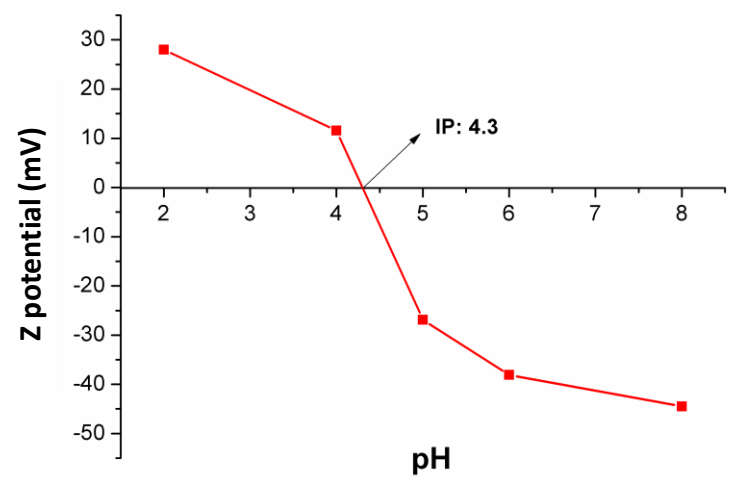


Figure IV.20.- Change in the Pz as a function of pH of m-NPs@pDA with a polymerisation time of 6 h. The cutt-off point with the x-axis was calculated to determine the isoelectric point.

Finally, and taking into account that the coating of the NPs with the polymeric film may alter the magnetic properties of the nanomaterial, a VSM was used to measure the magnetism of the final sorbent. As can be observed in Figure IV.21, both NPs and m-NPs exhibited a superparamagnetic behaviour at room temperature, with no remaining effect from the hysteresis loops when the applied magnetic field was removed. However, the saturation magnetisation of the pDA-modified NPs was reduced in 28 % (from 41.6 to 29.8 emu/g) after coating with the polymeric film. This reduction could be associated with the mass increase of the pDA-modified NPs. However, no influence in the deposition steps of the m-µ-dSPE procedure was experimentally observed.

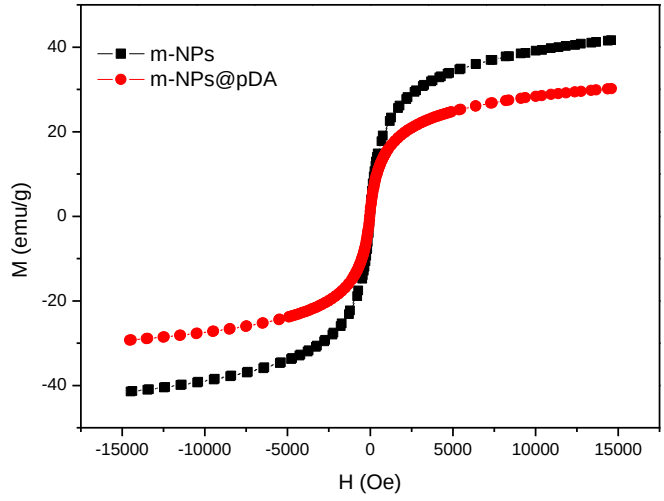


Figure IV.21.- Magnetisation curves of m-NPs and m-NPs@pDA (obtained with a polymerisation time of 6 h) at 298 K.

IV.3.4.- HPLC-MS/MS analysis

As starting point, and before carrying out the optimisation of the chromatographic separation, nebulisation and ionisation parameters of the MS detector were studied in order to obtain the best conditions for the determination of the selected oestrogens. With this aim, a mixture of the analytes in ACN at a concentration of 1 mg/L was directly infused in the MS system at a flow rate of 20 µL/min. Initially, IT acquisition conditions were established (the ICC was set at 10000, the maximum accumulation time at 200 ms with 6 averages for each experiment) for a suitable monitoring of the deprotonated molecule intensities, based on a

previous work in which a similar group of compounds was analysed by CE-MS (D'Orazio et al., 2014). In this sense, and taking into account the molecular mass of the studied analytes and possible m/z ratios of the deprotonated molecules, the studied m/z ratio range was 200-350 m/z, while 291 m/z was set as target mass. The parameters were optimised working in both positive and negative mode. The capillary voltage was modified between 3000 and 6000 V, the end plate offset between -500 and -5500 V, the nebulisation gas pressure (N_2) between 2 and 80 psi, the dry gas flow (N_2) between 0.5 and 12 L/min, and the dry gas temperature in the range 150 and 350 °C. The highest sensitivity for all analytes was reached working in the negative mode, with a capillary voltage of 5500 V, an end plate offset voltage of -600 V, a nebulisation gas pressure of 20 psi and a dry gas flow and temperature of 8 L/min and 300 °C, respectively. It should be remarked that although these compounds are generally uncharged in a wide pH range, they can be easily ionised under soft conditions in the ESI. In fact, as it was mentioned in the Introduction of this PhD Thesis, they have been previously determined both in negative (Sørensen and Elbk, 2005; Wang et al., 2010) and positive mode (di Mavungu et al., 2009; Kushnir et al., 2010). Finally, and keeping the previous optimised nebulisation conditions, the ICC, the maximum accumulation time and the average scans were modified, taking as optimal values 60000, 200 ms and 10, respectively.

Once nebulisation and detection parameters were studied, the chromatographic separation of the selected compounds was optimised. For this purpose, an X-Bridge C_{18} (100 mm × 4.6 mm × 3.5 µm) column and a pre-column (20 mm × 4.6 mm × 3.5 µm) filled with the same stationary phase, were used. Based on previous publications in which a basic media was used employing MS detection in the negative mode (Aufartová et al., 2011; Gentili et al., 2008; LaFleur and Schung, 2011), different ACN/H_2O mixtures were tested as mobile phases, containing or not small amounts of ammonia. Initially, isocratic and gradient elutions at room temperature were studied with and without ammonia addition, observing a decrease in the sensitivity of ZEN and its derivatives when ammonia was added. For this reason, this additive was not considered as part of the mobile phase in this case. The best separation was achieved using the gradient elution described in Section III.5.2 using ACN/H_2O mixtures. Finally, the influence of the temperature on the separation was studied up to 30 °C. It could be observed that a slight improvement of the resolution and efficiency was achieved when the column and precolumn were thermostated at 30 °C. As a result, such temperature was selected for further experiments. As can be seen in Figure IV.22, an adequate separation of the twelve selected

analytes and IS was achieved when the optimised conditions were applied.

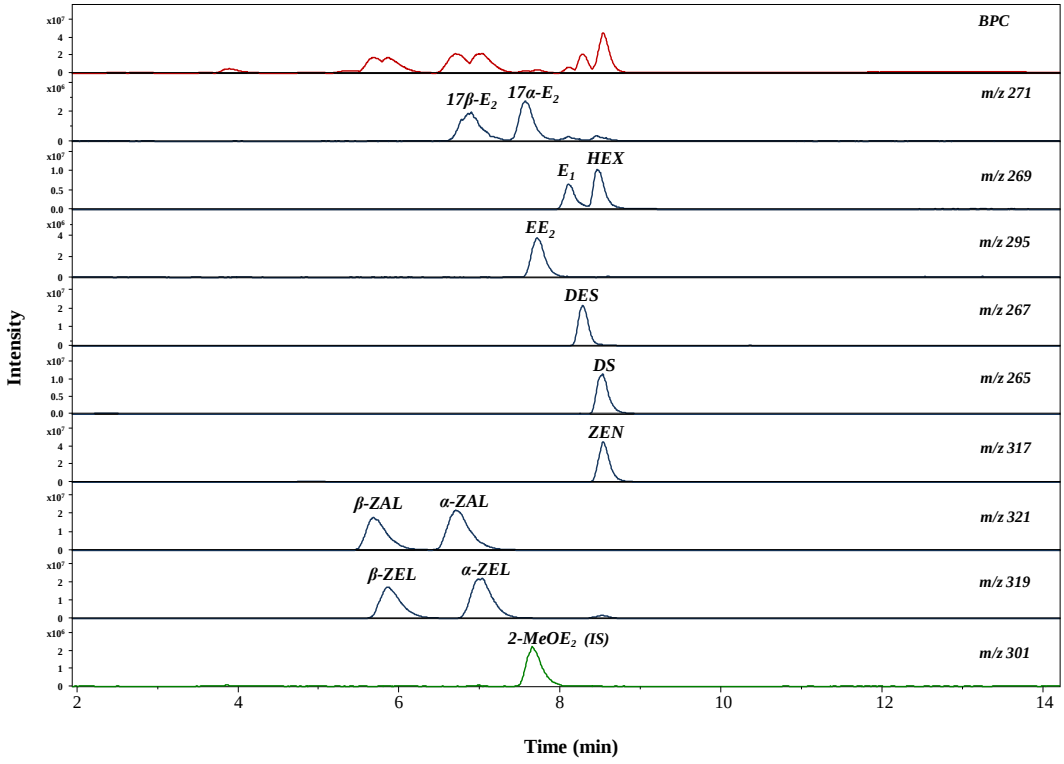


Figure IV.22.- HPLC-MS base peak chromatogram (BPC) and extracted ion chromatograms of the target analytes. Flow rate: 0.4 mL/min. Injection volume: 20 µL. Sample dissolved in 500 µL of 50/50 (v/v) ACN/H_2O. Separation at 30 °C. Concentration: 24 µg/L of IS (2-$MeOE_2$) and 8.0 µg/L of the target analytes. Gradient described in Section III.5.2.

Afterwards, and with the aim of carrying out the correct confirmation of the analytes, MS/MS parameters were evaluated. With this purpose, the direct infusion of individual analytes at a concentration of 5 mg/L was carried out, applying the nebulisation and detection conditions previously optimised and modifying the fragmentation amplitude for each compound. The optimum fragmentation parameters as well as the precursor and product ions of each analyte are shown in Table IV. 9.

Once the HPLC-MS/MS method was optimised, a repeatability study of the instrument performance was carried out at two different concentration levels obtaining RSD values lower

than 13.1 % for peak areas and below 0.6 % for retention times. Following, instrumental calibration curves based on the analyte/IS peak area ratio were obtained for each oestrogenic compound injecting seven increasing concentration levels in triplicate (n = 7). The IS (2-$MeOE_2$), which has a similar structure and behaviour than that of the target analytes, was selected in order to correct the possible errors during sample preparation procedure and to improve its reproducibility (Chen et al., 2014; Koot et al., 2013). As shown in Table IV.10, R^2 values were higher than 0.9943 and instrumental LODs and LOQs, calculated as the concentration which provided a S/N of 3 and 10, respectively, were between 0.21 and 4.5 µg/L for the LODs and 0.71 and 15 µg/L for the LOQs.

Table IV.9.- MS/MS fragmentation parameters of the selected oestrogenic compounds.

Analyte	Precursor ion (m/z)	Product ion* (m/z)	Fragmentation amplitude (V)
β-ZAL	321	277	0.70
β-ZEL	319	275	0.70
α-ZAL	321	277	0.70
17β-E_2	271	274	0.50
α-ZEL	319	275	0.70
17α-E_2	271	253	0.70
EE_2	295	267	0.70
E_1	269	272	0.70
DES	267	238	0.70
HEX	269	135	0.60
ZEN	317	274	0.60
DS	265	236	0.60

* The most intense product ion.

IV.3.5.- Optimisation of the m-µ-dSPE procedure

After m-NPs@pDA synthesis and characterisation, the sorbent was directly applied to the extraction of oestrogens from different water samples. However, a previous step by step optimisation of the influencing factors (polymerisation time, pH of the water sample, sorbent amount, deposition time after extraction, elution solvent and deposition time after elution) was developed using Milli-Q water with the aim of not introducing matrix effects and having a better vision of the influence of each factor in the extraction process. All the experiments developed during the optimisation procedure were carried out in duplicate, using 25 mL of spiked Milli-Q water containing a concentration of 0.02 mg/L of each target analyte.

Table IV.10.- Instrumental calibration data of the selected compounds.

Analyte	Retention time (min)	Calibration data (n = 7)				LOD[a)] (µg/L)	LOQ[b)] (µg/L)
		Range of concentration tested (µg/L)	Slope	Intercept	R^2		
β-ZAL	5.68	6-500	0.140 ± 0.004	0.095 ± 0.836	0.9988	0.71	2.4
β-ZEL	5.86	6-500	0.142 ± 0.004	0.131 ± 0.901	0.9987	0.34	1.1
α-ZAL	6.88	6-500	0.186 ± 0.006	0.255 ± 1.204	0.9986	0.55	1.8
17β-E_2	6.90	16-500	0.013 ± 0.001	0.026 ± 0.219	0.9943	4.5	15
α-ZEL	7.03	6-500	0.170 ± 0.007	0.238 ± 1.683	0.9971	0.25	0.84
17α-E_2	7.56	16-500	0.013 ± 0.001	-0.092 ± 0.189	0.9957	4.2	14
EE_2	7.71	16-500	0.017 ± 0.001	-0.110 ± 0.171	0.9980	3.7	12
E_1	8.10	10-500	0.026 ± 0.001	-0.161 ± 0.184	0.9988	2.4	8.1
DES	8.28	6-500	0.081 ± 0.002	-0.417 ± 0.425	0.9991	0.66	2.2
HEX	8.46	6-500	0.039 ± 0.002	0.238 ± 0.444	0.9963	1.0	3.5
DS	8.52	6-500	0.044 ± 0.002	0.321 ± 0.592	0.9959	0.48	1.6
ZEN	8.53	6-500	0.174 ± 0.010	1.208 ± 2.187	0.9954	0.21	0.71

R^2: Determination coefficient. [a)] Calculated as the concentration associated with a S/N of 3.
[b)] Calculated as the concentration associated with a S/N of 10.

IV.3.5.1.- Selection of the polymerisation time

As described in Section IV.3.3, the coating thickness increases during the first hours of polymerisation while the growth is more slowly afterwards. Since the extraction of oestrogens may be caused by the possible π-π staking interactions and hydrogen bonds that can be established between the pDA coating and the target analytes (Qiao et al., 2014;Wang et al., 2013), the thickness of the pDA coating should have a relevant role in the extraction procedure and it should be carefully evaluated. For this purpose, polymerisation time was increased up to 12 h, choosing as starting point the following extraction conditions: 25 mL of Milli-Q water at pH 5, 40 mg of sorbent, 10 min of magnetic deposition time after extraction, 6 mL of ACN as elution solvent and 5 min of magnetic deposition after elution. Figure IV.23 shows the recovery obtained in each case. As can be seen, the highest values (between 38 and 70 %) were obtained with 6 h of polymerisation. However, it could also be observed that recovery decreased at higher polimerisation time, which could be associated with the agglomeration of NPs due to the increase of the thicknesss of the pDA coating that causes a reduction of the surface area. Besides that, a thicker coating could also decrease the extraction kinetic since the diffusion of

the analytes is more difficult in this case. Taking this fact into account, 6 h of polymerisation was maintained for further experiments.

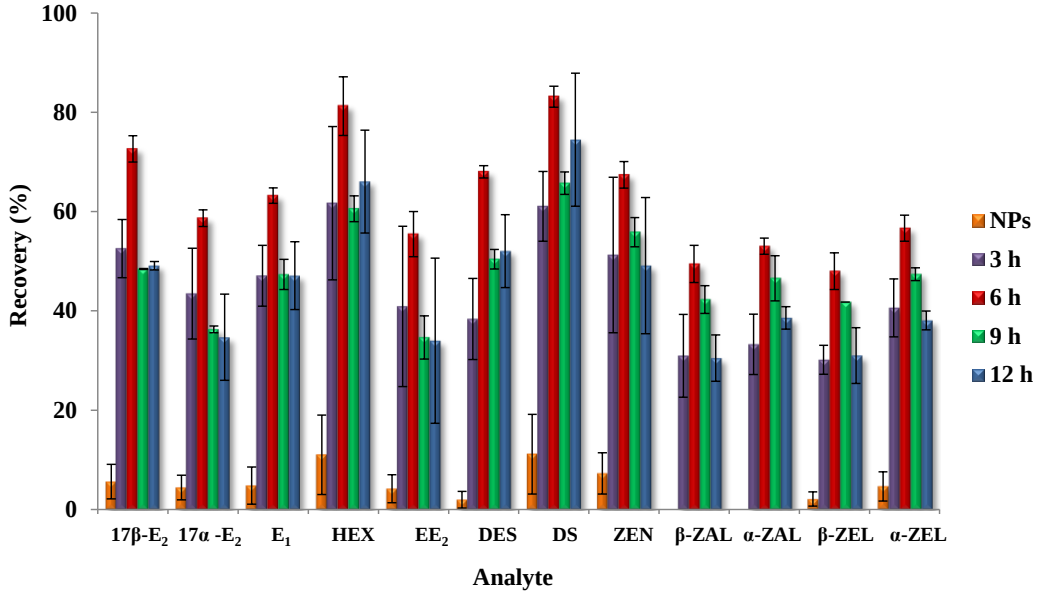


Figure IV.23.- Effect of the polymerisation time on the recovery of the target analytes after the Fe_3O_4@pDA m-µ-dSPE procedure. Extraction conditions: 25 mL of spiked Milli-Q water sample at pH 5, 40 mg of sorbent, 10 min of magnetic deposition time after extraction, 6 mL of ACN as elution solvent and 5 min of magnetic deposition after elution. Two extractions (n = 2) were carried out in each case. Concentration of the target analytes: 0.02 mg/L.

IV.3.5.2.- Influence of the pH

From the characterisation study of the synthesised NPs it could be appreciated that the IP of m-NPs@pDA was 4.3. Consequently, the pDA film is positively charged at pH below 4 and hence it displays some permeability and favourable interactions with anionic species. However, the film is permeable to cations and presents repulsions for anionic species at higher pH values. Based on this affirmation and taking into account that the pK_a values for oestrogens are around 8-11 (See Table I.1 and I.2), the optimal sample pH was expected below 8. To study this issue, the effect of the pH on the recovery of oestrogens was investigated in the range 3-11 while the rest of the conditions were maintained as follows: 25 mL of Milli-Q water, 40 mg of m-NPs@pDA (6 h polimerisation time), 10 min of magnetic deposition time after extraction,

6 mL of ACN as elution solvent and 5 min of magnetic deposition after elution. In general, the differences between the developed experiments were not important between pH 3 and 7 (recovery in the range 30-70 %), whereas at pH values higher than 8 an important decrease was observed (recovery in the range 4-62 %) which was caused by the deprotonating of the target analytes due to their pK_a values. At these pH values, their affinity for the aqueous phase is higher and the negatively charged analytes are also repulsed by the negative charge of the pDA coating. Thus, pH 7 was selected in the following experiments.

IV.3.5.3.- Effect of the sorbent amount and deposition time

In order to improve the extraction efficiency of the procedure, different amounts of m-NPs@pDA ranging from 20 to 80 mg were used. Figure IV.24 shows the results obtained in each case, in which an enhancement of the recovery values between 20 and 60 mg (recovery in the range 32-70 %) can be appreciated for most of the target analytes. Nevertheless, larger amounts of sorbent provided worse results, an aspect that could be related to a higher agglomeration

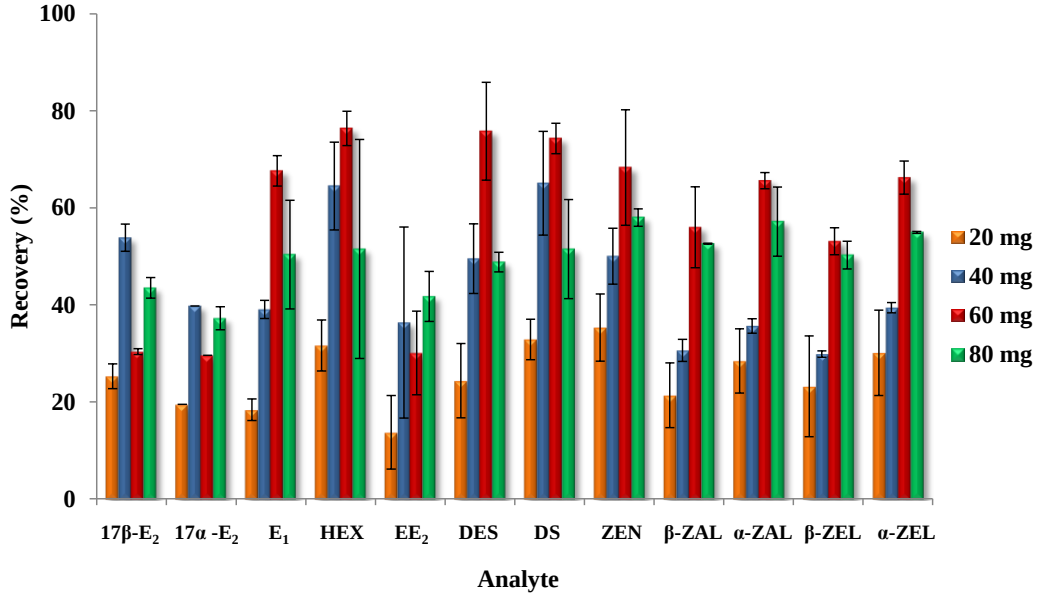


Figure IV.24.- Effect of the sorbent amount on the recovery of the target analytes after the Fe_3O_4@pDA m-μ-dSPE procedure. Extraction conditions: 25 mL of spiked Milli-Q water sample at pH 7, 10 min of magnetic deposition time after extraction, 6 mL of ACN as elution solvent and 5 min of magnetic deposition after elution. Two extractions (n = 2) were carried out in each case. Concentration of the target analytes: 0.02 mg/L.

of the particles under these circumstances. That is why 60 mg of sorbent was selected for further studies. After that, diverse experiments were carried out in order to select the optimum deposition time after extraction which was evaluated from 0 to 20 min observing that 10 min was enough to achieve the complete deposition of the sorbent.

IV.3.5.4.- Selection of the elution conditions

The effect of the different parameters involved in the elution process was also evaluated to guarantee the effectiveness of the procedure. For this purpose, the composition of the elution solvent was studied using different solvents such as ACN, MeOH and acetone. As it is shown in Figure IV.25, important differences between these solvents were not observed for the recovery values. However, MeOH was chosen due to its higher reproducibility in the extraction process compared to the rest of the tested solvents. Apart from these three solvents, different percentages of acetic acid were also added to them as suggested in the literature (Qiao et al., 2014) but no improvement in the recovery values was observed. Following, a brief study of the elution solvent volume and deposition time after elution was developed, finding that 6 mL of MeOH and 5 min of deposition were adequate to obtain an efficient desorption. Regarding the sample volume, different extractions using 50 and 100 mL of sample were carried out finding that the recovery values decreased in both cases: in the range 18-59 % for the 50 mL sample and 9-56 % for 100 mL. Nevertheless, if needed, the procedure can be scaled using higher amounts of sorbents with higher amounts of samples but considering that an adequate magnet may also be necessary.

IV.3.6.- Validation of the methodology

After the optimisation of the methodology was accomplished in Milli-Q water, the method was also applied and validated in different water samples.

First of all, and in order to demonstrate the reproducibility of the synthesis of Fe_3O_4@pDA NPs, a recovery study was carried out using several batches of NPs prepared in different days. As can be appreciated in Table IV.11, RSD values between batches were lower than 15 % in all cases which clearly demonstrates the reproducibility of the synthetic procedure.

Afterwards, the extraction efficiency and reproducibility of the whole method were evaluated in Milli-Q water. For this purpose, four replicated analyses were carried out using 25 mL of sample spiked at two concentration levels (0.8 and 8 µg/L) with the target analytes and

the IS (24 µg/L). Besides, a blank matrix was also prepared and spiked at the same concentration at the end of the procedure in order to calculate the relative recovery values taking into account the possible matrix effect, that is to say, comparing samples spiked at the beginning and at the end of the method. Recovery and RSD values as well as the LODs and LOQs of the whole method are shown in Table IV.12. As can be seen, recovery ranged between 78 and 113 % with RSD values below 13 %, which clearly demonstrate the reproducibility of the procedure. In addition, the low LODs and LOQs of the method, which were in the range 0.02-0.22 µg/L and 0.06-0.74 µg/L, respectively, also demonstrate the high sensitivity of the methodology.

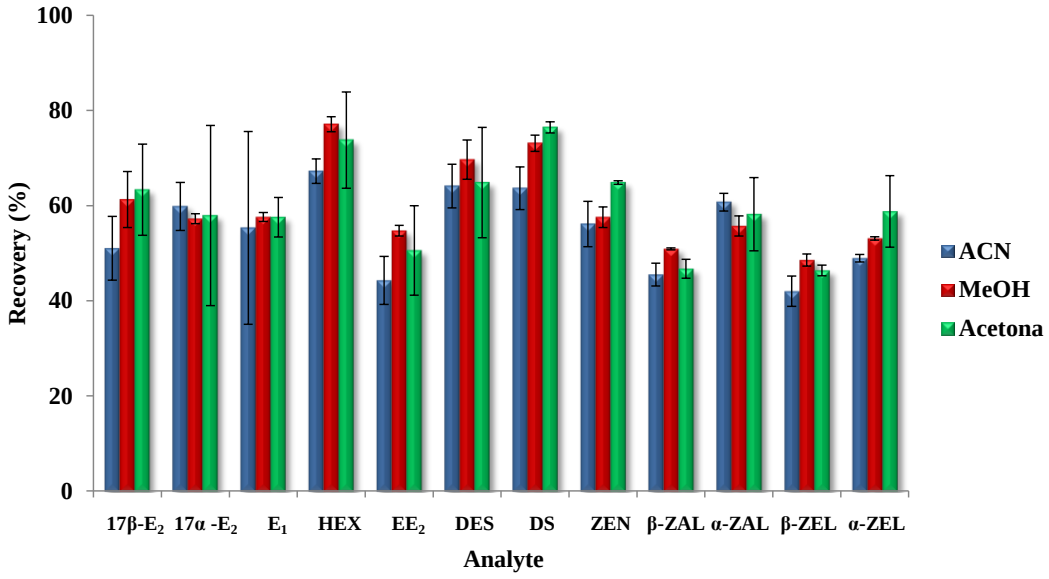


Figure IV.25.- Effect of the elution solvent on the recovery of the target analytes after the Fe_3O_4@pDA m-µ-dSPE procedure. Extraction conditions: 25 mL of spiked Milli-Q water sample at pH 7, 60 mg of sorbent, 10 min of magnetic deposition time after extraction, 6 mL of elution solvent and 5 min of magnetic deposition after elution. Two extractions (n = 2) were carried out in each case. Concentration of the target analytes: 0.02 mg/L.

Finally, the validation of the entire method was also extended to the analysis of mineral, tap and wastewater. As shown in Table IV.12, recovery values ranged between 70-119 % for mineral, between 70-119 % for tap and between 75-114 % for wastewater. Regarding LODs and LOQs they were also in the range 0.01-0.23 µg/L and 0.03-0.77 µg/L for mineral

water, 0.02-0.22 µg/L and 0.06-0.74 µg/L for tap samples and 0.07-0.34 µg/L and 0.22-1.1 µg/L for wastewater.

Table IV.11.- Reproducibility data of Fe_3O_4@pDA m-NPs synthetic procedure.

Analyte	Relative recovery %[a), b)]				
	Batch 1	Batch 2	Batch 3	Batch 4	RSD %
17β-E_2	110	94	116	110	9
17α-E_2	98	87	113	105	11
E_1	115	89	108	118	12
HEX	114	99	108	104	6
EE_2	83	96	108	85	13
DES	111	87	119	106	13
DS	114	112	118	105	5
ZEN	118	98	116	118	9
β-ZAL	90	93	103	80	10
α-ZAL	97	92	113	93	10
β-ZEL	86	80	107	79	15
α-ZEL	99	87	113	92	11

[a)] Concentration of the analytes: 0.02 mg/L.[b)] Data obtained as the average of two extractions (n = 2).

As an example of the successful application of the developed procedure to the analysis of water samples, Figure IV.26 shows the extracted ion chromatograms of a spiked and a blank wastewater. As can be appreciated, such sample did not contain the selected analytes and no interferences that precluded the correct determination of the analytes were found demonstrating the great extraction efficiency and selectivity of the proposed process for the analysis of the selected oestrogens.

IV.3.7.- Comparison with other methods

Although MRLs have not been established for this type of analytes in environmental water matrices yet, the evaluation and comparison of the LODs of the procedure with other previously developed methods in which NPs have been used for the extraction of oestrogens in water results of great interest in the search of new methodologies for the determination of these kinds of contaminants. In this sense, and as it was indicated at the beginning of this section, only few natural and synthetic oestrogens have been studied using m-dSPE with NPs (Chiu et al., 2008; Guan et al., 2010; Li et al., 2010; Liu and Jia, 2008; Pérez et al., 2014). In such cases, specific interaction sites provided by different types of modifiers such as MWCNTs-OH (Guan, et al., 2010), palmitic acid (Pérez et al., 2014), C_{18} (Liu and Jia, 2008) or poly(divinylbenzene-co-methacrylic acid) (Li et al., 2010) were used. However, none of them

Table IV.12.- Results of the recovery study (n = 4) of the m-µSPE-HPLC-MS method for the selected compounds in the different matrices at two levels of concentration.

Analyte	Type of water sample	Level 1[a] (n = 4) Recovery % (RSD, %)	Level 2[b] (n = 4) Recovery % (RSD, %)	LOD_{method}[c] (µg/L)	LOQ_{method}[d] (µg/L)	Analyte	Type of water sample	Level 1[a] (n = 4) Recovery % (RSD, %)	Level 2[b] (n = 4) Recovery % (RSD, %)	LOD_{method}[c] (µg/L)	LOQ_{method}[d] (µg/L)
β-ZAL	Milli-Q	106 (7)	90 (13)	0.13	0.43	EE_2	Milli-Q	110 (13)	102 (8)	0.18	0.59
	Mineral	84 (12)	98 (4)	0.11	0.37		Mineral	116 (17)	107 (5)	0.20	0.67
	Tap	119 (13)	75 (13)	0.07	0.23		Tap	116 (9)	98 (2)	0.19	0.65
	Wastewater	75 (15)	108 (2)	0.18	0.58		Wastewater	84 (8)	99 (3)	0.33	1.1
β-ZEL	Milli-Q	105 (7)	89 (6)	0.09	0.30	E_1	Milli-Q	111 (8)	106 (7)	0.17	0.58
	Mineral	86 (9)	101 (7)	0.06	0.19		Mineral	86 (16)	115 (6)	0.12	0.40
	Tap	106 (14)	70 (6)	0.04	0.15		Tap	105 (12)	111 (12)	0.22	0.74
	Wastewater	75 (11)	113 (11)	0.13	0.43		Wastewater	88 (10)	99 (5)	0.32	1.1
α-ZAL	Milli-Q	108 (8)	98 (13)	0.08	0.27	DES	Milli-Q	101 (11)	108 (7)	0.06	0.18
	Mineral	75 (9)	108 (4)	0.08	0.27		Mineral	84 (16)	111 (8)	0.02	0.05
	Tap	87 (18)	82 (4)	0.04	0.12		Tap	85 (16)	77 (3)	0.07	0.23
	Wastewater	79 (9)	81 (12)	0.12	0.39		Wastewater	89 (12)	96 (3)	0.08	0.25
17β-E_2	Milli-Q	113 (5)	108 (3)	0.21	0.70	HEX	Milli-Q	101 (11)	110 (1)	0.18	0.60
	Mineral	89 (14)	119 (4)	0.23	0.77		Mineral	87 (13)	110 (9)	0.04	0.14
	Tap	79 (19)	95 (3)	0.21	0.70		Tap	96 (6)	96 (19)	0.15	0.49
	Wastewater	77 (7)	96 (12)	0.34	1.1		Wastewater	98 (1)	114 (3)	0.15	0.49
α-ZEL	Milli-Q	109 (4)	98 (7)	0.05	0.16	DS	Milli-Q	95 (9)	106 (1)	0.07	0.24
	Mineral	70 (12)	118 (7)	0.03	0.10		Mineral	72 (14)	106 (9)	0.03	0.09
	Tap	107 (5)	91 (6)	0.03	0.09		Tap	86 (9)	92 (14)	0.13	0.42
	Wastewater	91 (7)	98 (8)	0.07	0.23		Wastewater	84 (10)	105 (7)	0.07	0.22
17α-E_2	Milli-Q	78 (10)	109 (8)	0.22	0.74	ZEN	Milli-Q	107 (6)	108 (7)	0.02	0.06
	Mineral	81 (18)	117 (5)	0.16	0.54		Mineral	70 (12)	117 (1)	0.01	0.03
	Tap	117 (9)	78 (3)	0.10	0.33		Tap	119 (13)	73 (14)	0.02	0.06
	Wastewater	75 (12)	97 (15)	0.23	0.78		Wastewater	108 (11)	95 (10)	0.08	0.26

[a] Concentration of the analytes in the samples: 0.8 µg/L except in wastewater which was 1.5 µg/L. [b] Concentration of the analytes in the samples: 8 µg/L.

[c] Calculated as the concentration associated with a S/N of 3. [d] Calculated as the concentration associated with a S/N of 10.

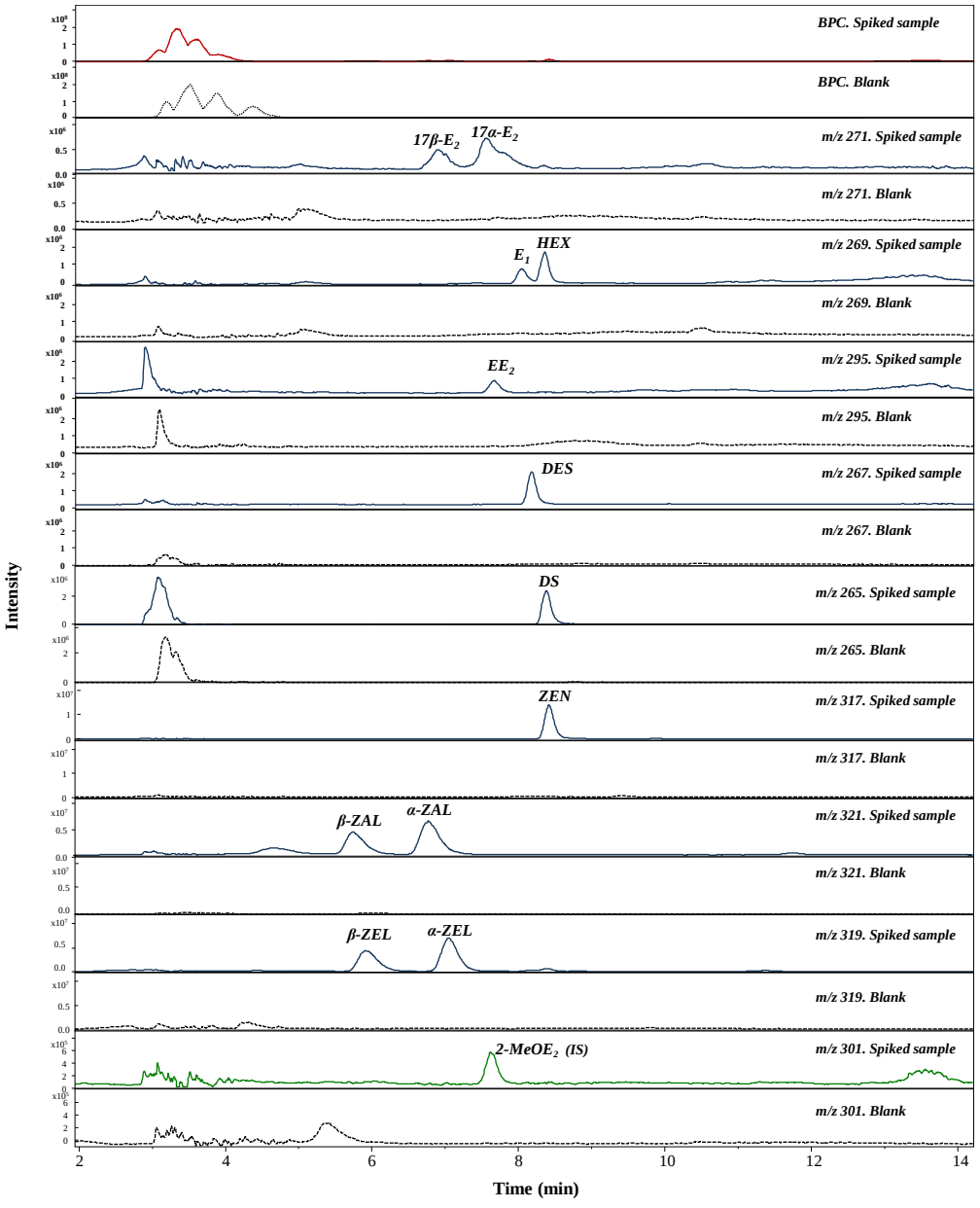


Figure IV.26.- HPLC-MS BPC and extracted ion chromatograms of the target analytes and the IS (2-MeOE$_2$) of a blank and a spiked wastewater sample after the Fe_3O_4@pDA m-µ-dSPE procedure. Flow rate: 0.4 mL/min. Injection volume: 20 µL. Sample dissolved in 500 µL of 50/50 (v/v) ACN/H_2O. Separation at 30 °C. Concentration in the water sample: 24 µg/L of IS (2-MeOE$_2$) and 8.0 µg/L of the target analytes.

used Fe_3O_4@pDA particles. In general terms, the LODs obtained in the present work are in the same order of magnitude than those obtained by other authors using similar or even higher sample volumes.

IV.3.8.- Conclusions

From the results obtained in this section, the following conclusions can be drawn:

- Fe_3O_4 NPs coated with a pDA layer have been synthesised, characterised and proposed as sorbents for the m-μ-dSPE of different oestrogenic compounds (17β-E_2, 17α-E_2, E_1, HEX, EE_2, DES, DS, ZEN, β-ZAL, α-ZAL, β-ZEL and α-ZEL) from several environmental water samples.
- Chromatographic separation and determination by HPLC-MS/MS of the oestrogens of interest was optimised and validated in terms of linearity and repeatability with R^2 higher to 0.9943 for all analytes and RSDs between areas and retention times lower than 13.1 and 0.6 %, respectively. Sensitivity was also evaluated obtaining instrumental LODs and LOQs below 4.5 and 15 μg/L, respectively.
- The m-μ-dSPE procedure was optimised using a step by step approach with Milli-Q water and the best results were achieved using 25 mL of sample at pH 7, 60 mg of Fe_3O_4@pDA NPs as extraction sorbent and 10 min of magnetic deposition as well as 6 mL of ACN as elution solvent and 5 min of magnetic deposition after this last step.
- The extraction efficiency and reproducibility of the methodology were evaluated by the recovery studies. The methodology, which was also applied to the extraction of mineral, tap and wastewater, resulted to be simple, effective, sensitive, selective and environmentally friendly for all samples.
- This methodology constitutes the first application of Fe_3O_4@pDA NPs as sorbent for the m-μ-dSPE of oestrogenic compounds from water samples and the first work in which this group of three different types of oestrogenic compounds (natural, synthetic and mycoestrogens) is extracted from water samples using dSPE.
- According to the good results obtained in the present study, this type of NPs represents an interesting alternative as extraction sorbents for the determination of oestrogens in different matrices. Future work could be developed to extend their application to the analysis of other compounds and samples. The methodology could also be applied for routine analysis.

IV.4.- Application of multiwalled carbon nanotubes as sorbents for the determination of oestrogenic mycotoxins in water samples and infant milk formulae prior to high-performance liquid chromatography-mass spectrometry analysis

In this section, a simple and environmentally friendly methodology was developed for the analysis of a group of six mycotoxins with remarkable oestrogenic activity (i.e. ZAN, ZEN, α-ZAL, β-ZAL, α-ZEL and β-ZEL) produced by *Fusarium* species, in environmental and food samples. The extraction was developed by the application of μ-dSPE using MWCNTs as sorbent. Separation, determination and quantification were achieved by HPLC coupled to an IT-MS with an ESI. Parameters affecting the efficiency of the extraction procedure such as pH of the sample, amount of MWCNTs, type and volume of the elution solvent and sample volume were studied and optimised by a step by step approach. Finally, the methodology was validated in different samples of interest including mineral, pond and wastewater as well as powdered infant milk using 17β-E_2-D_5 as IS.

IV.4.1.- Background

As indicated in the Introduction Section of this PhD Thesis, resorcycle lactones such as ZEN and its derivatives present an important oestrogenic character due to their similarity with 17β-E_2 which allows them to bond with the specific receptors of this natural oestrogen (Benzoni et al., 2008). Special relevance have the derivatives α-ZAL and α-ZEL which present an oestrogenic potential much higher than that of the rest of the mycoestrogens. Indeed, their oestrogenic activity is of the same order of magnitude than the most potent oestrogen: 17β-E_2 (Meucci et al., 2011).

Despite the fact that mycoestrogens are potentially known pollutants, their evaluation in the environment is scarce (Kolpin et al., 2014). However, it has been confirmed in several studies that they can appear in environmental water (Gromadzka et al., 2009; Kolpin et al., 2014) as a result of the excretion coming from cattle which have been treated with them as anabolic substances or which have ingested contaminated feed (Laganà et al., 2004) or also from runoff water from agricultural fields or effluents from wastewater treatment plants, among others (Emídio et al., 2015). In addition, these compounds have also been found in food products destined to vulnerable groups as, for example, milk formulae used for the feeding of new-borns, infants and young children (Meucci et al., 2011; Ok et al., 2014), which can bring about important disorders in their endocrine system as, for example, premature thelarche,

pubarche and breast enlargement (Ok et al., 2014).

Based on the harmful effects that have been attributed to mycoestrogens, the European Commission (EC) established a MRL of ZEN in different cereal-based food products (Commission Regulation 1881/2006) while the EFSA later fixed in 2014 in the Panel on Contaminants in the Food Chain a tolerable daily intake of 0.25 µg/kg body weight for the same compound. However, there is no limit established for environmental water or other foodstuff different than cereals such as infant formulae despite the negative health effects that they can produce in consumers and in the rest of the population.

Regarding the analysis of mycoestrogens in these types of samples, a reduced number of new strategies have been developed. In the case of infant formulae, the studies have been focus on the extraction of ZEN using LLE (Zhang et al., 2013), QuEChERS (Desmarchelie et al., 2014) or conventional SPE procedures using inmunoaffinity columns (Desmarchelie et al., 2014; Meucci et al., 2011; Ok et al., 2014). In the case of water samples, the extraction procedures applied have been conventional SPE (González-Sálamo et al., 2015; Gromadzka et al., 2009; Hartmann et al., 2007; Kolpin et al., 2014; Laganà et al., 2001, 2004; Lundgren and Novak, 2009) and DLLME (D'Orazio et al., 2014). Taking into account the low number of developed methodologies and the importance of the determination of this type of compounds, the search and development of new analytical methods are nowadays of important relevance.

MWCNTs are interesting alternative sorbents for the extraction of mycoestrogens. In fact, they have been widely used for the extraction of a great number of analytes with different chemical structures from diverse matrices by either SPE, SPME, membrane-based microextractions, SBSE, MSPD or, particularly, dSPE (Herrera-Herrera et al., 2012; Ravelo-Pérez et al., 2010). The last of these techniques is characterised by its simplicity, speed and high extraction efficiency as well as the use of only few grams of sorbent, especially, when the µ-dSPE approach is applied, in which sorbent amounts lower than 100 mg are frequently applied. Nevertheless, in spite of the above numbered advantages, the extraction of ZEN and its derivatives from water or infant formula using MWCNTs as sorbent has not been carried out until the development of this work. Furthermore, only one work has been proposed for their analysis in other types of samples, i.e. cereals samples (Ying et al., 2013).

IV.4.2.- Specific objectives

In view of the foregoing, the following specific objectives have been established for this work:

- The development of a new analytical methodology based on the combined use of HPLC-MS/MS and µ-dSPE to carry out the determination of a group of six mycotoxins with an important oestrogenic activity (i.e. ZAN, ZEN, α-ZAL, β-ZAL, α-ZEL and β-ZEL) in infant milk formula, mineral, pond and wastewater samples.
- The evaluation of the influence of the parameters affecting the µ-dSPE procedure using a step by step approach (i.e. pH of the aqueous sample, amount of sorbent, type and volume of elution solvent and sample volume) to achieve the best extraction efficiency.
- The validation of the whole methodology in terms of calibration, recovery and reproducibility at two concentration levels as well as the obtaininig of the LODs and LOQs of the method for each sample.
- The study of the applicability of the methodology to the analysis of different environmental samples such as mineral, pond and wastewater as well as food products like infant formulae which are intended for vulnerable groups like children.

IV.4.3.- HPLC-MS/MS method

With the aim of carrying out the LC separation of the target analytes, HPLC-MS/MS separation conditions were applied as described in Section IV.3.4. For this purpose, an X-Bridge C_{18} column was employed, together with the gradient described in Section III.5.2 as well as nebulisation and detection conditions. MS/MS experiments were also performed by fragmentation of the deprotonated molecule $[M-H]^-$, which was selected as the precursor ion. Results were found successful with a very good separation of all analytes and an analysis time of 10 min. As previously indicated, the repeatability of the separation was also evaluated obtaining good results for the peak areas and retention times of all analytes (results were similar to those indicated in Section IV.3.4).

IV.4.4.- Optimisation of the MWCNTs µ-dSPE method

In order to carry out the optimisation of the extraction procedure, a step by step approach of the main parameters that influence the extraction (pH of the sample, amount of

MWCNTs, type and volume of elution solvent and sample volume) was considered. For this purpose, Milli-Q water was used in order to avoid matrix effects and to evaluate the influence of each factor in the extraction process. All the experiments developed during this optimisation step were carried out in duplicate using 25 mL of spiked Milli-Q water (except for the evaluation of the sample volume) containing a concentration of 5.6 µg/L of each target analyte. In all cases, once MWCNTs were dispersed in Milli-Q water, the dispersion was passed through a glass SPE tube that contained inside two PTFE frits. Then, another frit was located onto the sorbent and vacuum was applied for 30 min in order to dry the stationary phase. Afterwards, the retained mycotoxins were eluted as it was indicated in Section III.8.4.

IV.4.4.1.- Influence of sample pH

It is widely known that changes in pH values produce modifications in the CNTs charge. Indeed, there exists a pH value called "isoelectric point" or "point of zero charge" at which this material does not have any charge on its surface and, consequently, it is not possible to establish electrostatic interactions with charged species (Gilbertson et al., 2016; Ravelo-Pérez et al., 2010). In addition, the pK_a values of this group of analytes are around 7-8. Based on that, a full study of the effect of the pH on the recovery of oestrogenic compounds was developed in the range 2-8 while the rest of the conditions were maintained as follows: 25 mL of Milli-Q water, 80 mg of MWCNTs, 1 min of shaking, 30 min of drying using vacuum and 25 mL of MeOH as elution solvent. As can be seen in Figure IV.27, in general terms, the highest recovery values were obtained at pH values below the pK_a of the target analytes, which indicates that the extraction is not associated with electrostatic interactions but should be mainly related to π-π staking interactions between the aromatic structures of CNTs and mycoestrogens. Taking this result into account, and also that pH 3 provided the highest recovery for almost all analytes, this value was applied in the following experiments.

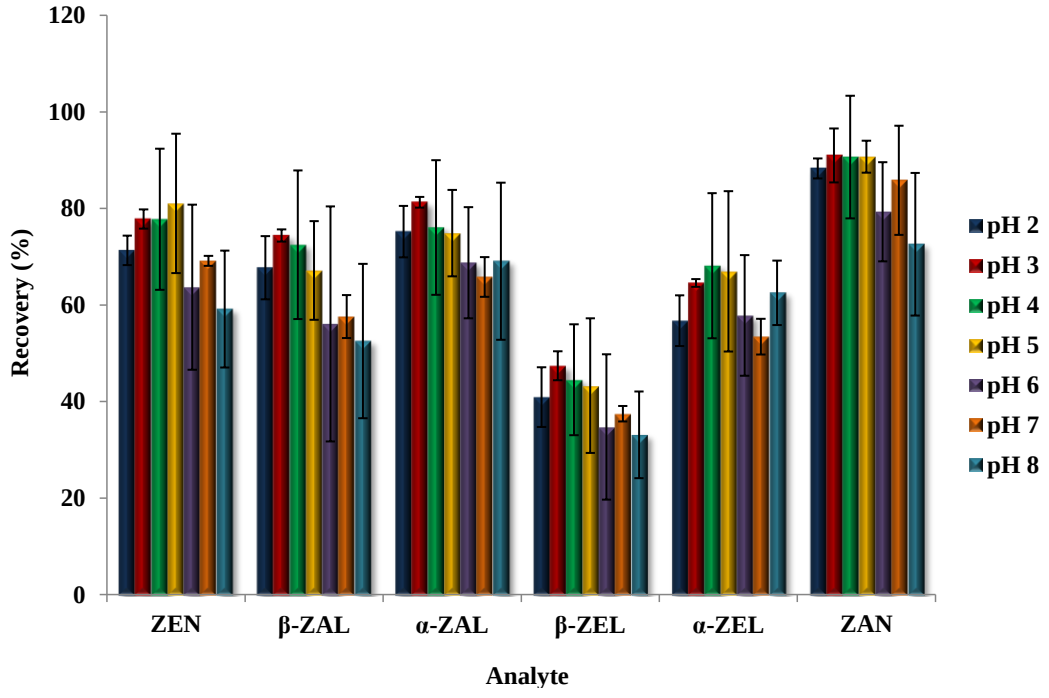


Figure IV.27.- Effect of the pH of the sample on the recovery of the selected analytes after the MWCNTs µ-dSPE procedure. Extraction conditions: 25 mL of Milli-Q water, 80 mg of MWCNTs, 1 min of shaking, 30 min of drying using vacuum and 25 mL of MeOH as elution solvent. Two extractions (n = 2) were carried out in each case. Concentration of the target analytes in the sample: 5.6 µg/L.

IV.4.4.2.- Study of the sorbent amount

In order to obtain the highest recovery values using the lowest amount of sorbent and, consequently, to reduce solvent consumption and to simplify the procedure, different amounts of MWCNTs ranging from 60 to 125 mg were applied maintaining the rest of the extraction conditions without changes. In Figure IV.28 it can be appreciated that no relevant differences were found in this study, obtaining recovery values in the range 70-100 % except for the case of β-ZEL for which recovery was around 50 %. However, 80 mg were selected for further studies, since a better repeatability of the extraction compared to that obtained with 60 mg of sorbent was achieved, probably associated with an ineffective dispersion of CNTs, or to a slight loss of the extraction material during manipulation, when lower amounts were applied.

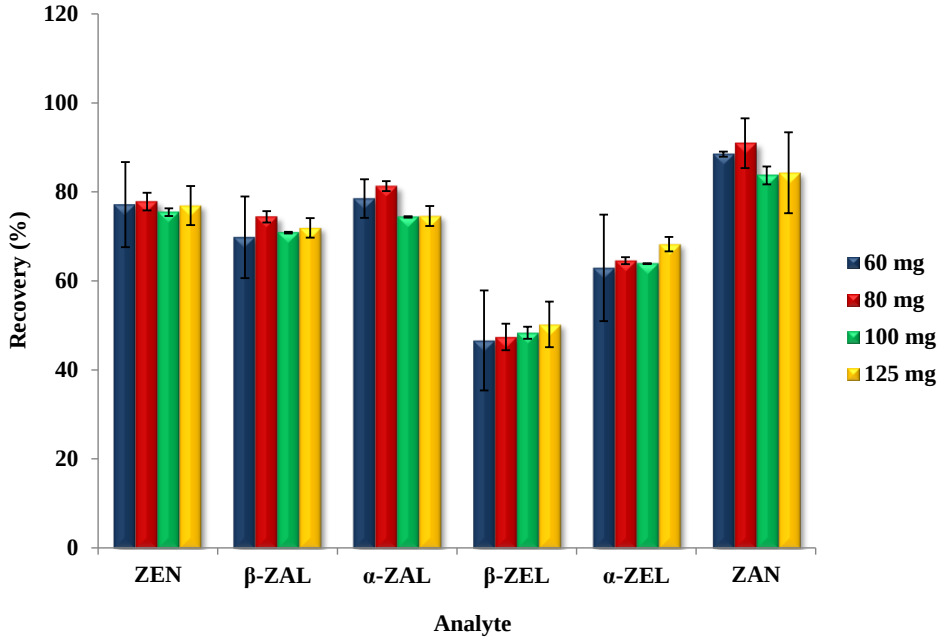


Figure IV.28.- Effect of the sorbent amount on the recovery of the selected analytes after the MWCNTs µ-dSPE procedure. Extraction conditions: 25 mL of Milli-Q water, pH 3, 1 min of shaking, 30 min of drying using vacuum and 25 mL of MeOH as elution solvent. Two extractions (n = 2) were carried out in each case. Concentration of the target analytes in the sample: 5.6 µg/L.

IV.4.4.3.- Selection of the type and volume of elution solvent

The elution step is a critical part of the methodology and, therefore, an adequate selection of the parameters is necessary to obtain an efficient desorption of the target analytes. Taking this fact into account, the nature of the elution solvent as well as its volume were studied. In this sense, DCM, MeOH, ACN, acetone and a mixture of MeOH/acetone 50/50 (v/v) were evaluated (25 mL of solvent were used in each case). Figure IV.29 shows the recovery values obtained in these experiments. As can be seen, the use of DCM provided the worst results, whereas the mixture MeOH/acetone provided an important improvement in the recovery with values between 90 and 106 %. A special enhancement of the results was found in the case of β-ZEL for which the addition of acetone in the desorption process increased the results around 40-50 % compared with the use of MeOH alone. This aspect could be associated with the compromise in terms of polarity and volatility of the solvent mixture.

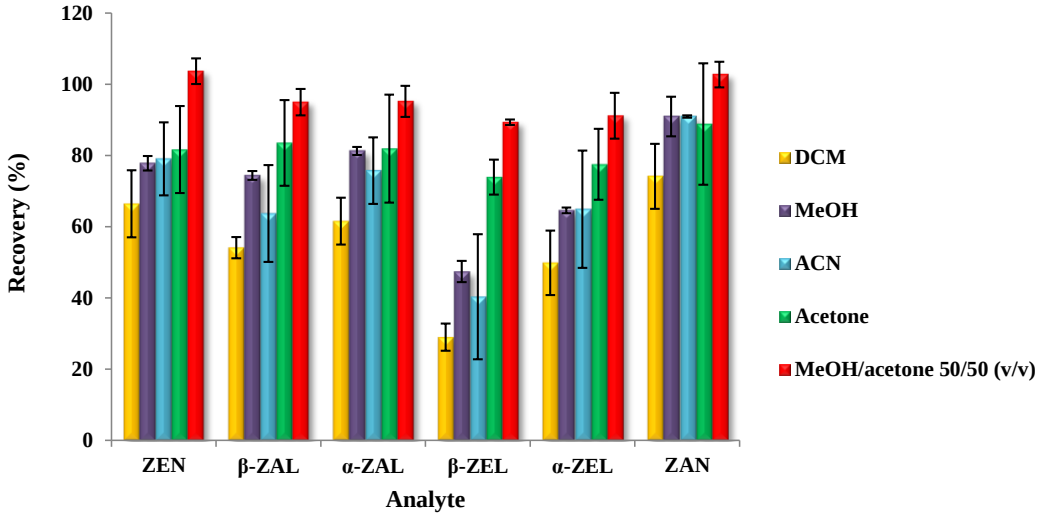


Figure IV.29.- Effect of the elution solvent nature on the recovery of the selected analytes after the MWCNTs µ-dSPE procedure. Extraction conditions: 25 mL of Milli-Q water, pH 3, 80 mg of MWCNTs, 1 min of shaking, 30 min of drying using vacuum and 25 mL of elution solvent. Two extractions (n = 2) were carried out in each case. Concentration of the target analytes in the sample: 5.6 µg/L.

Afterwards, a study of the volume used for the elution step was carried out using the selected mixture of solvents. With this aim, extractions using volumes of the mixture between 15 and 30 mL were performed. As can be seen in Figure IV.30, the best results were obtaining when 30 mL of MeOH/acetone 50/50 (v/v) were used. Therefore, this value was applied in subsequent experiments.

IV.4.4.4.- Study of the sample volume

With the aim of evaluating the influence of the sample volume used on the efficiency of the extraction, different experiments were carried out using 25, 50 and 100 mL maintaining the rest of conditions as previously optimised. Results did not show important changes in the recovery values when the different volumes were extracted, from what it can be concluded that volumes between 25 to 100 mL can be analysed without variation in the results. Finally, 50 mL were used in the case of water samples (since appropriate LODs of the method were obtained) and 25 mL when infant milk formula extracts were analysed as it will be later shown.

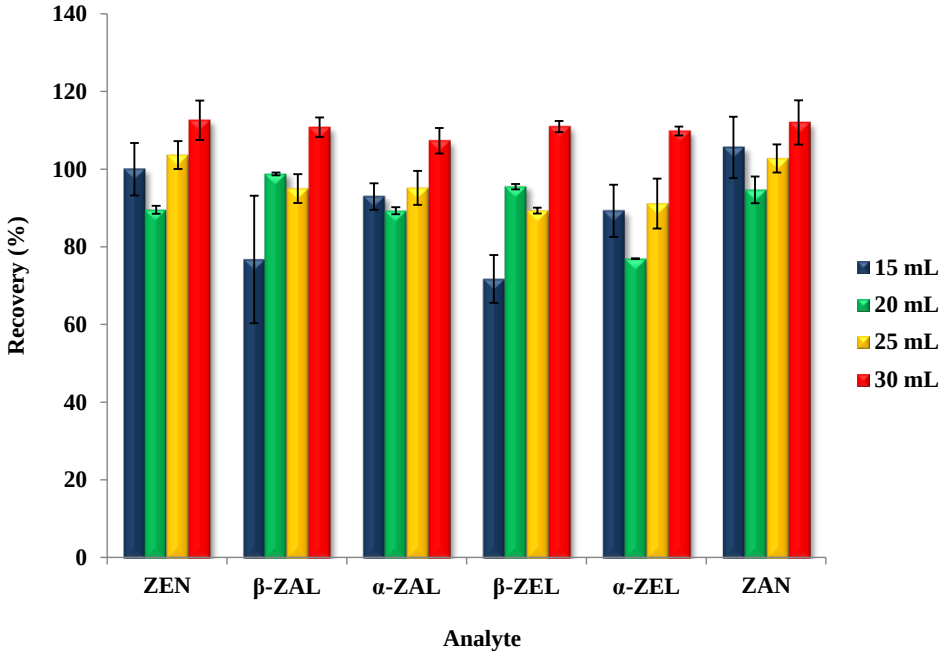


Figure IV.30.- Effect of the volume of the elution solvent on the recovery of the selected analytes after the MWCNTs µ-dSPE procedure. Extraction conditions: 25 mL of Milli-Q water, pH 3, 80 mg of MWCNTs, 1 min of shaking, 30 min of drying using vacuum and MeOH/acetone 50/50 (v/v) as elution solvent. Two extractions (n = 2) were carried out in each case. Concentration of the target analytes in the sample: 5.6 µg/L.

IV.4.5.- Validation of the µ-dSPE in water samples

Firstly, and before carrying out the validation of the methodology in more complex real samples, a recovery study using Milli-Q water was performed in order to avoid the matrix effect. For this purpose, five extractions (n = 5) of this type of water spiked with all the analytes at a concentration of 2.4 µg/L and the IS (17β-E_2-D_5) at 11 µg/L were developed. The results showed that very good extraction efficiency was obtained with recovery values in the range 97-110 % and excellent reproducibility with RSD values below 10 % between replicates.

Once the methodology was validated in Milli-Q water, its applicability in mineral, pond and wastewater samples was also evaluated. With this aim, matrix-matched calibration curves were developed for each sample based on the ratio between the analyte and the IS peak area by injecting seven different levels of concentration (n = 7) in triplicate (see Table IV.13).

Table IV.13.- Matrix-matched calibration data of the selected compounds in water samples.

Analyte	Water sample	Calibration data (n = 7)				LOD[a] (µg/L)	LOQ[b] (µg/L)
		Range of concentration tested (µg/L)	Slope	Intercept	R^2		
ZEN	Mineral	16-500	0.055 ± 0.003	0.707 ± 0.916	0.9974	1.7	5.6
	Pond	40-500	0.046 ± 0.002	-0.109 ± 0.641	0.9978	7.7	26
	Wastewater	25-500	0.036 ± 0.002	0.299 ± 0.491	0.9982	6.6	32
β-ZAL	Mineral	48-500	0.036 ± 0.001	-0.475 ± 0.356	0.9989	10	34
	Pond	160-500	0.021 ± 0.001	-1.557 ± 0.400	0.9973	35	115
	Wastewater	200-400	0.014 ± 0.001	-0.023 ± 0.404	0.9954	58	192
α-ZAL	Mineral	28-500	0.066 ± 0.002	-0.042 ± 0.590	0.9991	6.0	20
	Pond	80-500	0.049 ± 0.002	-0.736 ± 0.491	0.9989	15	51
	Wastewater	80-500	0.033 ± 0.001	0.288 ± 0.428	0.9984	20	68
β-ZEL	Mineral	16-500	0.059 ± 0.003	-0.272 ± 0.750	0.9981	3.3	11
	Pond	80-500	0.038 ± 0.002	-1.193 ± 0.372	0.9988	20	67
	Wastewater	80-500	0.027 ± 0.001	-0.177 ± 0.143	0.9997	21	68
α-ZAL	Mineral	16-500	0.041 ± 0.001	-0.207 ± 0.305	0.9993	4.0	13
	Pond	80-500	0.030 ± 0.001	-0.585 ± 0.479	0.9973	20	66
	Wastewater	80-500	0.022 ± 0.001	-0.157 ± 0.323	0.9980	20	67
ZAN	Mineral	16-500	0.079 ± 0.004	1.228 ± 1.216	0.9977	1.2	3.9
	Pond	40-500	0.063 ± 0.002	0.602 ± 0.567	0.9991	5.6	19
	Wastewater	25-500	0.052 ± 0.003	0.718 ± 0.992	0.9967	3.7	12

R^2: Determination coefficient. [a] Calculated as the concentration associated with a S/N of 3.

[b] Calculated as the concentration associated with a S/N of 10.

The IS, 17β-E_2-D_5, was used in order to correct the possible errors during sample preparation and to improve its reproducibility (Hartmann et al., 2007; Koot et al., 2013). The selection of the IS was based on its similar structure and behaviour to that of the target analytes. R^2 values obtained were higher than 0.9954 in all cases. Instrumental LODs, calculated as the concentration that provided a S/N of 3, ranged between 1.2 and 58 µg/L while LOQs, calculated as the concentration that provided a S/N of 10, were in the range 3.9-192 µg/L.

Afterwards, the reproducibility and extraction efficiency of the methodology were evaluated by the development of the recovery study in each water matrix at two different levels of concentration developing five replicates at each level (n = 5). Besides, a blank matrix of each sample was extracted and spiked at the same concentration level at the end of the extraction procedure (concentrations in the range 0.8-4 µg/L for the low level and in the range 4-8 µg/L for the high level). Relative recovery values were calculated taking into account the possible matrix effect, that is to say, comparing samples spiked at the beginning and at the end of the methodology. The obtained results, shown in Table IV.14, demonstrated the excellent reproducibility as well as the good efficiency of the extraction procedure. Relative recovery values were in the range 101-120 %, 88-119 %, 85-119 %, for mineral, pond and wastewater, respectively, while RSD values were lower than 10 % for all samples. Concerning the LODs of the method, they were in the range 0.01-0.13 µg/L for mineral, 0.08-0.60 µg /L for pond, and 0.05-0.87 µg /L for wastewater whereas the LOQs ranged between 0.05 and 0.42 µg /L for mineral, 0.26 and 2.0 µg/L for pond and 0.18 and 2.9 µg/L for wastewater.

As can be seen in Figure IV.31, in which the extracted ion chromatograms obtained for each analyte when pond water was analysed are shown, no chromatographic interference was found in any case. Similar chromatograms were also obtained for the other two types of water samples without the presence of interferences.

IV.4.6.- Validation of the µ-dSPE in powdered infant milk

In order to extend the application of the methodology to other complex samples of interest, the method was also validated in powdered milk devoted to the infant population in which the contamination with mycotoxins has been previously reported (Meucci et al., 2011; Ok et al., 2014). For this purpose, matrix-matched calibration curves were also prepared

applying the MWCNTs µ-dSPE procedure after a deproteinisation step as described in Section III.8.4. In this case, the sample was previously prepared as indicated by the manufacturer and, once the deproteinisation was developed and centrifugation took place, the suspernant was evaporated in a rotavapor and the residue obtained was redissolved with Milli-Q water up to 25 mL. R^2 obtained were higher than 0.9946 for all analytes and the LODs and LOQs were in the ranges 2.4-41 µg/L and 8.0-137 µg/L, respectively, as shown in Table IV.15.

Table IV.14.- Results of the recovery study (n = 5) of the MWCNTs µ-dSPE-HPLC-MS method for the selected compounds in different water samples at two levels of concentration.

Analyte	Water sample	Level 1[a] (n = 5)	Level 2[b] (n = 5)	LOD_{method}[c] (µg/L)	LOQ_{method}[d] (µg/L)
		Recovery % (RSD, %)	Recovery % (RSD, %)		
ZEN	Mineral	120 (4)	107 (6)	0.02	0.07
	Pond	119 (9)	116 (7)	0.10	0.35
	Wastewater	114 (4)	102 (10)	0.09	0.29
β-ZAL	Mineral	111 (7)	118 (6)	0.13	0.42
	Pond	96 (7)	88 (5)	0.60	2.0
	Wastewater	109 (10)	85 (5)	0.87	2.9
α-ZAL	Mineral	118 (5)	104 (6)	0.08	0.25
	Pond	105 (4)	101 (5)	0.23	0.78
	Wastewater	101 (9)	101 (10)	0.28	0.93
β-ZEL	Mineral	106 (7)	101 (6)	0.05	0.15
	Pond	104 (7)	105 (7)	0.31	1.0
	Wastewater	114 (3)	89 (9)	0.29	0.98
α-ZAL	Mineral	107 (4)	106 (6)	0.05	0.18
	Pond	107 (8)	106 (8)	0.29	0.98
	Wastewater	119 (6)	100 (8)	0.26	0.88
ZAN	Mineral	120 (6)	115 (5)	0.01	0.05
	Pond	109 (8)	109 (6)	0.08	0.26
	Wastewater	98 (3)	94 (10)	0.05	0.18

[a] Concentration of the analytes in the samples: 0.8-4 µg/L. [b] Concentration of the analytes in the samples: 4 µg/L in mineral water and 8 µg/L in the rest of the water samples. [c] Calculated as the concentration associated with a S/N of 3. [d] Calculated as the concentration associated with a S/N of 10.

In addition, a recovery study was also developed at two levels of concentration (5-50 µg/L and 80 µg/L) obtaining, once more, good relative recovery in the range 77-115 %, as can be seen in Table IV.16, with RSDs lower than 10 %. Concerning de limits of the method they were in the range 0.60-9.6 µg/L (LODs) and 2.0-32 µg/L (LOQs).

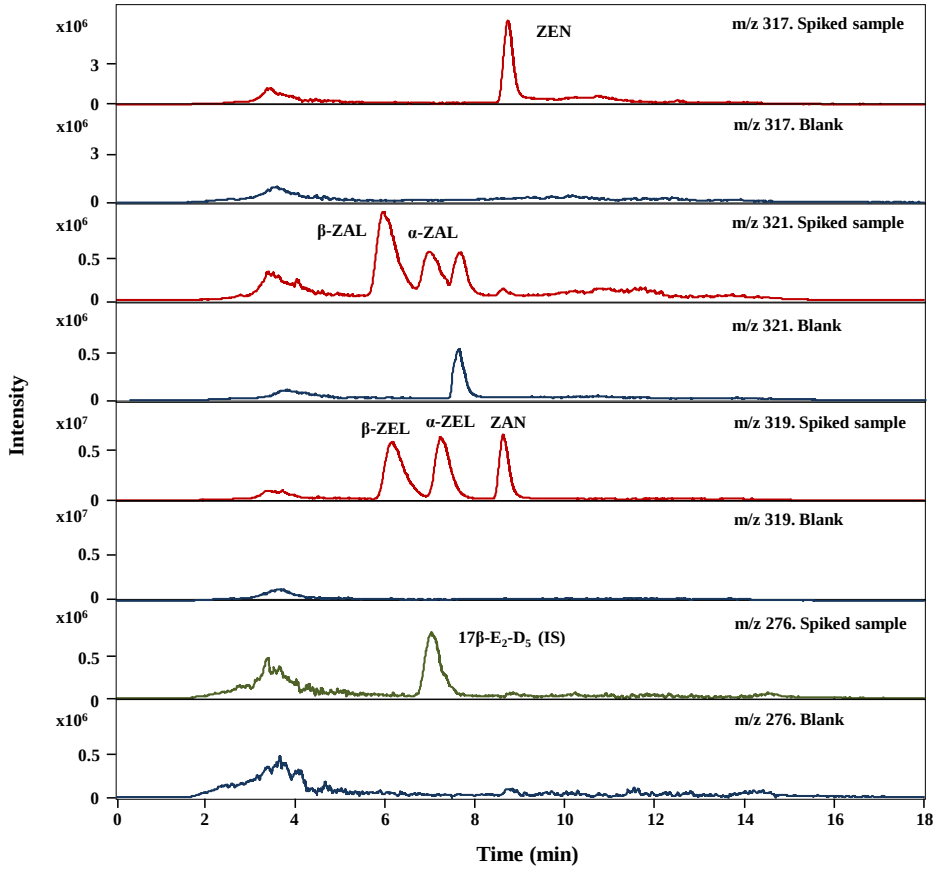


Figure IV.31.- HPLC-MS extracted ion chromatograms of ZEN, ZAN, β-ZAL, α-ZAL, β-ZEL, α-ZEL and 17β-E_2-D_5 (IS) of a blank and a spiked pond water sample after the MWCNTs µ-dSPE procedure. Mobile phase flow rate: 0.4 mL/min. Injection volume: 20 µL. Sample dissolved in 500 µL of 50/50 (v/v) ACN/H_2O. Separation at 30 °C. Concentration in the water sample: 11 µg/L of IS (17β-E_2-D_5), 0.8 µg/L of ZEN, ZAN and α-ZAL and 4 µg/L of β-ZAL, β-ZEL and α-ZEL.

Table IV.15.- Matrix-matched calibration data of the selected compounds in powdered infant formula.

Analyte	Calibration data (n = 7)				LOD[a] (µg/L)	LOQ[b] (µg/L)
	Range of concentration tested (µg/L)	Slope	Intercept	R^2		
ZEN	10-500	0.324 ± 0.018	-0.914 ± 4.707	0.9978	2.4	8.0
β-ZAL	138-500	0.115 ± 0.004	0.741 ± 1.104	0.9994	41	137
α-ZAL	85-500	0.142 ± 0.012	-1.605 ± 3.148	0.9946	24	81
β-ZEL	60-500	0.139 ± 0.006	1.467 ± 1.599	0.9980	16	53
α-ZAL	60-500	0.115 ± 0.008	-0.237 ± 2.087	0.9951	18	59
ZAN	10-500	0.436 ± 0.021	-0.679 ± 5.735	0.9978	3.0	9.9

R^2: Determination coefficient. [a] Calculated as the concentration associated with a S/N of 3.
[b] Calculated as the concentration associated with a S/N of 10.

Table IV.16.- Results of the recovery study (n = 5) of the MWCNTs µ-dSPE-HPLC-MS method for the selected compounds in powdered infant formula at two levels of concentration.

Analyte	Level 1[a] (n = 5)	Level 2[b] (n = 5)	LOD_{method}[c] (µg/L)	LOQ_{method}[d] (µg/L)
	Recovery % (RSD, %)	Recovery % (RSD, %)		
ZEN	77 (8)	113 (5)	0.60	2.0
β-ZAL	81 (9)	109 (10)	9.6	32
α-ZAL	91 (7)	113 (9)	5.2	17
β-ZEL	110 (9)	108 (4)	3.2	11
α-ZAL	107 (10)	114 (6)	3.6	12
ZAN	88 (10)	115 (7)	0.71	2.4

[a] Concentration of the analytes in the samples: 5-50 µg/L. [b] Concentration of the analytes in the samples: 80 µg/L. [c] Calculated as the concentration associated with a S/N of 3. [d] Calculated as the concentration associated with a S/N of 10.

Figure IV.32 shows the extracted ion chromatograms obtained for a spiked and a non-spiked powdered infant milk sample. As can be seen, none of the selected analytes presented interferences as it also happened for the rest of the analysed samples.

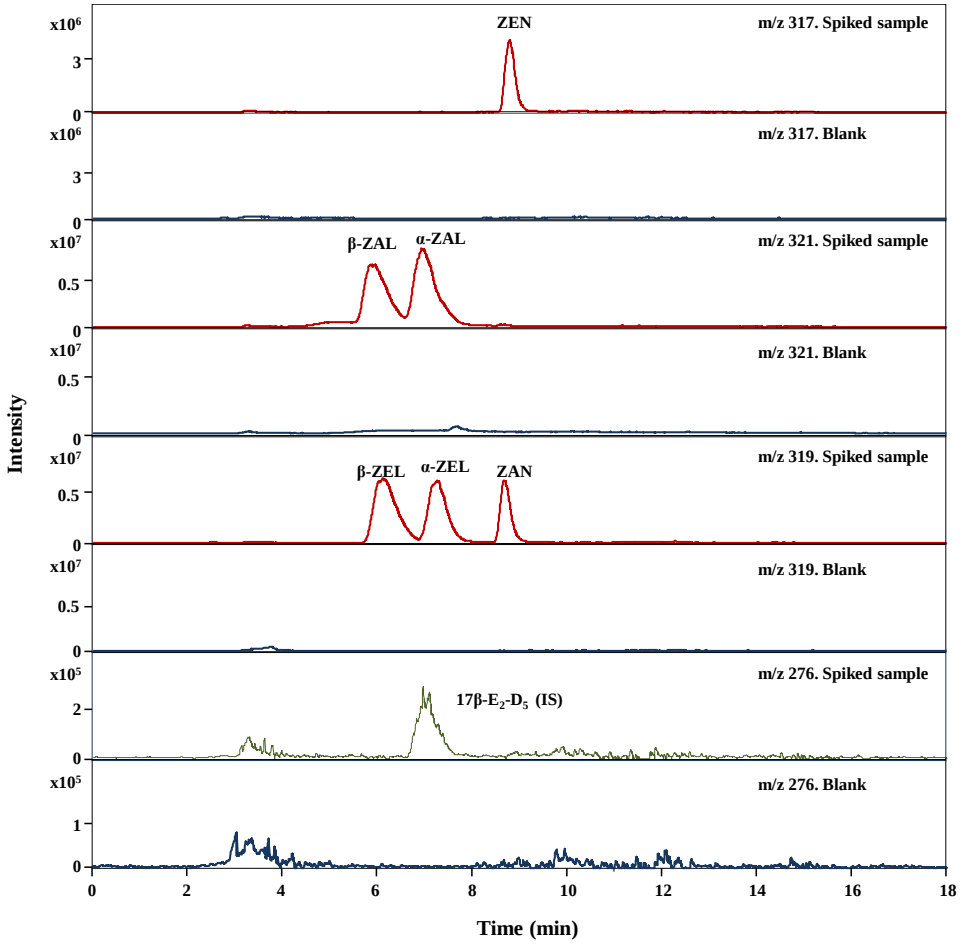


Figure IV.32.- HPLC-MS extracted ion chromatograms of ZEN, ZAN, β-ZAL, α-ZAL, β-ZEL, α-ZEL and 17β-E_2-D_5 (IS) of a blank and a spiked powdered infant milk sample after the MWCNTs μ-dSPE procedure. Mobile phase flow rate: 0.4 mL/min. Injection volume: 20 μL. Sample dissolved in 500 μL of 50/50 (v/v) ACN/H_2O. Separation at 30 °C. Concentration in the water sample: 90 μg/L of IS (17β-E_2-D_5) and 14 μg/L of ZEN and ZAN, 83 μg/L of β-ZEL and α-ZEL, 117 μg/L of α-ZAL and 191 μg/L of α-ZAL.

IV.4.7.- Comparison with other methods

As it was indicated at the beginning of this section, the application of MWCNTs for the analysis of mycoestrogens had only been carried out once before the development of this

work (Ying et al., 2013). In that case, the application was developed in cereal samples and, even though a higher amount of MWCNTs was necessary to achieve an efficient extraction (100 mg), the LODs were similar to those obtained in this work. In addition, and after developing an exhaustive revision of the methodologies developed for the determination of these contaminants in water (D'Orazio et al., 2014; González-Sálamo et al., 2015; Gromadzka et al., 2009; Hartmann et al., 2007; Laganà et al., 2001; Lundgren and Novak, 2009) and infant milk formulae (Desmarchelie et al., 2014; Meucci et al., 2011; Ok et al., 2014; Zhang et al., 2013), it is possible to conclude that the LOQs obtained in such cases were in the same order of magnitude than those obtained in our work, except for some manuscripts in which wastewater was analysed (Gromadzka et al., 2009; Hartmann et al., 2007; Kolpin et al., 2014). In these cases the LOQs were lower because the sample volume was between 20 and 100 times higher than the one applied in our case. Therefore, the application of HPLC-IT-MS has allowed obtaining better results than other conventional systems such as DAD or FD, previously used. Indeed, similar values of sensitivity as other sensitive analysers such as QqQ or triple quadrupole/linear ion trap (Qtrap) were obtained in this case using an IT, which shows the good sensitivity and the potential of the developed methodology.

IV.4.8.- Conclusions

From the data obtained in this section the following conclusions can be drawn:

- A methodology based on a µ-dSPE procedure using MWCNTs as sorbent followed by the determination by HPLC-MS/MS has been proposed for the analysis of the selected compounds in mineral, pond and wastewater as well as powdered infant milk.
- The chromatographic separation and determination by HPLC-MS/MS of the six mycotoxins with known oestrogenic activity was successfully carried out in less than 10 minutes.
- The µ-dSPE procedure was optimised using a step by step approach with Milli-Q water. The best results were achieved using 50 mL of sample at pH 3, 80 mg of MWCNTs as extraction sorbent, 1 min of shaking, 30 min of drying using vacuum and 30 mL of MeOH/acetone 50/50 (v/v) as elution solvent.
- The linearity of the methodology was evaluated by the preparation of matrix-matched calibration curves obtaining R^2 higher to 0.9946 in all cases.
- The extraction efficiency and reproducibility of the methodology was evaluated by recovery

studies obtaining excellent results with recovery between 77-120 % and RSDs below 10 % for all analytes.

- This methodology constitutes the first application of MWCNTs as sorbent for the extraction of the selected analytes from environmental water samples and infant milk using dSPE and, in general, the second time in which MWCNTs were used for the extraction of mycotoxins.
- The proposed methodology could be used for the routine analysis of the selected compounds in the studied matrices, although it could also be applied to other types of samples.
- This work demonstrates that CNTs can be successfully used as selective sorbents, also in the dSPE modality, for the analysis of this group of mycotoxins in complex samples as an alternative to their use in conventional SPE.

IV.5.- Development of a multiclass analytical method for the determination of natural, synthetic, myco- and phytoestrogens in milk and dairy products using ultra-high-performance liquid chromatography-tandem mass spectrometry

In this section, a simple, cheap and fast methodology is proposed for the determination of a group of twenty four oestrogenic compounds potentially occurring in milk and dairy products with different physico-chemical properties and biological activity: four natural (E_3, 17α-E_2, 17β-E_2 and E_1) and four synthetic (EE_2, DES, DS and HEX) oestrogens, six mycotoxins (α-ZAL, α-ZEL, β-ZAL, β-ZEL, ZAN and ZEN) and ten phytoestrogens (daidzein, enterodiol, glycitein, enterolactone, genistein, formononetin, prunetin, biochanin A, equol and coumestrol). Extraction was carried out using the QuEChERS method while separation, determination and quantification of the target analytes were achieved by UHPLC coupled to QqQ-MS/MS using an ESI. The methodology was validated in six dairy product samples with relevant interest for the population including whole cow milk, yogurt, skimmed and whole cow cheese and goat and cow kefir. Finally, the developed procedure was applied for the investigation of the presence of oestrogenic compounds in milk, yogurt, cheese and kefir samples from the Czech and Spanish retail markets finding the presence of biochanin A, daidzein, equol, formononetin, genistein, glycitein, coumestrol, enterolactone and E_1 in some of the studied samples.

IV.5.1.- Background

One of the sample preparation procedures most frequently used worldwide, though mainly applied for pesticide residue analysis in vegetables and fruit matrices, is the so-called QuEChERS method (Anastassiades et al., 2003). This extraction procedure is commonly used in official laboratories that require multiresidue methods in order to maximise sample throughput by minimising sample preparation, to ensure short analysis time and to carry out an effective control. Such approach is easily adaptable and different versions have been independently developed and applied in monitoring laboratories, mainly in combination with GC and LC coupled to MS. Consequently, its excellent and inherent advantages combined with both separation techniques have brought about its extremely high popularity. Apart from its application for pesticide residue analysis, the method has also been successfully applied to the extraction of other groups of compounds such as PAHs (Naícher-Mestre et al., 2014), pharmaceuticals (Peysson and Vulliet, 2013), PCBs (Norli et al., 2011), etc., also from matrices

different than fruit or vegetables like mussels (Li et al., 2012a; Madureira et al., 2014), sewage sludge (Peysson and Vulliet, 2013) or fish (Li et al., 2012a), among others, with outstanding results.

As previously indicated, myco-, natural and synthetic oestrogens constitute a group of compounds with oestrogenic activity of great relevance. Another group of importance that should be added to them is that known as phytoestrogens which, as it occurs with mycoestrogens, have a natural origin since they are secondary metabolites of plants. In the same way to the rest of exoestrogens, these analytes can mimic the activity of endoestrogens but can also act as anti-oestrogens bringing about diverse endocrine disorders (Křišová et al., 2011; Kuhnle et al., 2008), though the appearance of cancer has also been suggested (Kuhnle et al., 2008). As a result of their vegetable origin, they have been widely evaluated in plant based food (Kuhnle et al., 2009; Mulligan et al., 2012) but they can also appear in products of animal origin like milk and dairy as a consequence of the feeding of livestock with vegetable species that have a high content of them. However, the studies in these kinds of matrices are reduced which produce an underestimation of the intake of such compounds.

As a result and, as previously commented, there is a great interest in the determination of oestrogenic compounds in milk and dairy products and the development of efficient methodologies is of special concern for the scientific community. However, and probably due to the complexity of these samples, the analysis of such compounds has not been so widely tackled as for other simpler matrices like water samples (Lafleur and Schug, 2011). In fact, although the development of methodologies for the analysis of specific groups of oestrogens in milk samples have been widely studied (Adamusova et al., 2014), the development of multiresidue analysis in this matrix is less extended. In this sense, and before the development of this work, only SPE using graphite carbon (Capriotti et al., 2015), HF-LPME (D'Orazio et al., 2016b) and DLLME (D'Orazio et al., 2015) have been applied for the simultaneous extraction of natural oestrogens and mycotoxins as well as some synthetic compounds, while SPE using hydrophilic-lipophilic-balanced (HLB) cartridges has been used for the extraction of some myco- and phyto oestrogens together with other organic pollutants (Wielogórska et al., 2015). Besides, only some phyto-, natural and synthetic oestrogens have been analysed in few occasions in cheese samples (cow cheese) and yogurt using SLE (Křišová et al., 2011; Li et al., 2014) and SLE combined with SPE (Cavaliere et al., 2015), while the analysis of the presence of these groups of compounds in kefir samples had not been reported in the bibliography until

the development of this work.

Regarding the specific application of the QuEChERS method for the analysis of oestrogenic compounds, up to now, and to the best of our knowledge, it has only been used for the extraction of some oestrogenic compounds from milk or dairy products such as yogurt in very few occasions (Ehling and Reddy, 2013; Jia et al., 2014; Rubert et al., 2014). However, none of them have determined such analytes in cheese or kefir samples. For this reason, the evaluation of a new procedure based on the extraction of different groups of oestrogenic compounds using the QuEChERS method followed with their separation and analysis by a sensitive and selective UHPLC-QqQ-MS/MS system constitutes a challenge of great interest.

IV.5.2.- Specific objectives

In view of the foregoing, the following specific objectives have been established for this work:

- The determination of a group of twenty four oestrogenic compounds with potential occurrence in milk and dairy products, including four natural (17α-E_2, 17β-E_2, E_1 and E_3) and four synthetic (HEX, EE_2, DES and DS) oestrogens as well as six mycoestrogens (ZAN, ZEN, α-ZAL, β-ZAL, α-ZEL and β-ZEL) and ten phytoestrogens (daidzein, enterodiol, glycitein, enterolactone, genistein, formononetin, prunetin, biochanin A, equol and coumestrol) using a QuEChERS-UHPLC-MS/MS method.
- The optimisation of UHPLC separation as well as ionisation and MS/MS fragmentation conditions using a QqQ as analyser and an ESI.
- The optimisation of the dSPE clean up step of the QuEChERS method with the aim of achieving the highest extraction efficiency and minimising the amount of co-extracted material to reduce matrix effects.
- The validation of the whole methodology in terms of linearity, recovery and reproducibility at different concentration levels, as well as the obtaining of the LODs and LOQs of the method for milk, yogurt, whole and skimmed cheese as well as kefir with cow and goat origin.
- The application of the proposed methodology for the monitoring of the target oestrogenic compounds in different samples commercially available from Czech and Spanish supermarkets.

IV.5.3.- UHPLC-MS/MS method

Initially, working conditions for the simultaneous analysis of the twenty four selected compounds were established using an Acquity UPLC system coupled to MS Xevo TQ-S QqQ detector. As a starting point, optimisation of MS conditions was carried out through the direct infusion of each analyte separately in a mixture of MeOH/H_2O 50/50 (v/v) at 1 mg/L, using the automatic tunning of MS/MS parameters. The deprotonated $[M-H]^-$ or protonate $[M-H]^+$ molecule, depending on each analyte, were determined and selected as the precursor ions. The most intense transition was used for quantification, whereas the second transition was used for confirmation. Optimised MRM transitions as well as the values of cone voltage and collision energy of the target analytes and ISs are listed in Table IV.17.

Afterwards, an exhaustive study of the mobile phase used for the correct separation of the analytes was developed. With this aim, an Acquity UPLC BEH C_{18} column (100 mm × 2.1 mm, 1.7 µm) and different mixtures of ACN/H_2O and MeOH/H_2O were tested. However, and although the separation of the different epimers (i.e. 17α-E_2, 17β-E_2, α-ZAL, β-ZAL, α-ZEL and β-ZEL) was achieved (they show the same mass spectra), good sensitivity was only obtained for the group of phyto- and mycoestrogens but not for natural and synthetic oestrogens. Taking this fact into account, and that the use of acidic (Malekinejad et al., 2006) and basic (Farke et al., 2011) mobile phases had also been previously proposed in the literature, the addition of formic or acetic acid, ammonium hydroxide and ammonium acetate to the aqueous phase was tested. As can be seen in Figure IV.33, the addition of ammonium hydroxide to the water phase (A) significantly improved the deprotonation of the very weak acidic natural and synthetic oestrogens. The signals of the analytes were 4-5 times higher compared to the same mobile phase without additives for both groups of compounds. Regarding the organic phase (B), the highest sensitivity for these oestrogens was achieved when the mixture MeOH/ACN 50/50 (v/v) was used. In the case of myco- and phytoestrogens, a decrease of their signals was observed when the mobile phase additives were tested. Based on the above mentioned results, two different phases were finally used for the analyses: (A) 2 mM ammonium hydroxide in water and (B) MeOH/ACN 50/50 (v/v) for natural and synthetic oestrogens, and (A) water and (B) MeOH for myco- and phytoestrogens analysis. Under these conditions, a good separation of the selected compounds in terms of efficiency and analysis time ($t_{analysis}$< 5 min) was achieved using the gradients described in Sections III.5.3 and III.5.4 since this work was carried out in two different laboratories.

Table IV.17.- QqQ-MS/MS parameters of the selected compounds and ISs.

Analyte	MW (g/mol)	MRM (m/z)	Cone voltage (V)	Collision energy (V)	Analyte	MW (g/mol)	MRM (m/z)	Cone voltage (V)	Collision energy (V)
Daidzein	254.2	253.0 > 208.0	45	35	ZAN	320.4	321.2 > 303.2	36	12
		253.0 > 223.0	45	40					
Enterodiol	302.4	301.1 > 106.3	62	32	ZEN	318.4	317.2 > 130.0	66	28
		301.1 > 271.1	76	22			317.2 > 175.0	66	26
Glycitein	284.3	283.1 > 240.0	62	26	EE_2	296.4	295.2 > 145.0	60	34
		283.1 > 268.0	62	18			295.2 > 159.0	60	36
Enterolactone	298.3	297.2 > 107.0	70	24	DES	268.4	267.1 > 237.1	68	28
		297.2 > 253.1	70	20			267.1 > 251.1	68	24
Genistein	270.2	269.2 > 133.0	72	32	DS	266.3	265.3 > 93.0	26	24
		269.2 > 159.0	72	28			265.3 > 235.3	26	20
Formononetin	268.3	252.2 > 223.2	80	22	HEX	270.4	269.3 > 119.1	22	38
		267.2 > 252.0	84	20			269.3 > 134.1	22	14
Prunetin	284.3	285.1 > 91.0	60	40	E_3	288.4	287.3 > 145.2	72	36
		285.1 > 167.1	60	30			287.3 > 183.1	72	38

Table IV.17.- (Continued).

Analyte	MW (g/mol)	MRM (m/z)	Cone voltage (V)	Collision energy (V)	Analyte	MW (g/mol)	MRM (m/z)	Cone voltage (V)	Collision energy (V)
Biochanin A	284.3	268.2 > 239.2	96	22	17β-E_2	272.4	271.3 > 145.1	96	38
		283.2 > 268.0	96	22			271.3 > 183.1	96	34
Equol	242.3	241.4 > 135.0	50	18	17α-E_2	272.4	271.3 > 145.1	96	34
		241.4 > 118.6	50	16			271.3 > 183.1	96	36
Coumestrol	268.2	267.1 > 211.0	50	28	E_1	270.4	269.3 > 145.1	70	32
		267.1 > 239.0	50	24			269.3 > 159.0	70	32
β-ZAL	322.4	321.2 > 277.2	66	22	Chrysin (IS)	254.1	253.1 > 143	66	30
		321.2 > 303.2	66	20			253.1 > 209	66	24
β-ZEL	320.4	319.2 > 159.9	62	32	$^{13}C_{18}$-ZEN (IS)	336.3	335.3 > 185	24	26
		319.2 > 275.1	62	30			335.3 > 140	24	32
α-ZAL	322.4	321.1 > 277.2	66	22	17β-E_2-D_5 (IS)	277.4	276.1 > 147.1	78	42
		321.1 > 303.1	66	22			276.1 > 187.1	78	40
α-ZEL	320.4	319.2 > 159.9	62	32	β-ZAL-D_5 (IS)	327.4	326.2 > 282.2	58	24
		319.2 > 275.1	62	30			326.2 > 308.2	58	22

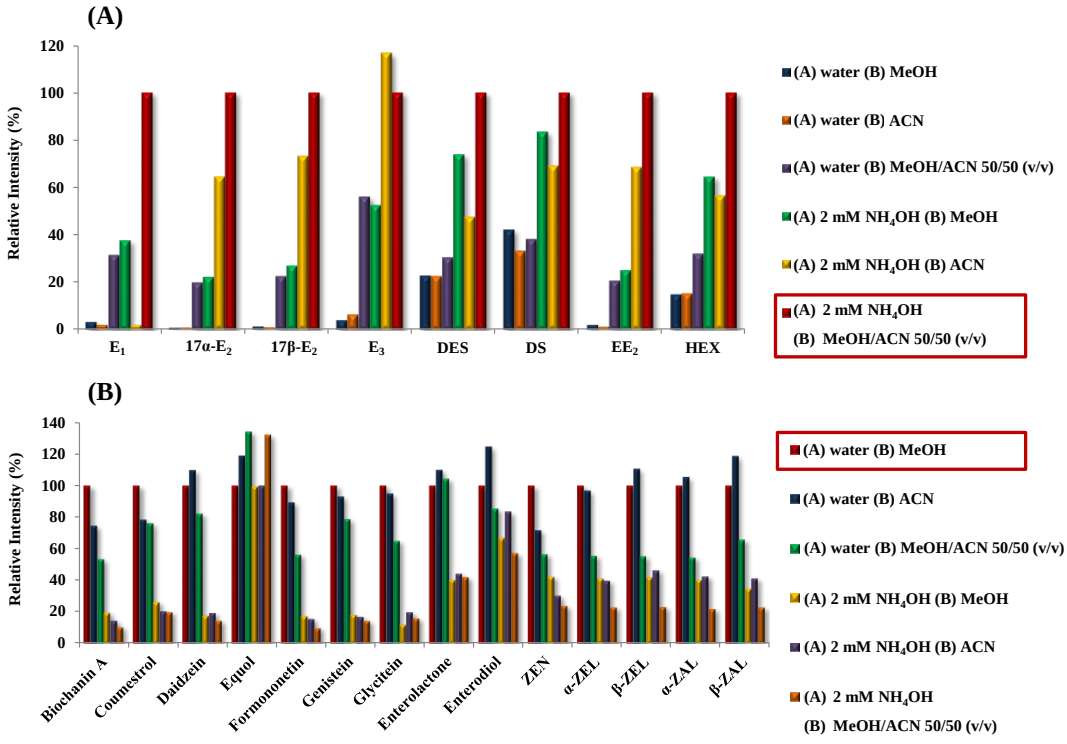


Figure IV.33.- Comparison between the signal intensities obtained when the composition of the mobile phase was varied.

In addition, repeatability and calibration studies were carried out. For repeatability evaluation, six consecutive injections (n = 6) of a mixture of the analytes at three levels of concentration 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 %, respectively, and between days, with RSDs below 0.5 % and 16 %, respectively. Regarding the calibration study, seven concentration levels (n = 7) were injected in quadruplicate. Calibration curves were based on the ratio of each analyte and the IS peak areas used in each case. R^2 values were higher than 0.9968 for all analytes in the range of concentration tested.

IV.5.4.- Application of the QuEChERS method

The methodology applied for the extraction of the selected oestrogenic compounds was based on a previous work in which perfluoroalkyl substances, brominated flame retardants and

their metabolites were extracted from breast milk and powder infant formulae (Lanková et al., 2013). Due to the complexity of the matrices that were evaluated, special attention was focused on the dSPE clean up step.

In this sense, several clean up sorbents which included not only conventional C_{18} but also Z-Sep+ and their mixture C_{18}/Z-Sep+ 50/50 (w/w) were examined to remove the co-extracted lipids and other lipophilic compounds from the crude ACN extract. Z-Sep+ is a material constituted by hybrid particles containing a silica carrier coated with zirconium dioxide and C_{18} groups which has been recently introduced as clean up sorbent for the effective removal of lipids from fatty biological samples (Rajski et al., 2013). In addition to hydrophobic interactions, Lewis acid-base interactions between the sample components and this sorbent also take place, which improves the effectiveness of the clean up step. It has been previously applied in the analysis of different types of matrices with excellent results including the multiresidue determination of contaminants in fish (Sapozhnikova and Lehotay, 2013) or pesticide analysis in high fat commodities like avocadoes (Rajski et al., 2013).

The best results were obtained when C_{18} was used, with recovery values in the range 91-112 %. However, in the case of Z-Sep+ and C_{18}/Z-Sep+ mixtures, recovery was lower (around 20-30 % lower compared to C_{18}), due to the retention of the analytes onto the sorbent. That is why this material was excluded and C_{18} was finally used as clean up sorbent in this study.

IV.5.5.- Validation of the methodology

IV.5.5.1.- Evaluation of the matrix effect

To assess the efficiency of the clean up step based on C_{18} dSPE, matrix effects for oestrogens were determined using the Matuszewski method (Matuszewski et al., 2003). The matrix effect was calculated as the percentage of the ratio of the peak area of the analytes in the matrix-matched standards and the area for solvent prepared standards at different levels of concentration.

In general, matrix effects for most natural/synthetic oestrogens and mycoestrogens in milk and yogurt samples were in the range of 28-104 % whereas for phytoestrogens the values in these matrices were in average 90 and 82 %, respectively. In the case of cheese and kefir samples, as it is shown in Table IV.18, results varied between samples and analytes although

Table IV.18.- 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

Table IV.18.- (Continued).

Analyte	Type of matrix	ME[a), b)] (%)	RSD (%)	Analyte	Type of matrix	ME[a), b)] (%)	RSD (%)
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 (n = 15) at three different concentration levels.

[b)] Calculated following the Matuszewski method (Matuszewski et al., 2003).

values lower than 80 % were found for the majority of cases with RSDs below 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 percentages were higher than 80 %. These results show a clear ion suppression effect for almost all cases, except for phytoestrogens in milk and yogurt samples.

Based on these results, it was concluded that compensation of the matrix effects through the use of a matrix-matched calibration is needed for the accurate quantification of natural/synthetic oestrogens and mycoestrogens in all matrices as well as for phytoestrogens in cheese and kefir, while the use of solvent calibration is feasible for the analysis of this last group in milk and yogurt samples.

IV.5.5.2.- Matrix-matched calibration and recovery study

The QuEChERS-UHPLC-MS/MS method was also validated by developing linearity, recovery and reproducibility studies. With this aim, and taking into account the results obtained from the matrix effect evaluation, matrix-matched calibration curves were developed in each case, except for phytoestrogens in milk and yogurt for which solvent calibration curves were adequate. Samples were spiked at the end of the process and the curves were prepared based on the ratio between the analyte and the IS peak areas chosen for each group of oestrogens, by injecting different levels of concentration in quadruplicate.

For milk and yogurt samples, calibration curves were developed injecting eight different concentration levels in the blank matrix extract in the range of 0.5-100 µg/L (only for natural/synthetic oestrogens and mycoestrogens). For the quantification of phytoestrogens, solvent calibration (MeOH) was also prepared at the same concentration levels as matrix-matched calibration points. LOQs were estimated as the lowest calibration standard for which the S/N was higher than 10 for the first MRM transition and S/N was higher than 3 for the second MRM transition. As it is shown in Table IV.19, LOQs of the target analytes ranged from 0.02 to 0.6 µg/L for milk (0.20-6.0 µg/kg in dw sample) and 0.02 to 0.90 µg/kg for yogurt (0.20-6.0 µg/kg in dw sample), which are slightly lower than those reported in recently published studies, which have employed the QuEChERS method for the extraction of synthetic oestrogens from milk powder (Ehling and Reddy, 2013) or mycoestrogens from human breast milk (Rubert et al., 2014) and dairy products (Jia et al., 2014). In addition, LOQs were comparable to the ones obtained by similar studies recently published for the determination of natural oestrogens in milk samples (Capriotti et al., 2015; Cavaliere et al., 2015).

Table IV.19.- Results of the recovery study (n = 6) and LOQs of the QuEChERS-UHPLC-MS/MS method for the selected compounds in milk and yogurt matrices at two levels of concentration.

Analyte	Type of matrix	Level 1[a] (n = 6) Recovery % (RSD, %)	Level 2[b] (n = 6) Recovery % (RSD, %)	LOQ_{method}[c), d)]	Analyte	Type of matrix	Level 1[a] (n = 6) Recovery % (RSD, %)	Level 2[b] (n = 6) Recovery % (RSD, %)	LOQ_{method}[c), d)]
Daidzein	Milk	111 (9)	75 (9)	0.02	α-ZAL	Milk	105 (9)	99 (12)	0.02
	Yogurt	73 (4)	71 (3)	0.02		Yogurt	111 (6)	106 (11)	0.02
Enterodiol	Milk	106 (8)	88 (10)	0.02	α-ZEL	Milk	103 (20)	85 (12)	0.02
	Yogurt	76 (5)	74 (6)	0.02		Yogurt	119 (12)	87 (4)	0.02
Glycitein	Milk	112 (7)	80 (6)	0.02	ZEN	Milk	92 (20)	85 (13)	0.02
	Yogurt	74 (4)	74 (3)	0.02		Yogurt	120 (5)	107 (3)	0.02
Enterolactone	Milk	106 (6)	85 (10)	0.02	EE_2	Milk	104 (12)	112 (10)	0.60
	Yogurt	119 (4)	93 (3)	0.02		Yogurt	101 (14)	109 (8)	0.90
Genistein	Milk	95 (8)	70 (10)	0.02	DES	Milk	114 (13)	100 (14)	0.15
	Yogurt	91 (6)	82 (1)	0.02		Yogurt	108 (5)	119 (9)	0.20
Formononetin	Milk	118 (7)	83 (6)	0.02	DS	Milk	121 (8)	119 (5)	0.02
	Yogurt	81 (2)	74 (2)	0.02		Yogurt	120 (5)	105 (9)	0.02
Biochanin A	Milk	105 (2)	84 (6)	0.02	HEX	Milk	118 (6)	120 (8)	0.02
	Yogurt	97 (3)	93 (6)	0.02		Yogurt	120 (4)	110 (7)	0.02
Equol	Milk	97 (16)	96 (19)	0.08	E_3	Milk	114 (17)	119 (17)	0.20
	Yogurt	79 (3)	70 (7)	0.10		Yogurt	81 (5)	103 (17)	0.20
Coumestrol	Milk	120 (7)	85 (4)	0.02	17β-E_2	Milk	120 (5)	120 (7)	0.03
	Yogurt	77 (3)	72 (7)	0.02		Yogurt	103 (5)	110 (9)	0.05
β-ZAL	Milk	119 (6)	106 (11)	0.02	17α-E_2	Milk	118 (19)	114 (8)	0.03
	Yogurt	102 (13)	70 (6)	0.02		Yogurt	120 (6)	100 (9)	0.05
β-ZEL	Milk	95 (20)	76 (11)	0.02	E_1	Milk	94 (20)	94 (18)	0.02
	Yogurt	118 (10)	96 (4)	0.02		Yogurt	108 (13)	106 (8)	0.02

[a)] Concentrations of the analytes in the samples: 0.2 μg/L of milk (0.2 μg/kg of yogurt) for natural, synthetic and mycoestrogens and 1 μg/L of milk (1 μg/kg of yogurt) for phytoestrogens and EE_2. [b)] Concentrations of the analytes in the samples: 1 μg/L of milk (1 μg/kg of yogurt) for natural, synthetic and mycoestrogens and 10 μg/L of milk (10 μg/kg of yogurt) for phytoestrogens and 5 μg/L of milk (5 μg/kg of yogurt) for EE_2. [c)] Defined as the lowest calibration concentration which provided a S/N higher than 10 for the quantification transition and at least 3 for the confirmation transition (if it was available). [d)] μg/kg for yogurt samples and μg/L for milk samples.

Regarding kefir and cheese samples, matrix-matched calibration data was obtained injecting seven different concentration levels in the range of 0.5-750 µg/L (see Table IV.20). R^2 values were higher than 0.9905 in all cases, which demonstrates the linearity of the methodology in the range of concentration studied. LOQs of the method, defined as the lowest matrix-matched calibration concentration which provided a S/N higher than 10 for the quantification transition and at least 3 for the confirmation transition (if it was available), were in the range 0.025-0.25 µg/kg for skimmed cheese, 0.050-0.50 µg/kg for whole cheese and in the ranges 0.050-2.5 µg/kg and 0.050-0.50 µg/kg for cow and goat kefir, respectively (see Table IV.21).

Afterwards, experiments were performed with the aim of evaluating the reproducibility and recovery efficiency of the whole methodology. Concerning milk and yogurt samples, six spiked bovine milk (3.5 % fat content) and six natural yogurt samples (3 % fat content), were analysed. The spiked concentrations for milk were 0.2 and 1 µg/L for natural, synthetic and mycoestrogens and 1 and 10 µg/L for phytoestrogens. For yogurt, concentrations were the same as for milk, 0.2 and 1 µg/kg for natural, synthetic and mycoestrogens and 1 and 10 µg/kg for phytoestrogens. As a result of the significantly lower signal intensity of EE_2 compared to other synthetic oestrogens, the spiked concentrations were higher for this analyte: 1 µg/L of milk (1 µg/kg of yogurt) and 5 µg/L of milk (5 µg/kg of yogurt). The whole optimised procedure was validated by employing chrysin as IS for natural, synthetic and phytoestrogens and $^{13}C_{18}$-ZEN for mycoestrogens. The recovery values of all target compounds were in the range of 70-121 % with RSDs below 20 % (see Table IV.19).

Concerning kefir and cheese samples, recovery studies were developed at three levels of concentration with five replicate extractions at each level and matrix, using β-ZAL-D_5 as IS for mycoestrogens and 17β-E_2-D_5 for the rest of compounds. In addition, a blank of each type of sample was also extracted and spiked at the same concentration level at the end of the extraction procedure. Relative recovery values (see Table IV.21) 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 15 % for all samples, demonstrating the excellent reproducibility as well as the good efficiency of the extraction procedure developed.

Table IV.20.- Matrix-matched calibration data of the selected compounds in the different matrices.

Analyte	Type of matrix	Calibration data (n = 7)			
		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
	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
	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
	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
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
	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
	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
	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
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
	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
	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
	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
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
	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
	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
	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
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
	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
	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
	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
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
	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
	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
	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

Table IV.20.- (Continued).

Analyte	Type of matrix	Calibration data (n = 7)			
		Range of concentration studied (µg/L)	Slope	Intercept	R^2
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
	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
	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
	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
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
	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
	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
	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
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
	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
	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
	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
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
	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
	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
	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
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
	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
	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
	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
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	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	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	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

Table IV.20.- (Continued).

Analyte	Type of matrix	Calibration data (n = 7)			
		Range of concentration studied (µg/L)	Slope	Intercept	R^2
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	$1.51 \cdot 10^{-2} \pm 4.03 \cdot 10^{-4}$	$7.51 \cdot 10^{-2} \pm 1.28 \cdot 10^{-1}$	0.9995
	Cow kefir	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	$6.20 \cdot 10^{-2} \pm 2.27 \cdot 10^{-3}$	$5.97 \cdot 10^{-2} \pm 7.75 \cdot 10^{-1}$	0.9990
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	$1.90 \cdot 10^{-3} \pm 8.65 \cdot 10^{-5}$	$-1.75 \cdot 10^{-2} \pm 2.64 \cdot 10^{-2}$	0.9984
	Cow kefir	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	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
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	$1.42 \cdot 10^{-3} \pm 1.16 \cdot 10^{-4}$	$-3.11 \cdot 10^{-2} \pm 3.42 \cdot 10^{-2}$	0.9950
	Cow kefir	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	$2.07 \cdot 10^{-3} \pm 9.61 \cdot 10^{-5}$	$-3.18 \cdot 10^{-2} \pm 2.96 \cdot 10^{-2}$	0.9984
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	$7.20 \cdot 10^{-3} \pm 4.93 \cdot 10^{-4}$	$-1.23 \cdot 10^{-1} \pm 1.46 \cdot 10^{-1}$	0.9965
	Cow kefir	5-750	$7.12 \cdot 10^{-3} \pm 1.79 \cdot 10^{-4}$	$-7.76 \cdot 10^{-2} \pm 5.66 \cdot 10^{-2}$	0.9995
	Goat kefir	5-750	$1.26 \cdot 10^{-2} \pm 8.31 \cdot 10^{-4}$	$-1.25 \cdot 10^{-1} \pm 2.56 \cdot 10^{-1}$	0.9967
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	$1.02 \cdot 10^{-2} \pm 7.38 \cdot 10^{-4}$	$-2.21 \cdot 10^{-1} \pm 2.34 \cdot 10^{-1}$	0.9960
	Cow kefir	5-750	$9.03 \cdot 10^{-3} \pm 2.55 \cdot 10^{-4}$	$-5.62 \cdot 10^{-2} \pm 8.07 \cdot 10^{-2}$	0.9994
	Goat kefir	5-750	$1.35 \cdot 10^{-2} \pm 9.39 \cdot 10^{-4}$	$-1.77 \cdot 10^{-1} \pm 2.98 \cdot 10^{-1}$	0.9963
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	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	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	$1.73 \cdot 10^{-2} \pm 1.40 \cdot 10^{-3}$	$-1.70 \cdot 10^{-1} \pm 4.90 \cdot 10^{-1}$	0.9951

Table IV.20.- (Continued).

Analyte	Type of matrix	Calibration data (n = 7)			
		Range of concentration studied (µg/L)	Slope	Intercept	R^2
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	$3.69 \cdot 10^{-3} \pm 3.71 \cdot 10^{-4}$	$-7.39 \cdot 10^{-2} \pm 1.10 \cdot 10^{-1}$	0.9924
	Cow kefir	5-750	$3.16 \cdot 10^{-3} \pm 8.76 \cdot 10^{-5}$	$-2.35 \cdot 10^{-2} \pm 3.06 \cdot 10^{-2}$	0.9994
	Goat kefir	5-750	$2.63 \cdot 10^{-3} \pm 1.45 \cdot 10^{-4}$	$-1.75 \cdot 10^{-2} \pm 4.28 \cdot 10^{-2}$	0.9977
17β-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	$1.66 \cdot 10^{-3} \pm 1.61 \cdot 10^{-4}$	$-4.20 \cdot 10^{-2} \pm 5.82 \cdot 10^{-2}$	0.9930
	Cow kefir	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	$2.12 \cdot 10^{-3} \pm 1.44 \cdot 10^{-4}$	$-4.45 \cdot 10^{-2} \pm 4.55 \cdot 10^{-2}$	0.9965
17α-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	$2.02 \cdot 10^{-3} \pm 1.87 \cdot 10^{-4}$	$-5.54 \cdot 10^{-2} \pm 5.54 \cdot 10^{-2}$	0.9936
	Cow kefir	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	$2.48 \cdot 10^{-3} \pm 1.29 \cdot 10^{-4}$	$-3.16 \cdot 10^{-2} \pm 3.98 \cdot 10^{-2}$	0.9980
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	$1.03 \cdot 10^{-2} \pm 9.87 \cdot 10^{-4}$	$-2.40 \cdot 10^{-1} \pm 3.13 \cdot 10^{-1}$	0.9931
	Cow kefir	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	$1.07 \cdot 10^{-2} \pm 5.05 \cdot 10^{-4}$	$-6.92 \cdot 10^{-2} \pm 1.49 \cdot 10^{-1}$	0.9983

R^2: Determination coefficient.

Table IV.21.- Results of the recovery study (n = 5) of the QuEChERS-UHPLC-MS/MS method for the selected compounds in the cheese and kefir matrices at three levels of concentration.

Analyte	Type of matrix	Level 1[a] (n = 5)	Level 2[b] (n = 5)	Level 3[c] (n = 5)	LOQ_{method}[d] (µg/kg)
		Recovery % (RSD, %)	Recovery % (RSD, %)	Recovery % (RSD, %)	
Daidzein	Skimmed cheese	109 (5)	94 (10)	92 (4)	0.025
	Whole cheese	109 (8)	84 (6)	90 (3)	0.050
	Cow kefir	119 (13)	119 (4)	114 (7)	0.050
	Goat kefir	100 (8)	103 (3)	99 (5)	0.050
Enterodiol	Skimmed cheese	97 (10)	94 (9)	91 (4)	0.25
	Whole cheese	90 (14)	80 (6)	90 (7)	0.25
	Cow kefir	103 (6)	93 (8)	99 (5)	0.50
	Goat kefir	107 (1)	84 (6)	91 (8)	0.25
Glycitein	Skimmed cheese	102 (9)	100 (8)	101 (3)	0.050
	Whole cheese	106 (6)	81 (6)	90 (7)	0.25
	Cow kefir	98 (11)	109 (10)	97 (7)	0.50
	Goat kefir	106 (7)	96 (6)	101 (8)	0.50
Enterolactone	Skimmed cheese	109 (10)	115 (7)	110 (2)	0.050
	Whole cheese	98 (7)	119 (7)	113 (10)	0.25
	Cow kefir	108 (6)	118 (6)	106 (10)	0.25
	Goat kefir	109 (11)	112 (6)	78 (11)	0.25
Genistein	Skimmed cheese	109 (8)	98 (9)	92 (2)	0.050
	Whole cheese	102 (6)	78 (6)	82 (4)	0.25
	Cow kefir	100 (9)	107 (12)	108 (11)	0.25
	Goat kefir	100 (8)	118 (8)	82 (8)	0.25
Formononetin	Skimmed cheese	88 (9)	96 (15)	92 (4)	0.25
	Whole cheese	87 (10)	70 (3)	77 (12)	0.25
	Cow kefir	103 (10)	103 (9)	100 (10)	0.25
	Goat kefir	112 (9)	112 (9)	74 (8)	0.25
Prunetin	Skimmed cheese	99 (7)	90 (9)	96 (8)	0.25
	Whole cheese	79 (11)	76 (5)	70 (5)	0.50
	Cow kefir	77 (10)	77 (4)	86 (7)	0.75
	Goat kefir	102 (8)	94 (3)	78 (4)	0.25
Biochanin A	Skimmed cheese	97 (6)	89 (8)	92 (10)	0.25
	Whole cheese	78 (4)	82 (9)	92 (10)	0.25
	Cow kefir	94 (5)	83 (3)	85 (4)	0.25
	Goat kefir	105 (14)	71 (11)	76 (6)	0.25

Table IV.21.- (Continued).

Analyte	Type of matrix	Level 1[a] (n = 5)	Level 2[b] (n = 5)	Level 3[c] (n = 5)	LOQ_{method}[d] (µg/kg)
		Recovery % (RSD, %)	Recovery % (RSD, %)	Recovery % (RSD, %)	
β-ZAL	Skimmed cheese	90 (7)	112 (6)	92 (2)	0.25
	Whole cheese	94 (11)	92 (1)	84 (3)	0.25
	Cow kefir	98 (10)	99 (6)	99 (6)	0.50
	Goat kefir	86 (10)	103 (5)	94 (3)	0.25
β-ZEL	Skimmed cheese	98 (8)	111 (8)	89 (3)	0.25
	Whole cheese	84 (10)	71 (3)	81 (4)	0.25
	Cow kefir	73 (4)	93 (10)	99 (6)	0.50
	Goat kefir	78 (9)	81 (11)	76 (6)	0.25
α-ZAL	Skimmed cheese	84 (10)	106 (9)	97 (6)	0.050
	Whole cheese	90 (8)	78 (5)	71 (5)	0.25
	Cow kefir	102 (11)	90 (9)	106 (8)	0.50
	Goat kefir	100 (4)	89 (1)	109 (9)	0.25
α-ZEL	Skimmed cheese	96 (8)	112 (8)	93 (8)	0.25
	Whole cheese	83 (11)	83 (9)	80 (2)	0.25
	Cow kefir	93 (11)	73 (5)	94 (7)	0.25
	Goat kefir	91 (4)	78 (11)	80 (4)	0.50
ZAN	Skimmed cheese	78 (9)	119 (4)	99 (8)	0.25
	Whole cheese	84 (9)	78 (6)	88 (8)	0.25
	Cow kefir	111 (9)	74 (6)	84 (8)	2.5
	Goat kefir	110 (7)	86 (6)	84 (5)	0.25
ZEN	Skimmed cheese	97 (10)	116 (3)	112 (9)	0.050
	Whole cheese	98 (15)	71 (3)	78 (3)	0.25
	Cow kefir	93 (7)	77 (9)	82 (5)	0.25
	Goat kefir	102 (9)	76 (8)	55 (8)	0.25
EE_2	Skimmed cheese	80 (6)	102 (8)	103 (6)	0.25
	Whole cheese	97 (6)	90 (12)	102 (9)	0.25
	Cow kefir	93 (7)	92 (8)	88 (8)	0.50
	Goat kefir	85 (13)	100 (9	87 (4)	0.25
DES	Skimmed cheese	87 (9)	86 (7)	97 (10)	0.25
	Whole cheese	105 (5)	108 (9)	86 (9)	0.25
	Cow kefir	101 (10)	80 (7)	79 (6)	0.25
	Goat kefir	81 (8)	83 (6)	91 (8)	0.25

Table IV.21.- (Continued).

Analyte	Type of matrix	Level 1[a] (n = 5)	Level 2[b] (n = 5)	Level 3[c] (n = 5)	LOQ_{method}[d] (µg/kg)
		Recovery % (RSD, %)	Recovery % (RSD, %)	Recovery % (RSD, %)	
DS	Skimmed cheese	85 (9)	90 (9)	107 (2)	0.25
	Whole cheese	86 (7)	97 (10)	83 (9)	0.25
	Cow kefir	88 (4)	81 (6)	75 (6)	0.25
	Goat kefir	81 (14)	88 (3)	84 (5)	0.25
HEX	Skimmed cheese	100 (5)	108 (5)	114 (2)	0.25
	Whole cheese	75 (9)	106 (7)	93 (9)	0.25
	Cow kefir	109 (5)	96 (8)	81 (6)	0.25
	Goat kefir	84 (13)	101 (4)	95 (4)	0.25
E_3	Skimmed cheese	109 (10)	96 (9)	103 (13)	0.25
	Whole cheese	89 (12)	97 (4)	85 (8)	0.25
	Cow kefir	102 (6)	119 (7)	105 (6)	0.25
	Goat kefir	96 (14)	100 (4)	96 (2)	0.25
17β-E_2	Skimmed cheese	92 (8)	114 (6)	94 (9)	0.25
	Whole cheese	92 (10)	80 (10)	84 (13)	0.25
	Cow kefir	77 (9)	93 (6)	96 (8)	0.25
	Goat kefir	95 (9)	86 (9)	91 (4)	0.25
17α-E_2	Skimmed cheese	107 (9)	110 (5)	100 (8)	0.25
	Whole cheese	81 (11)	95 (12)	93 (12)	0.25
	Cow kefir	99 (6)	103 (10)	97 (5)	0.25
	Goat kefir	96 (4)	89 (6)	92 (3)	0.25
E_1	Skimmed cheese	112 (6)	107 (3)	101 (8)	0.25
	Whole cheese	96 (9)	100 (6)	91 (5)	0.25
	Cow kefir	96 (5)	109 (9)	99 (5)	0.25
	Goat kefir	85 (10)	103 (2)	99 (3)	0.25

[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 oestrogens 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 S/N higher than 10 for the quantification transition and at least 3 for the confirmation transition (if it was available).

In Figure IV.34, UHPLC-MS/MS chromatograms of the quantification transition obtained for each analyte when a spiked cow kefir sample was analysed are presented. Similar chromatograms were obtained for the rest of the samples while no interferences were found in any of them when blanks of the matrices were evaluated.

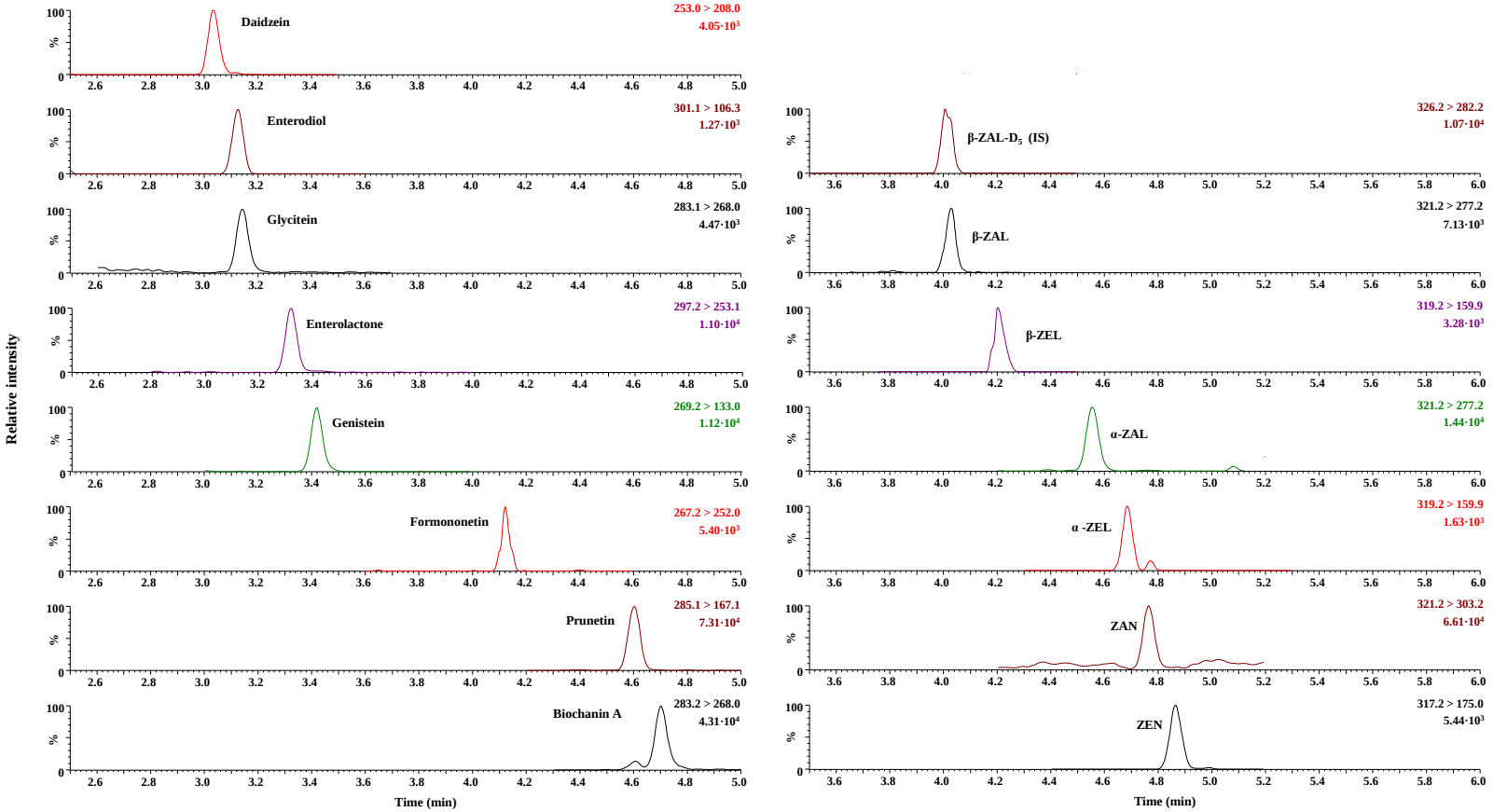
However, the presence of some of the target analytes including equol, daidzein, glycitein, enterolactone and genistein could be observed when the blank matrices were analysed, as can be seen in Figure IV.35 for the whole cheese matrix in which daidzein, glycitein, enterolactone and genistein were detected. Taking this into account, and with the aim of validating the methodology correctly, the peak areas of such analytes present in the matrices were subtracted during the study.

IV.5.5.3.- Analysis of real samples

Once the methodology was validated, it was initially applied to the analysis of the target analytes in 11 milk and 13 yogurt samples bought at the Czech retail market. The results obtained are summarised in Table IV.22. Among the target oestrogenic compounds, only eight phytoestrogens (biochanin A, daidzein, equol, formononetin, genistein, glycitein, coumestrol and enterolactone) and one natural oestrogen (E_1) were found while neither mycoestrogen nor synthetic oestrogen was determined in any of the evaluated samples.

Regarding phytoestrogens, the concentrations found varied considerably between the analytes being equol and enterolactone the most abundant with concentrations 10 times higher than those of the rest of the compounds. In fact, these two substances constitute around 40-90 % of the total analysed oestrogenic content of the samples. These results are consistent with previous studies (Antignac et al., 2004; Tsen et al., 2014) in which it was indicated that the content of equol and enterolactone in milk and dairy products is usually higher than that of the phytoestrogens initially present in feeding stuff because they are the products of rumen and gut microorganism metabolisation of some of these compounds such as formononetin and daidzein (equol precursors) and enterodiol (enterolactone precursor).

(A)


Relative intensity
Daidzein
253.0 > 208.0
4.05·10³
Enterodiol
301.1 > 106.3
1.27·10³
Glycitein
283.1 > 268.0
4.47·10³
Enterolactone
297.2 > 253.1
1.10·10⁴
Genistein
269.2 > 133.0
1.12·10⁴
Formononetin
267.2 > 252.0
5.40·10³
Prunetin
285.1 > 167.1
7.31·10⁴
Biochanin A
283.2 > 268.0
4.31·10⁴
β-ZAL-D5 (IS)
326.2 > 282.2
1.07·10⁴
β-ZAL
321.2 > 277.2
7.13·10³
β-ZEL
319.2 > 159.9
3.28·10³
α-ZAL
321.2 > 277.2
1.44·10⁴
α -ZEL
319.2 > 159.9
1.63·10³
ZAN
321.2 > 303.2
6.61·10⁴
ZEN
317.2 > 175.0
5.44·10³
Time (min)
Time (min)

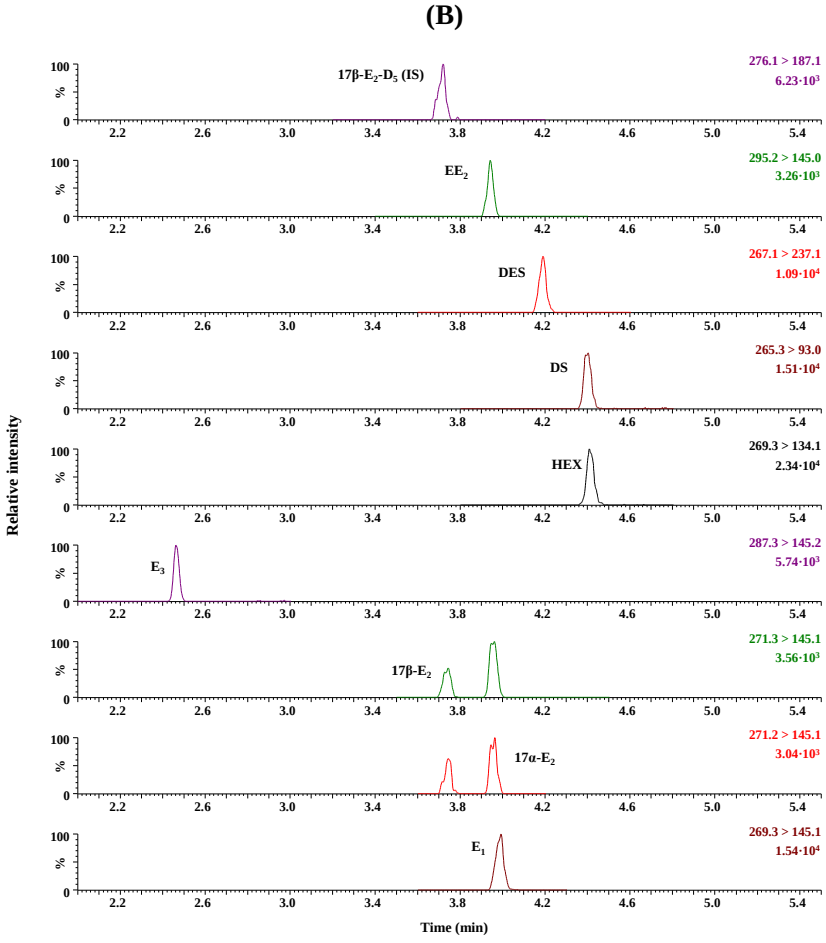


Figure IV.34.- UHPLC-MS/MS chromatograms of (A) phytoestrogens and mycoestrogens and (B) natural and synthetic oestrogens and their ISs of a spiked goat kefir sample after the QuEChERS method. Injection volume: 5 µL. Sample dissolved in 500 µL of 50/50 (v/v) MeOH/H_2O. Separation at 40 °C. Concentration in the sample: 25 µg/kg of IS and 17.5 µg/kg of the target analytes.

Regarding natural oestrogens, only E_1 was detected above the LOQ of the method in one goat milk and one yogurt produced from goat milk. The occurrence of these substances is very different in comparison with similar studies (Courant et al., 2008; Tso and Aga, 2010). These variations could be attributed to several factors. As an example, the collection period of the milk is very important because there is a direct correlation between the oestrogen levels in milk and the oestrus cycle of cattle (Křížová et al., 2011).

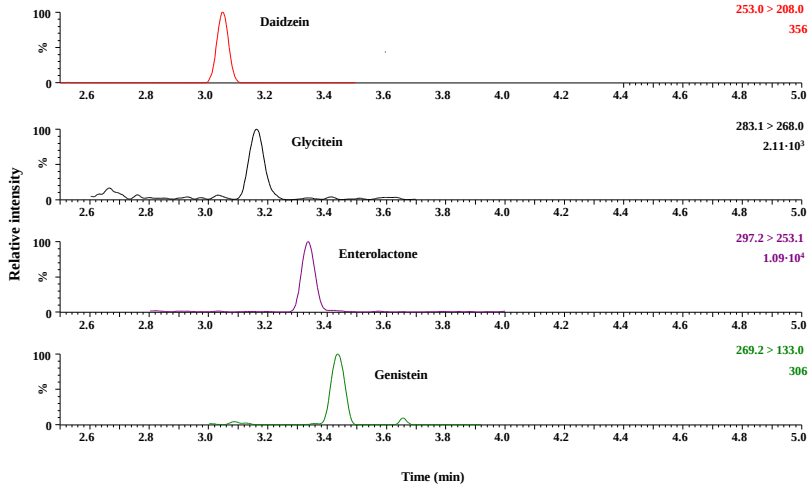


Figure IV.35.- UHPLC-MS/MS chromatograms of the analytes found in a blank of whole cheese after the application of the QuEChERS method. Injection volume: 5 µL. Sample dissolved in 500 µL of MeOH/H_2O 50/50 (v/v). Separation at 40 °C.

Table IV.22.- Concentration (µg/kg dw) of the detected oestrogenic compounds in bovine milk and yogurt samples[a), b)].

Analyte	Milk (n = 11)				Yogurt (n = 13)			
	Positive samples	µg/kg dw			Positive samples	µg/kg dw		
		Mean	Minimum	Maximum		Mean	Minimum	Maximum
Biochanin A	6	1.98	< 0.20	9.92	5	-	< 0.20	2.07
Daidzein	9	0.79	< 0.20	6.00	11	0.80	< 0.20	2.46
Equol	11	10.9	1.07	54.7	13	15.4	1.33	70.0
Formononetin	6	1.28	< 0.20	12.1	7	-	< 0.20	4.02
Genistein	9	0.62	< 0.20	4.70	9	0.41	< 0.20	1.10
Glycitein	10	1.55	< 0.20	9.10	12	2.0	0.28	4.10
Coumestrol	8	0.56	< 0.20	2.71	9	0.70	< 0.20	3.20
Enterolactone	11	3.13	< 0.20	10.0	13	14.2	1.06	27.3
E_1	1	-	< 0.20	0.25	1	-	< 0.20	1.27

[a)] Mean values were calculated when more than 50 % of the samples were positively detected at concentrations above the LOQ. [b)] For results below LOQ, one-half the LOQ value was used.

Concerning kefir and cheese, a group of 8 real samples from diverse commercial brands, purchased in different supermarkets of Tenerife, were analysed using the developed QuEChERS-UHPLC-MS/MS method. Results, which are presented in Table IV.23, show similar contents of phytoestrogens to those obtained for milk and yogurt samples. In this case, the presence of mycoestrogens, natural or synthetic oestrogens was not observed in any of the samples whereas phytoestrogens (daidzein, glycitein, enterolactone and genistein) could be detected and even quantified in some of the products since they were present at concentrations above the LOQ of the method (in the range 1.14-46.7 µg/kg).

As previously indicated, the content of phytoestrogens in kefir samples had not been previously reported up to now. In the case of 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. (Křížová et al., 2011) who reported values around 11.7-30.5 µg/kg for daidzein, genistein and glycitein. Our results are also slightly lower than the data reported by Kuhnle et al. (Kuhnle et al., 2008) who found enterolactone at concentrations of 30-230 µg/kg in different type of cheese products. As demonstrated by Křížová et al. (Křížová et al., 2011), these differences can be associated with the animal diet as well as the type of process applied to each product which can considerably modify the initial level of phytoestrogens present in the raw material used for its preparation.

Table IV.23.- Analysis of real samples (cheese and kefir) 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	1.76 ± 0.87	3.26 ± 0.87	< LOQ	< LOQ	n.d.	n.d.	< LOQ	4.78 ± 1.32
Glycitein	13.6 ± 0.4	25.1 ± 0.5	1.86 ± 0.75	3.37 ± 0.74	n.d.	n.d.	5.88 ± 1.40	11.4 ± 1.4
Enterolactone	34.7 ± 0.7	46.7 ± 0.8	23.3 ± 1.0	24.5 ± 1.1	8.80 ± 0.24	n.d.	< LOQ	3.97 ± 1.11
Genistein	< LOQ	< LOQ	< LOQ	< LOQ	n.d.	n.d.	< LOQ	1.14 ± 0.56

[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.

IV.5.6.- Conclusions

From the results obtained in this section the following conclusions can be drawn:

- A fast and low cost methodology based on the QuEChERS extraction method followed by UHPLC-QqQ-MS/MS determination has been developed for the analysis of twenty four oestrogenic compounds including ten phytoestrogens (daidzein, enterodiol, glycitein, enterolactone, genistein, formononetin, prunetin, biochanin A, equol and coumestrol), six mycoestrogens (ZAN, ZEN, α-ZAL, β-ZAL, α-ZEL and β-ZEL), four natural (17α-E_2, 17β-E_2, E_1 and E_3) and four synthetic oestrogens (HEX, EE_2, DES and DS) in different milk and dairy products.

- Chromatographic separation and determination by UHPLC-QqQ-MS/MS of the selected analytes was optimised and validated in terms of linearity and repeatability with good results in the ranges of concentration studied and with low LOQs in all cases.

- The clean up step of the QuEChERS method was carefully checked and different sorbents were tested obtaining that C_{18} offers the best matrix removal capacity maintaining an adequate extraction of the target oestrogenic compounds from the selected samples.

- Matrix effect was evaluated for all matrices finding an important influence of the sample matrix in almost all cases, except for phytoestrogens in milk and yogurt samples for which matrix effect percentages, calculated using the Matuszewski method, were higher than 80 %.

- The whole method was validated in terms of linearity by the preparation of matrix-matched calibration curves except for phytoestrogens in milk and yogurt for which solvent calibration curves (MeOH) were adequate for the correct quantification of the analytes, taking into account the results obtained from the matrix effect evaluation. R^2 were higher than 0.9905.

- The methodology was also validated by means of a suitable recovery study obtaining values between 70-121 % in all cases with RSDs below 20 % which demonstrates the great extraction capacity of the developed methodology and its excellent reproducibility.

- LOQs of the method in the range 0.02-0.6 µg/L and 0.02-2.5 µg/kg were obtained for milk and for the rest of dairy products, respectively. These results show the excellent linearity of the method in the range of concentrations studied as well as the outstanding sensitivity that the combination of the QuEChERS method with UHPLC-QqQ-MS/MS system offers

which is better than the data previously reported for the analysis of such compounds in the indicated matrices.

- The methodology was successfully applied to the determination of oestrogenic compounds in milk, yogurt, cheese and kefir products finding the presence of several phytoestrogens (biochanin A, daidzein, equol, formononetin, genistein, glycitein, coumestrol and enterolactone) and one natural oestrogen (E_1) in some of the samples. The highest occurrence was found for equol and enterolactone at concentrations in the range (1.06-70.0 µg/kg dw) for milk and yogurt samples and between 3.97-46.7 µg/kg for cheese and kefir. The contents determined in this work were similar to those previously found in the literature, although with some slight differences possibly associated with the different periods of milking for each samples as well as the variations in livestock feeding and the processing applied to each product.
- This methodology constitutes one the first applications of the QuEChERS method for the analysis of such variety of analytes from milk and dairy products and the first work reported in which oestrogenic compounds have been analysed in kefir samples. Due to the simplicity of the procedure and the good results obtained, this methodology could be applied for the routine analysis of the studied compounds in the validated matrices and could also be extended to the analysis of samples of different nature than the ones studied in this work.

IV.6.- Determination of oestrogenic compounds in milk samples using core-shell poly(dopamine) magnetic nanoparticles as micro-dispersive solid-phase extraction sorbent combined with ultra-high-performance liquid chromatography-tandem mass spectrometry

In this section, the potential of core-shell Fe_3O_4@pDA NPs as extraction sorbent was evaluated and validated for the extraction of twenty one different oestrogenic compounds including four natural (E_3, 17β-E_2, 17α-E_2 and E_1) and four synthetic (EE_2, DES, DS and HEX) oestrogens, six mycotoxins with oestrogenic activity (β-ZAL, β-ZEL, α-ZAL, α-ZEL, ZAN and ZEN) and seven phytoestrogens (daidzein, enterodiol, glycitein, enterolactone, genistein, formononetin and biochanin A) in milk samples. The methodology involves a prior deproteinisation step with ACN and acetic acid followed by m-μ-dSPE using Fe_3O_4@pDA NPs as sorbent. Separation, determination and quantification were achieved by UHPLC-QqQ-MS/MS with an ESI. The methodology was validated for five different milk samples (i.e. whole and skimmed cow milk, semi-skimmed goat and sheep milk and human breast milk), using 17β-E_2-D_5, as IS for natural and synthetic oestrogens, β-ZAL-D_5 for mycotoxins and prunetin for phytoestrogens.

IV.6.1.- Background

As it has been previously indicated, oestrogenic compounds including natural, synthetic, myco- and phytoestrogens are a group of EDCs of special concern which can appear in milk products (Capriotti et al., 2015; Křišová et al., 2011; Kuhnle et al., 2008; Sørensen and Elbæk, 2005). However, despite the important impact of these analytes in the consumers, the simultaneous analysis of some of these groups in milk samples have been carried out only in few occasions (D'Orazio et al., 2015, 2016b; Capriotti et al., 2015, Wielogórska et al., 2015) and in none of these cases their evaluation in milk samples with sheep origin has been carried out.

In Section IV.3, the potential of iron oxide m-NPs coated with pDA as sorbent in μ-dSPE was demonstrated for the extraction of natural, synthetic and mycoestrogens from different water samples. As it was indicated in that occasion, m-NPs, and especially iron oxide NPs, have been widely applied as sorbents in sample preparation due to their particular characteristics since, in addition to their high stability and large ratio area-to-volume, they considerably simplify the extraction process, reducing the time consumed due to their capacity

to be easily isolated from the sample matrix by the action of an external magnetic field. Therefore, the predisposition of these materials to be modified by their interaction with other nanomaterials, specific groups or polymeric coatings such as pDA (Qiao et al., 2014; Wang et al., 2013), increases their stability and avoids the possible aggregation problems associated with their very small size (González-Sálamo et al., 2016a). In addition, this possibility of modification also favours the specificity of the interaction between the sorbent and the target analytes increasing the selectivity of the procedure which constitutes an issue of special concern when complex samples like milk products are analysed.

Despite the great advantages that the use of Fe_3O_4@pDA NPs presents in their application as sorbent for the extraction of organic pollutants, their use for the extraction of oestrogenic compounds is still reduced. Prior to the development of this work, only few studies had developed methodologies based on that sorbent for the analysis of such compounds. They were fundamentally focused on the evaluation of water samples. In this sense, it has been only carried out the determination of natural and synthetic oestrogens in tap, drain and mineral water (Huang and Lee, 2015) and the simultaneous extraction of natural, synthetic, myco- and phytoestrogens from different surface water matrices (Capriotti et al., 2016b). Regarding their use in more complex samples, only one work has been published in which Fe_3O_4@pDA NPs have been applied for the extraction of mycoestrogens from milk and yogurt samples applying the methodology discussed in Section IV.3 with slight modifications (González-Sálamo et al., 2017). However, the simultaneous analysis of oestrogenic compounds in such complex matrices has not been previously reported in the literature. Besides, to the best of our knowledge, it has not been developed any methodology for the analysis of any type of oestrogenic compounds in milk with sheep origin.

IV.6.2.- Specific objectives

In view of the foregoing, the following specific goals have been established for this work:

- The development of a fast and environmentally friendly methodology based on Fe_3O_4@pDA NPs m-µ-dSPE followed by UHPLC-MS/MS for the determination of a wide group of oestrogenic compounds of great interest, constituted by four natural (E_3, 17β-E_2, 17α-E_2 and E_1) and four synthetic (EE_2, DES, DS and HEX) oestrogens, six mycotoxins (β-ZAL, β-ZEL, α-ZAL, α-ZEL, ZAN and ZEN) and seven phytoestrogens (daidzein,

enterodiol, glycitein, enterolactone, genistein, formononetin and biochanin A) in milk samples.

- The validation of the separation and determination of the target analytes by UHPLC-MS/MS using 17β-E_2-D_5 as IS for natural and synthetic oestrogens, β-ZAL-D_5 for mycotoxins and prunetin for phytoestrogens.
- The application of the Fe_3O_4@pDA NPs m-μ-dSPE procedure to the extraction of the target analytes from whole and skimmed cow milk, semi-skimmed goat and sheep milk and human breast milk samples, after a suitable deproteinisation step.
- The validation of the whole method by the development of matrix effect, calibration, recovery and reproducibility studies as well as the obtaining of the LOQs of the method for each matrix.

IV.6.3.- UHPLC-MS/MS method

UHPLC-MS/MS separation and detection conditions were the same to the ones previously optimised in Section IV.5 and also described in Section III.5.4. In this case, the ISs used were also 17β-E_2-D_5 for natural and synthetic oestrogens, β-ZAL-D_5 for mycoestrogens while prunetin was incorporated for the determination of phytoestrogens since such compound presents a similar behaviour to the group of phytoestrogens studied. In fact, it had been previously used for the analysis of phytoestrogens in milk samples by Antignac et al. (Antignac et al., 2004) with good results. Taking this modification into account, new calibration curves were prepared in order to verify the suitability of the separation and detection. For this reason, seven different levels of concentration (n = 7) were injected in the range 0.5-750 μg/L in quadruplicate. As it is shown in Table IV.24, R^2 values obtained were higher than 0.9901 for all analytes.

IV.6.4.- Application of Fe_3O_4@pDA NPs m-μ-dSPE for the extraction of milk samples

In this section, the application of the dSPE methodology proposed in Section IV.3 for the extraction of natural and synthetic oestrogens as well as mycotoxins from water samples, that was later slightly modified by González-Sálamo et al. for the extraction of six mycoestrogens in milk and yogurt samples (González-Sálamo et al., 2017), was proposed for the extraction of a higher number of oestrogenic compounds from milk samples.

Table IV.24.- 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	3.15	1.0-750	$1.29 \cdot 10^{-4} \pm 1.28 \cdot 10^{-5}$	$1.02 \cdot 10^{-3} \pm 4.09 \cdot 10^{-3}$	0.9925
Enterodiol	3.23	0.50-750	$6.18 \cdot 10^{-5} \pm 4.06 \cdot 10^{-6}$	$8.88 \cdot 10^{-4} \pm 1.42 \cdot 10^{-3}$	0.9960
Glycitein	3.28	0.50-750	$2.47 \cdot 10^{-4} \pm 2.47 \cdot 10^{-5}$	$4.63 \cdot 10^{-3} \pm 7.78 \cdot 10^{-3}$	0.9910
Enterolactone	3.43	0.50-750	$2.10 \cdot 10^{-4} \pm 1.71 \cdot 10^{-6}$	$-3.71 \cdot 10^{-5} \pm 5.97 \cdot 10^{-4}$	0.9999
Genistein	3.55	0.50-750	$2.81 \cdot 10^{-4} \pm 1.06 \cdot 10^{-5}$	$1.71 \cdot 10^{-3} \pm 3.69 \cdot 10^{-3}$	0.9987
Formononetin	4.25	1.0-750	$6.70 \cdot 10^{-4} \pm 3.05 \cdot 10^{-5}$	$-8.62 \cdot 10^{-3} \pm 1.10 \cdot 10^{-2}$	0.9938
Biochanin A	4.75	0.50-750	$2.65 \cdot 10^{-3} \pm 7.71 \cdot 10^{-5}$	$1.17 \cdot 10^{-1} \pm 2.79 \cdot 10^{-2}$	0.9992
Mycoestrogens					
β-ZAL	4.15	0.50-750	$2.36 \cdot 10^{-3} \pm 8.25 \cdot 10^{-5}$	$-8.30 \cdot 10^{-3} \pm 2.88 \cdot 10^{-2}$	0.9989
β-ZEL	4.33	0.50-750	$9.65 \cdot 10^{-4} \pm 6.30 \cdot 10^{-5}$	$4.94 \cdot 10^{-3} \pm 1.94 \cdot 10^{-2}$	0.9961
α-ZAL	4.68	0.50-750	$4.13 \cdot 10^{-3} \pm 1.34 \cdot 10^{-4}$	$-1.52 \cdot 10^{-2} \pm 4.68 \cdot 10^{-2}$	0.9990
α-ZEL	4.82	0.50-750	$6.17 \cdot 10^{-4} \pm 4.84 \cdot 10^{-5}$	$7.44 \cdot 10^{-3} \pm 1.69 \cdot 10^{-2}$	0.9944
ZAN	4.88	0.50-750	$2.21 \cdot 10^{-2} \pm 2.32 \cdot 10^{-3}$	$4.81 \cdot 10^{-1} \pm 7.16 \cdot 10^{-1}$	0.9901
ZEN	4.99	0.50-750	$2.55 \cdot 10^{-3} \pm 6.17 \cdot 10^{-5}$	$-5.91 \cdot 10^{-3} \pm 2.16 \cdot 10^{-2}$	0.9995
Synthetic estrogens					
EE_2	4.00	5.0-750	$1.80 \cdot 10^{-3} \pm 1.68 \cdot 10^{-4}$	$-3.39 \cdot 10^{-2} \pm 5.89 \cdot 10^{-2}$	0.9921
DES	4.25	5.0-750	$5.87 \cdot 10^{-3} \pm 3.16 \cdot 10^{-4}$	$-8.87 \cdot 10^{-2} \pm 1.11 \cdot 10^{-1}$	0.9974
DS	4.45	5.0-750	$1.38 \cdot 10^{-2} \pm 1.37 \cdot 10^{-3}$	$-1.96 \cdot 10^{-1} \pm 5.04 \cdot 10^{-1}$	0.9926
HEX	4.45	5.0-750	$1.61 \cdot 10^{-2} \pm 1.26 \cdot 10^{-3}$	$-2.67 \cdot 10^{-1} \pm 4.41 \cdot 10^{-1}$	0.9945
Natural estrogens					
E_3	2.49	5.0-750	$2.33 \cdot 10^{-3} \pm 1.61 \cdot 10^{-4}$	$-3.58 \cdot 10^{-2} \pm 5.82 \cdot 10^{-2}$	0.9957
17β-E_2	3.78	5.0-750	$1.19 \cdot 10^{-3} \pm 1.05 \cdot 10^{-4}$	$-2.93 \cdot 10^{-2} \pm 3.70 \cdot 10^{-2}$	0.9929
17α-E_2	4.00	5.0-750	$1.74 \cdot 10^{-3} \pm 1.72 \cdot 10^{-4}$	$-4.18 \cdot 10^{-2} \pm 6.06 \cdot 10^{-2}$	0.9912
E_1	4.04	5.0-750	$1.15 \cdot 10^{-2} \pm 1.99 \cdot 10^{-4}$	$-7.89 \cdot 10^{-2} \pm 6.981 \cdot 10^{-2}$	0.9997

R^2: Determination coefficient.

Since previous results demonstrated that most of the target analytes were efficiently extracted from water samples and that also this group of mycoestrogens were previously extracted from milk samples with good recovery, the method was directly applied to such samples after a suitable deproteinisation step and validated in terms of matrix effect, linearity, recovery and reproducibility as it will be described below.

IV.6.4.1.- Evaluation of the matrix effect

As previously commented, milk products are very complex samples with an important content of fat, carbohydrates and proteins that may have a great influence in the extraction efficiency of any developed methodology, despite the application of a previous deproteinisation step. Due to the fact that Fe_3O_4@pDA NPs have not been previously used for the extraction of such variety of compounds from this type of samples as well as the important differences between the nature of the milk samples evaluated (see Section III.4), an exhaustive study of the matrix effect was carried out for each matrix at two different concentration levels (13.4 and 250 µg/L) following the Matuszewski method (Matuszewski et al., 2003). For this purpose, five replicates of each matrix were spiked at each indicated concentration level at the end of the procedure and analysed by UHPLC-QqQ-MS/MS. Afterwards, the matrix effect was calculated as the percentage ratio of the peak areas of the analytes in the matrix and their peak areas in the pure solvent at the same concentration.

Table IV.25 shows the obtained results. As can be appreciated, matrix effect percentages were lower than 80 % in almost all cases with RSDs lower than 20 % which indicates a clear ion suppression effect of all the matrices evaluated. Special attention should be paid to the effect of human breast milk for natural and synthetic oestrogens for which matrix effect values in the ranged 15-22 % were found in almost all cases, demonstrating a great influence of this type of sample in such group of oestrogenic compounds. In addition, the impact of the same matrix on daidzein, glycitein and genistein response should also be highlighted since they could not be determined when human breast milk was analysed although no interferences were detected when non-spiked samples were studied. This aspect could be associated with the bonding of these analytes to proteins, among other type of molecules present in the samples, which were not efficiency removed during the deproteinisation procedure.

Table IV.25.- Average results of the matrix effect study (n = 10) of the m-µ-dSPE-UHPLC-MS/MS method for the selected compounds in the different matrices at two levels of concentration.

Analyte	Type of milk	ME[a), b)], %	RSD, %	Analyte	Type of matrix	ME[a), b)], %	RSD, %
Daidzein	Skimmed cow	88	11	ZAN	Skimmed cow	51	9
	Whole cow	73	1		Whole cow	71	10
	Goat	98	9		Goat	60	12
	Sheep	95	5		Sheep	67	12
	Human	-	-		Human	36	6
Enterodiol	Skimmed cow	88	16	ZEN	Skimmed cow	67	18
	Whole cow	67	8		Whole cow	63	0.6
	Goat	65	18		Goat	78	20
	Sheep	71	6		Sheep	67	16
	Human	91	8		Human	71	2
Glycitein	Skimmed cow	94	8	EE_2	Skimmed cow	73	9
	Whole cow	73	9		Whole cow	76	18
	Goat	68	3		Goat	45	14
	Sheep	70	14		Sheep	89	16
	Human	-	-		Human	15	18
Enterolactone	Skimmed cow	98	8	DES	Skimmed cow	95	7
	Whole cow	69	15		Whole cow	99	15
	Goat	75	11		Goat	80	17
	Sheep	90	16		Sheep	96	6
	Human	83	6		Human	22	12
Genistein	Skimmed cow	81	0.2	DS	Skimmed cow	79	13
	Whole cow	64	7		Whole cow	88	7
	Goat	84	14		Goat	49	19
	Sheep	94	14		Sheep	78	8
	Human	-	-		Human	16	9
Formononetin	Skimmed cow	91	3	HEX	Skimmed cow	77	6
	Whole cow	58	9		Whole cow	76	2
	Goat	80	10		Goat	79	13
	Sheep	92	14		Sheep	76	1
	Human	78	19		Human	17	11

Table IV.25.- (Continued).

Analyte	Type of milk	ME[a), b)], %	RSD, %	Analyte	Type of matrix	ME[a), b)], %	RSD, %
	Skimmed cow	58	5		Skimmed cow	89	2
	Whole cow	32	7		Whole cow	98	5
Biochanin A	Goat	48	7	E_3	Goat	61	8
	Sheep	59	16		Sheep	60	13
	Human	50	10		Human	62	9
	Skimmed cow	86	15		Skimmed cow	52	18
	Whole cow	73	15		Whole cow	99	14
β-ZAL	Goat	94	19	17β-E_2	Goat	76	21
	Sheep	87	13		Sheep	74	4
	Human	93	3		Human	22	13
	Skimmed cow	79	19		Skimmed cow	82	12
	Whole cow	39	6		Whole cow	80	10
β-ZEL	Goat	86	14	17α-E_2	Goat	54	10
	Sheep	84	11		Sheep	61	15
	Human	79	6		Human	21	14
	Skimmed cow	78	19		Skimmed cow	89	17
	Whole cow	72	15		Whole cow	91	14
α-ZAL	Goat	78	6	E_1	Goat	66	18
	Sheep	77	16		Sheep	86	5
	Human	73	10		Human	21	11
	Skimmed cow	74	12				
	Whole cow	62	19				
α-ZEL	Goat	71	24				
	Sheep	64	12				
	Human	96	6				

[a)] Results obtained as an average (n = 10) of each analyte at two different concentration levels.

[b)] Calculated following the Matuszewski method (Matuszewski et al., 2003).

In addition, the evaluation of non-spiked samples (blanks) showed the presence of glycitein in goat milk and enterolactone in goat and sheep milk (see Figure IV.36). Consequently, and in order to carry out an appropriate validation of the procedure for all the target analytes, the peak areas of these oestrogenic compounds were subtracted when necessary.

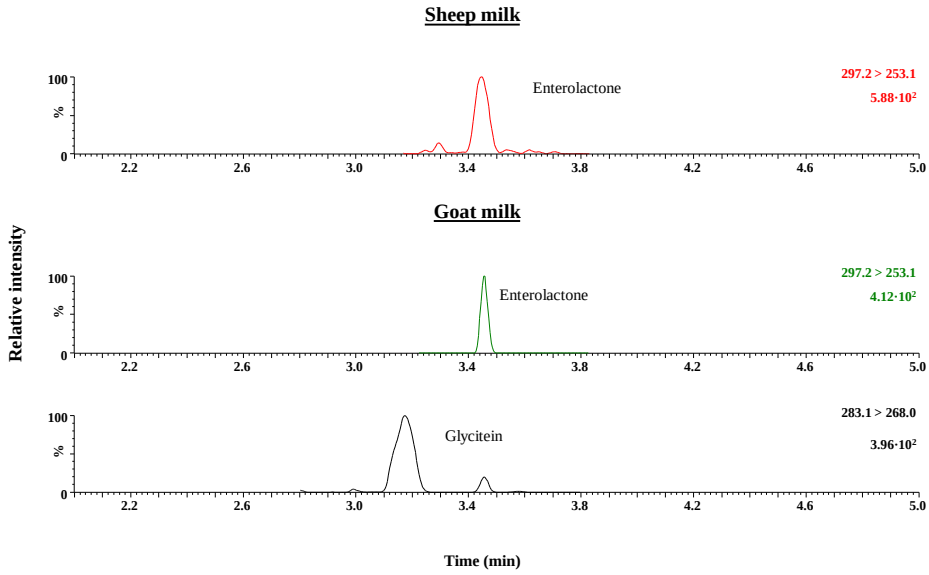


Figure IV.36.- UHPLC-MS/MS chromatograms of the analytes found in non-spiked sheep and goat milk samples (blanks) after the m-µ-dSPE procedure. Injection volume: 5 µL. Sample dissolved in 500 µL of 50/50 (v/v) MeOH/H_2O. Separation at 40 °C.

IV.6.4.2.- Matrix-matched calibration and recovery study

Taking into account the results obtained from the matrix effect study, matrix-matched calibration curves were prepared in each milk sample in order to validate the methodology. With this aim, the samples were 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 oestrogens, by injecting seven different levels of concentration in quadruplicate (n = 7). Table IV.26 shows the results of the study as well as the range of concentration evaluated and the lowest calibration level. As can be appreciated, the method presents an excellent linearity with R^2 higher than 0.9909 in all cases.

Table IV.26.- Matrix-matched calibration data of the selected compounds in the different matrices.

Analyte	Type of milk	Calibration data (n = 7)			
		Range of concentration studied (µg/L)	Slope	Intercept	R^2
Daidzein	Skimmed cow	1-750	$7.01 \cdot 10^{-4} \pm 6.87 \cdot 10^{-5}$	$9.23 \cdot 10^{-4} \pm 2.16 \cdot 10^{-2}$	0.9930
	Whole cow	1-750	$8.23 \cdot 10^{-4} \pm 5.87 \cdot 10^{-5}$	$-3.25 \cdot 10^{-3} \pm 1.85 \cdot 10^{-2}$	0.9962
	Goat	1-750	$1.02 \cdot 10^{-3} \pm 5.04 \cdot 10^{-5}$	$9.27 \cdot 10^{-3} \pm 1.55 \cdot 10^{-2}$	0.9982
	Sheep	1-750	$1.57 \cdot 10^{-3} \pm 9.56 \cdot 10^{-5}$	$-1.15 \cdot 10^{-3} \pm 3.01 \cdot 10^{-2}$	0.9972
	Human	-	-	-	-
Enterodiol	Skimmed cow	5-750	$4.63 \cdot 10^{-4} \pm 4.12 \cdot 10^{-5}$	$1.14 \cdot 10^{-2} \pm 1.44 \cdot 10^{-2}$	0.9941
	Whole cow	5-750	$5.61 \cdot 10^{-4} \pm 1.94 \cdot 10^{-5}$	$7.46 \cdot 10^{-3} \pm 7.11 \cdot 10^{-3}$	0.9991
	Goat	5-750	$7.25 \cdot 10^{-4} \pm 4.54 \cdot 10^{-5}$	$1.31 \cdot 10^{-2} \pm 1.43 \cdot 10^{-2}$	0.9970
	Sheep	5-750	$8.13 \cdot 10^{-4} \pm 6.43 \cdot 10^{-5}$	$1.79 \cdot 10^{-2} \pm 2.31 \cdot 10^{-2}$	0.9953
	Human	5-750	$4.79 \cdot 10^{-4} \pm 5.1 \cdot 10^{-5}$	$7.65 \cdot 10^{-3} \pm 1.74 \cdot 10^{-2}$	0.9914
Glycitein	Skimmed cow	1-750	$2.02 \cdot 10^{-3} \pm 1.65 \cdot 10^{-4}$	$2.88 \cdot 10^{-2} \pm 5.20 \cdot 10^{-2}$	0.9950
	Whole cow	1-750	$2.48 \cdot 10^{-3} \pm 1.30 \cdot 10^{-4}$	$2.85 \cdot 10^{-2} \pm 4.75 \cdot 10^{-2}$	0.9979
	Goat	1-750	$4.19 \cdot 10^{-3} \pm 2.63 \cdot 10^{-4}$	$6.50 \cdot 10^{-2} \pm 9.65 \cdot 10^{-2}$	0.9970
	Sheep	1-750	$3.89 \cdot 10^{-3} \pm 1.64 \cdot 10^{-4}$	$3.85 \cdot 10^{-2} \pm 5.16 \cdot 10^{-2}$	0.9987
	Human	-	-	-	-
Enterolactone	Skimmed cow	1-750	$1.22 \cdot 10^{-3} \pm 1.77 \cdot 10^{-5}$	$3.10 \cdot 10^{-3} \pm 9.71 \cdot 10^{-3}$	0.9996
	Whole cow	1-750	$1.71 \cdot 10^{-3} \pm 4.74 \cdot 10^{-5}$	$-4.74 \cdot 10^{-4} \pm 1.66 \cdot 10^{-2}$	0.9994
	Goat	1-750	$2.34 \cdot 10^{-3} \pm 9.91 \cdot 10^{-5}$	$2.57 \cdot 10^{-2} \pm 3.64 \cdot 10^{-2}$	0.9986
	Sheep	1-750	$2.66 \cdot 10^{-3} \pm 1.81 \cdot 10^{-4}$	$-5.55 \cdot 10^{-3} \pm 6.34 \cdot 10^{-2}$	0.9965
	Human	1-750	$1.61 \cdot 10^{-3} \pm 2.84 \cdot 10^{-5}$	$-1.41 \cdot 10^{-3} \pm 9.01 \cdot 10^{-3}$	0.9998
Genistein	Skimmed cow	1-750	$1.49 \cdot 10^{-3} \pm 2.63 \cdot 10^{-5}$	$-1.77 \cdot 10^{-3} \pm 8.34 \cdot 10^{-3}$	0.9998
	Whole cow	1-750	$1.82 \cdot 10^{-3} \pm 6.93 \cdot 10^{-5}$	$2.52 \cdot 10^{-3} \pm 2.43 \cdot 10^{-2}$	0.9989
	Goat	1-750	$2.35 \cdot 10^{-3} \pm 2.57 \cdot 10^{-5}$	$6.88 \cdot 10^{-3} \pm 9.01 \cdot 10^{-3}$	0.9999
	Sheep	1-750	$2.86 \cdot 10^{-3} \pm 1.06 \cdot 10^{-4}$	$6.46 \cdot 10^{-3} \pm 3.35 \cdot 10^{-2}$	0.9990
	Human	-	-	-	-
Formononetin	Skimmed cow	5-750	$1.61 \cdot 10^{-3} \pm 1.17 \cdot 10^{-4}$	$-2.17 \cdot 10^{-2} \pm 4.11 \cdot 10^{-2}$	0.9960
	Whole cow	5-750	$2.29 \cdot 10^{-3} \pm 2.45 \cdot 10^{-4}$	$-3.68 \cdot 10^{-2} \pm 8.59 \cdot 10^{-2}$	0.9914
	Goat	5-750	$3.45 \cdot 10^{-3} \pm 6.67 \cdot 10^{-5}$	$-1.05 \cdot 10^{-2} \pm 2.34 \cdot 10^{-2}$	0.9997
	Sheep	1-750	$4.37 \cdot 10^{-3} \pm 2.44 \cdot 10^{-4}$	$-3.84 \cdot 10^{-2} \pm 7.23 \cdot 10^{-2}$	0.9976
	Human	5-750	$2.05 \cdot 10^{-3} \pm 9.84 \cdot 10^{-5}$	$1.12 \cdot 10^{-3} \pm 3.12 \cdot 10^{-2}$	0.9983
Biochanin A	Skimmed cow	5-750	$9.85 \cdot 10^{-3} \pm 8.36 \cdot 10^{-4}$	$-1.30 \cdot 10^{-1} \pm 2.86 \cdot 10^{-1}$	0.9946
	Whole cow	5-750	$1.30 \cdot 10^{-2} \pm 1.31 \cdot 10^{-3}$	$-5.70 \cdot 10^{-2} \pm 4.50 \cdot 10^{-1}$	0.9923
	Goat	5-750	$1.54 \cdot 10^{-2} \pm 4.44 \cdot 10^{-4}$	$-1.43 \cdot 10^{-1} \pm 1.52 \cdot 10^{-1}$	0.9994
	Sheep	1-750	$2.29 \cdot 10^{-2} \pm 1.01 \cdot 10^{-3}$	$5.13 \cdot 10^{-1} \pm 3.13 \cdot 10^{-1}$	0.9985
	Human	5-750	$1.44 \cdot 10^{-2} \pm 1.46 \cdot 10^{-3}$	$6.12 \cdot 10^{-1} \pm 5.01 \cdot 10^{-1}$	0.9923

Table IV.26.- (Continued).

Analyte	Type of milk	Calibration data (n = 7)			
		Range of concentration studied (µg/L)	Slope	Intercept	R^2
β-ZAL	Skimmed cow	1-750	$2.93 \cdot 10^{-3} \pm 7.53 \cdot 10^{-5}$	$-1.44 \cdot 10^{-2} \pm 2.64 \cdot 10^{-2}$	0.9995
	Whole cow	1-750	$3.23 \cdot 10^{-3} \pm 2.05 \cdot 10^{-4}$	$-3.01 \cdot 10^{-2} \pm 7.20 \cdot 10^{-2}$	0.9970
	Goat	1-750	$3.12 \cdot 10^{-3} \pm 7.61 \cdot 10^{-5}$	$-1.63 \cdot 10^{-2} \pm 2.67 \cdot 10^{-2}$	0.9995
	Sheep	1-750	$2.70 \cdot 10^{-3} \pm 7.74 \cdot 10^{-5}$	$-1.66 \cdot 10^{-2} \pm 2.72 \cdot 10^{-2}$	0.9994
	Human	1-750	$2.95 \cdot 10^{-3} \pm 3.81 \cdot 10^{-5}$	$-1.08 \cdot 10^{-2} \pm 1.38 \cdot 10^{-2}$	0.9998
β-ZEL	Skimmed cow	1-750	$1.18 \cdot 10^{-3} \pm 5.03 \cdot 10^{-5}$	$7.59 \cdot 10^{-3} \pm 1.76 \cdot 10^{-2}$	0.9986
	Whole cow	1-750	$1.30 \cdot 10^{-3} \pm 2.09 \cdot 10^{-5}$	$3.39 \cdot 10^{-3} \pm 7.54 \cdot 10^{-3}$	0.9998
	Goat	1-750	$1.24 \cdot 10^{-3} \pm 5.81 \cdot 10^{-5}$	$9.78 \cdot 10^{-3} \pm 2.10 \cdot 10^{-2}$	0.9983
	Sheep	1-750	$1.26 \cdot 10^{-3} \pm 1.18 \cdot 10^{-5}$	$-4.37 \cdot 10^{-3} \pm 4.28 \cdot 10^{-3}$	0.9999
	Human	1-750	$1.54 \cdot 10^{-3} \pm 1.12 \cdot 10^{-4}$	$2.41 \cdot 10^{-3} \pm 4.04 \cdot 10^{-2}$	0.9960
α-ZAL	Skimmed cow	1-750	$5.04 \cdot 10^{-3} \pm 9.12 \cdot 10^{-5}$	$-1.24 \cdot 10^{-2} \pm 2.89 \cdot 10^{-2}$	0.9998
	Whole cow	1-750	$5.68 \cdot 10^{-3} \pm 2.29 \cdot 10^{-4}$	$-4.85 \cdot 10^{-2} \pm 7.24 \cdot 10^{-2}$	0.9988
	Goat	1-750	$5.35 \cdot 10^{-3} \pm 6.53 \cdot 10^{-5}$	$1.21 \cdot 10^{-2} \pm 2.07 \cdot 10^{-2}$	0.9999
	Sheep	1-750	$5.46 \cdot 10^{-3} \pm 2.42 \cdot 10^{-4}$	$-4.85 \cdot 10^{-2} \pm 8.75 \cdot 10^{-2}$	0.9985
	Human	1-750	$6.07 \cdot 10^{-3} \pm 1.54 \cdot 10^{-4}$	$3.05 \cdot 10^{-2} \pm 4.88 \cdot 10^{-2}$	0.9995
α-ZEL	Skimmed cow	5-750	$6.33 \cdot 10^{-4} \pm 4.19 \cdot 10^{-5}$	$5.70 \cdot 10^{-3} \pm 1.47 \cdot 10^{-2}$	0.9967
	Whole cow	5-750	$6.83 \cdot 10^{-4} \pm 5.44 \cdot 10^{-5}$	$1.04 \cdot 10^{-2} \pm 1.91 \cdot 10^{-2}$	0.9952
	Goat	5-750	$5.39 \cdot 10^{-4} \pm 3.92 \cdot 10^{-5}$	$9.18 \cdot 10^{-3} \pm 1.41 \cdot 10^{-2}$	0.9960
	Sheep	5-750	$7.95 \cdot 10^{-4} \pm 2.03 \cdot 10^{-5}$	$-1.90 \cdot 10^{-3} \pm 7.31 \cdot 10^{-3}$	0.9995
	Human	5-750	$8.35 \cdot 10^{-4} \pm 2.81 \cdot 10^{-5}$	$3.71 \cdot 10^{-3} \pm 8.85 \cdot 10^{-3}$	0.9991
ZAN	Skimmed cow	1-750	$1.89 \cdot 10^{-2} \pm 9.57 \cdot 10^{-4}$	$1.28 \cdot 10^{-1} \pm 3.28 \cdot 10^{-1}$	0.9981
	Whole cow	1-750	$1.44 \cdot 10^{-2} \pm 5.13 \cdot 10^{-4}$	$8.45 \cdot 10^{-2} \pm 1.76 \cdot 10^{-1}$	0.9990
	Goat	1-750	$1.26 \cdot 10^{-2} \pm 7.71 \cdot 10^{-4}$	$6.56 \cdot 10^{-2} \pm 2.37 \cdot 10^{-1}$	0.9972
	Sheep	1-750	$1.10 \cdot 10^{-2} \pm 4.08 \cdot 10^{-4}$	$-6.59 \cdot 10^{-2} \pm 1.43 \cdot 10^{-1}$	0.9990
	Human	10-750	$2.37 \cdot 10^{-2} \pm 2.44 \cdot 10^{-3}$	$-3.87 \cdot 10^{-1} \pm 7.50 \cdot 10^{-1}$	0.9921
ZEN	Skimmed cow	1-750	$2.68 \cdot 10^{-4} \pm 8.46 \cdot 10^{-5}$	$-1.64 \cdot 10^{-2} \pm 2.97 \cdot 10^{-2}$	0.9992
	Whole cow	1-750	$3.28 \cdot 10^{-3} \pm 1.83 \cdot 10^{-4}$	$-3.78 \cdot 10^{-2} \pm 5.79 \cdot 10^{-2}$	0.9972
	Goat	1-750	$2.76 \cdot 10^{-3} \pm 3.30 \cdot 10^{-5}$	$-7.16 \cdot 10^{-3} \pm 1.04 \cdot 10^{-2}$	0.9999
	Sheep	1-750	$2.76 \cdot 10^{-3} \pm 1.22 \cdot 10^{-4}$	$-2.74 \cdot 10^{-2} \pm 4.41 \cdot 10^{-2}$	0.9985
	Human	1-750	$3.24 \cdot 10^{-3} \pm 1.02 \cdot 10^{-4}$	$-1.62 \cdot 10^{-2} \pm 3.23 \cdot 10^{-2}$	0.9993
EE_2	Skimmed cow	5-750	$1.50 \cdot 10^{-3} \pm 6.77 \cdot 10^{-5}$	$-1.90 \cdot 10^{-2} \pm 2.45 \cdot 10^{-2}$	0.9984
	Whole cow	5-750	$1.45 \cdot 10^{-3} \pm 3.77 \cdot 10^{-5}$	$-7.41 \cdot 10^{-3} \pm 1.36 \cdot 10^{-2}$	0.9995
	Goat	5-750	$1.22 \cdot 10^{-3} \pm 8.31 \cdot 10^{-5}$	$-1.62 \cdot 10^{-2} \pm 2.56 \cdot 10^{-2}$	0.9965
	Sheep	10-750	$1.53 \cdot 10^{-3} \pm 3.99 \cdot 10^{-5}$	$-2.60 \cdot 10^{-2} \pm 1.26 \cdot 10^{-2}$	0.9995
	Human	10-750	$1.46 \cdot 10^{-3} \pm 1.42 \cdot 10^{-4}$	$-4.36 \cdot 10^{-2} \pm 4.37 \cdot 10^{-2}$	0.9930

Table IV.26.- (Continued).

Analyte	Type of milk	Calibration data (n = 7)			
		Range of concentration studied (µg/L)	Slope	Intercept	R^2
DES	Skimmed cow	5-750	$1.42 \cdot 10^{-2} \pm 1.16 \cdot 10^{-3}$	$-2.29 \cdot 10^{-1} \pm 4.07 \cdot 10^{-1}$	0.9950
	Whole cow	5-750	$1.24 \cdot 10^{-2} \pm 4.90 \cdot 10^{-4}$	$-7.25 \cdot 10^{-2} \pm 1.77 \cdot 10^{-1}$	0.9988
	Goat	5-750	$1.48 \cdot 10^{-2} \pm 7.40 \cdot 10^{-4}$	$-1.82 \cdot 10^{-1} \pm 2.60 \cdot 10^{-1}$	0.9981
	Sheep	5-750	$1.11 \cdot 10^{-2} \pm 1.62 \cdot 10^{-4}$	$-6.61 \cdot 10^{-2} \pm 5.94 \cdot 10^{-2}$	0.9998
	Human	5-750	$1.13 \cdot 10^{-2} \pm 2.91 \cdot 10^{-4}$	$-7.37 \cdot 10^{-2} \pm 9.17 \cdot 10^{-2}$	0.9995
DS	Skimmed cow	5-750	$1.07 \cdot 10^{-2} \pm 5.41 \cdot 10^{-4}$	$-1.03 \cdot 10^{-1} \pm 1.71 \cdot 10^{-1}$	0.9981
	Whole cow	5-750	$1.01 \cdot 10^{-2} \pm 3.27 \cdot 10^{-4}$	$4.34 \cdot 10^{-2} \pm 1.20 \cdot 10^{-1}$	0.9992
	Goat	5-750	$1.15 \cdot 10^{-2} \pm 5.22 \cdot 10^{-4}$	$-1.51 \cdot 10^{-1} \pm 1.92 \cdot 10^{-1}$	0.9984
	Sheep	5-750	$1.43 \cdot 10^{-2} \pm 2.64 \cdot 10^{-4}$	$-9.20 \cdot 10^{-2} \pm 8.37 \cdot 10^{-2}$	0.9997
	Human	5-750	$1.24 \cdot 10^{-2} \pm 2.03 \cdot 10^{-4}$	$-6.58 \cdot 10^{-2} \pm 7.48 \cdot 10^{-2}$	0.9998
HEX	Skimmed cow	5-750	$1.43 \cdot 10^{-2} \pm 8.97 \cdot 10^{-4}$	$-1.36 \cdot 10^{-1} \pm 3.15 \cdot 10^{-1}$	0.9970
	Whole cow	5-750	$1.43 \cdot 10^{-2} \pm 2.37 \cdot 10^{-4}$	$-2.41 \cdot 10^{-2} \pm 8.31 \cdot 10^{-2}$	0.9997
	Goat	5-750	$1.58 \cdot 10^{-2} \pm 7.76 \cdot 10^{-4}$	$-1.79 \cdot 10^{-1} \pm 2.73 \cdot 10^{-1}$	0.9982
	Sheep	5-750	$1.78 \cdot 10^{-2} \pm 1.27 \cdot 10^{-4}$	$-8.75 \cdot 10^{-2} \pm 4.01 \cdot 10^{-2}$	0.9999
	Human	5-750	$1.59 \cdot 10^{-2} \pm 4.18 \cdot 10^{-4}$	$-8.93 \cdot 10^{-2} \pm 1.51 \cdot 10^{-1}$	0.9995
E_3	Skimmed cow	5-750	$2.54 \cdot 10^{-3} \pm 1.66 \cdot 10^{-4}$	$-1.97 \cdot 10^{-2} \pm 5.81 \cdot 10^{-2}$	0.9966
	Whole cow	5-750	$2.18 \cdot 10^{-3} \pm 3.93 \cdot 10^{-5}$	$-3.04 \cdot 10^{-3} \pm 1.38 \cdot 10^{-2}$	0.9997
	Goat	5-750	$2.41 \cdot 10^{-3} \pm 7.98 \cdot 10^{-5}$	$-1.82 \cdot 10^{-2} \pm 2.88 \cdot 10^{-2}$	0.9992
	Sheep	5-750	$2.40 \cdot 10^{-3} \pm 1.28 \cdot 10^{-4}$	$-6.89 \cdot 10^{-4} \pm 4.72 \cdot 10^{-2}$	0.9978
	Human	5-750	$2.69 \cdot 10^{-3} \pm 1.51 \cdot 10^{-4}$	$-2.84 \cdot 10^{-2} \pm 5.45 \cdot 10^{-2}$	0.9976
17β-E_2	Skimmed cow	10-750	$1.90 \cdot 10^{-3} \pm 1.05 \cdot 10^{-4}$	$-3.93 \cdot 10^{-2} \pm 3.78 \cdot 10^{-2}$	0.9977
	Whole cow	10-750	$1.62 \cdot 10^{-3} \pm 6.00 \cdot 10^{-5}$	$-2.17 \cdot 10^{-2} \pm 2.17 \cdot 10^{-2}$	0.9990
	Goat	10-750	$2.25 \cdot 10^{-3} \pm 1.01 \cdot 10^{-4}$	$-2.94 \cdot 10^{-2} \pm 3.11 \cdot 10^{-2}$	0.9985
	Sheep	10-750	$1.30 \cdot 10^{-3} \pm 8.85 \cdot 10^{-5}$	$-3.83 \cdot 10^{-2} \pm 3.27 \cdot 10^{-2}$	0.9965
	Human	10-750	$1.75 \cdot 10^{-3} \pm 7.97 \cdot 10^{-5}$	$-3.47 \cdot 10^{-2} \pm 2.88 \cdot 10^{-2}$	0.9984
17α-E_2	Skimmed cow	5-750	$2.41 \cdot 10^{-3} \pm 7.87 \cdot 10^{-5}$	$-2.11 \cdot 10^{-2} \pm 2.75 \cdot 10^{-2}$	0.9992
	Whole cow	5-750	$2.04 \cdot 10^{-3} \pm 3.17 \cdot 10^{-5}$	$-8.05 \cdot 10^{-3} \pm 1.16 \cdot 10^{-2}$	0.9998
	Goat	5-750	$2.03 \cdot 10^{-3} \pm 9.53 \cdot 10^{-5}$	$-1.49 \cdot 10^{-2} \pm 3.51 \cdot 10^{-2}$	0.9983
	Sheep	10-750	$1.59 \cdot 10^{-3} \pm 3.64 \cdot 10^{-5}$	$-1.24 \cdot 10^{-2} \pm 1.16 \cdot 10^{-2}$	0.9996
	Human	10-750	$1.86 \cdot 10^{-3} \pm 1.88 \cdot 10^{-4}$	$-5.43 \cdot 10^{-2} \pm 5.56 \cdot 10^{-2}$	0.9924
E_1	Skimmed cow	5-750	$1.17 \cdot 10^{-2} \pm 7.70 \cdot 10^{-4}$	$-1.47 \cdot 10^{-1} \pm 2.71 \cdot 10^{-1}$	0.9967
	Whole cow	5-750	$1.05 \cdot 10^{-2} \pm 2.39 \cdot 10^{-4}$	$-7.75 \cdot 10^{-3} \pm 8.63 \cdot 10^{-2}$	0.9996
	Goat	5-750	$9.40 \cdot 10^{-3} \pm 4.14 \cdot 10^{-4}$	$4.03 \cdot 10^{-2} \pm 1.45 \cdot 10^{-1}$	0.9985
	Sheep	5-750	$1.21 \cdot 10^{-2} \pm 2.20 \cdot 10^{-4}$	$-5.93 \cdot 10^{-2} \pm 8.09 \cdot 10^{-2}$	0.9998
	Human	5-750	$9.44 \cdot 10^{-3} \pm 1.04 \cdot 10^{-3}$	$-2.29 \cdot 10^{-1} \pm 3.29 \cdot 10^{-1}$	0.9909

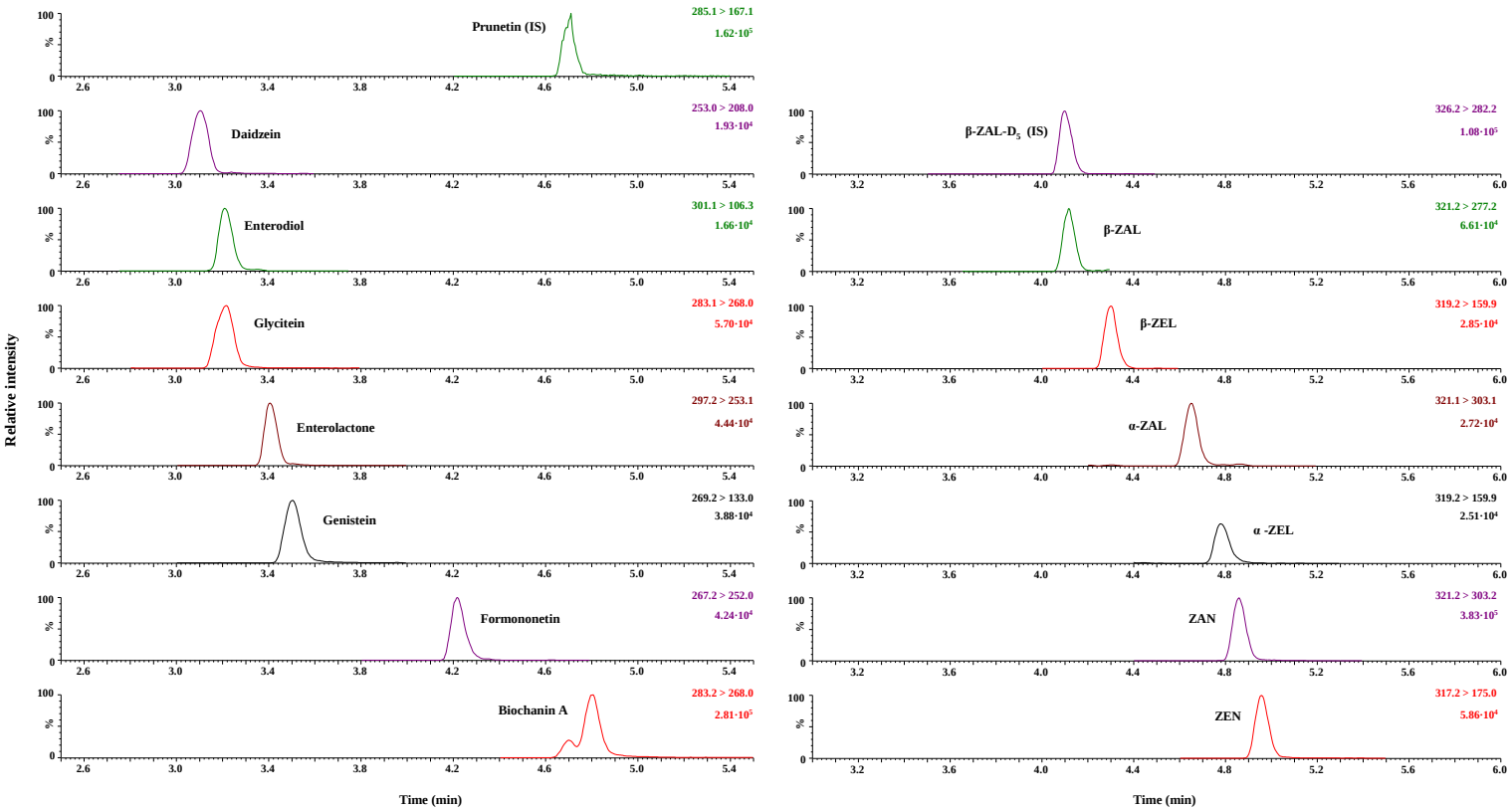
R^2: Determination coefficient.

In addition, the recovery and reproducibility of the method were also evaluated. For this purpose, five replicates of each matrix were spiked at two levels of concentration (13.4 and 250 µg/L), extracted and analysed following the developed methodology. Figure IV.37 shows the quantification transition obtained for each analyte when whole cow milk was analysed. Similar chromatograms were also obtained for the other types of milk products. At the same time, a blank of each sample was also extracted and spiked at the end of the procedure. 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.

Table IV.27 shows the results obtained in the recovery study. As can be seen, recovery values were in the range 70-120 % with RSDs lower than 18 % in all cases, which demonstrates the high extraction capacity of the applied sorbent as well as the excellent reproducibility of the whole method. The LOQs of the method, defined as the lowest matrix-matched calibration concentration which provided a S/N higher than 10 for the quantification transition and at least 3 for the confirmation transition (if it was available) were also calculated. The resulting values, in the range 0.34-3.3 µg/L for all samples, were lower than the ones previously obtained by González-Sálamo et al. for the extraction of mycotoxins from milk samples using the same type of sorbent (González-Sálamo et al., 2017), probably caused by the detection system used in this cased, which evidences the great sensitivity achieved with the developed method combining Fe_3O_4@pDA m-µ-dSPE with UHPLC-QqQ-MS/MS analysis.

Regarding the application of other types of NPs for the extraction of oestrogens from milk samples using dSPE and previous to the development of this work, most applications were focused on the determination of natural and synthetic oestrogens using a silica NPs-MIP sorbent (Yuan et al., 2012) or m-NPs coated with MIPs (Qiao et al., 2014), MWCNTs (Ding et al., 2011) or other polymers such as polypyrrole (Gao et al., 2011), although the analysis of mycotoxins was also carried out in one occasion using graphitised carbon black (GCB) with NPs, Fe_3O_4@GCB (Capriotti et al., 2016a). The limits achieved in this work are similar to the ones obtained in such publications (even in the cases in which specific MIPs were used) except when polypyrrole m-NPs and Fe_3O_4@GCB were applied for which LOQs were slightly lower. However, none of these works have determined the high number and variety of compounds evaluated in this work. Besides, the synthesis procedures used in these articles were considerably more complex than the one described in this work, demonstrating the simplicity and time-saving of the new proposed methodology.

(A)


Relative intensity
Time (min)
Prunetin (IS)
285.1 > 167.1
1.62·10⁵
Daidzein
253.0 > 208.0
1.93·10⁴
Enterodiol
301.1 > 106.3
1.66·10⁴
Glycitein
283.1 > 268.0
5.70·10⁴
Enterolactone
297.2 > 253.1
4.44·10⁴
Genistein
269.2 > 133.0
3.88·10⁴
Formononetin
267.2 > 252.0
4.24·10⁴
Biochanin A
283.2 > 268.0
2.81·10⁵
β-ZAL-D5 (IS)
326.2 > 282.2
1.08·10⁵
β-ZAL
321.2 > 277.2
6.61·10⁴
β-ZEL
319.2 > 159.9
2.85·10⁴
α-ZAL
321.1 > 303.1
2.72·10⁴
α -ZEL
319.2 > 159.9
2.51·10⁴
ZAN
321.2 > 303.2
3.83·10⁵
ZEN
317.2 > 175.0
5.86·10⁴

(B)

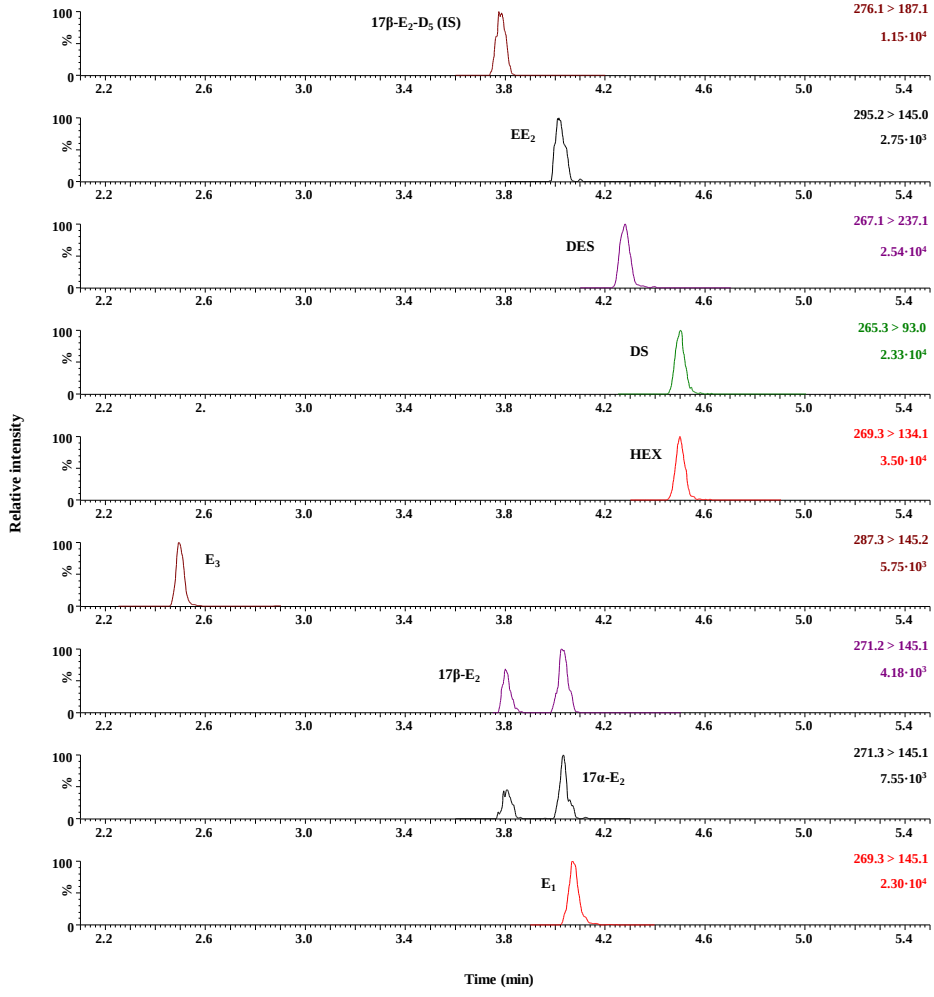


Figure IV.37.- UHPLC-MS/MS chromatograms of (A) phytoestrogens and mycoestrogens and (B) natural and synthetic oestrogens and their respective ISs of a spiked whole cow milk sample after the m-µ-dSPE procedure. Injection volume: 5 µL. Sample dissolved in 500 µL of 50/50 (v/v) MeOH/H_2O. Separation at 40 °C. Concentration in the sample: 125 µg/L of ISs and 16.7 µg/L of the target analytes.

Table IV.27.- Results of the recovery study (n = 5) of the m-µ-dSPE-UHPLC-MS/MS method for the selected compounds in the different matrices at two levels of concentration.

Analyte	Type of milk	Level 1[a] (n = 5)	Level 2[b] (n = 5)	LOQ_{method}[c] (µg/L)	Analyte	Type of milk	Level 1[a] (n = 5)	Level 2[b] (n = 5)	LOQ_{method}[c] (µg/L)
		Recovery % (RSD, %)	Recovery % (RSD, %)				Recovery % (RSD, %)	Recovery % (RSD, %)	
Daidzein	Skimmed cow	86 (8)	94 (9)	0.34	ZAN	Skimmed cow	106 (9)	102 (6)	0.34
	Whole cow	90 (7)	119 (10)	0.34		Whole cow	100 (2)	113 (3)	0.34
	Goat	83 (9)	99 (9)	0.34		Goat	107 (6)	118 (4)	0.34
	Sheep	106 (8)	105 (9)	0.34		Sheep	109 (12)	105 (5)	0.34
	Human	-	-	-		Human	115 (8)	111 (7)	3.3
Enterodiol	Skimmed cow	70 (10)	72 (13)	1.7	ZEN	Skimmed cow	115 (6)	112 (5)	0.34
	Whole cow	72 (13)	70 (5)	1.7		Whole cow	116 (7)	115 (5)	0.34
	Goat	79 (17)	77 (10)	1.7		Goat	104 (4)	112 (2)	0.34
	Sheep	76 (12)	88 (13)	1.7		Sheep	118 (12)	116 (5)	0.34
	Human	95 (11)	82 (7)	1.7		Human	82 (9)	77 (4)	0.34
Glycitein	Skimmed cow	81 (8)	90 (8)	0.34	EE_2	Skimmed cow	62 (15)	75 (15)	1.7
	Whole cow	92 (10)	99 (12)	0.34		Whole cow	81 (15)	84 (7)	1.7
	Goat	72 (15)	90 (10)	0.34		Goat	72 (13)	88 (14)	1.7
	Sheep	82 (10)	113 (9)	0.34		Sheep	98 (15)	91 (8)	3.3
	Human	-	-	-		Human	83 (16)	108 (9)	3.3
Enterolactone	Skimmed cow	87 (7)	81 (13)	0.34	DES	Skimmed cow	86 (15)	70 (6)	1.7
	Whole cow	85 (8)	94 (10)	0.34		Whole cow	71 (6)	76 (5)	1.7
	Goat	80 (11)	91 (8)	0.34		Goat	70 (12)	71 (9)	1.7
	Sheep	113 (13)	108 (10)	0.34		Sheep	82 (10)	83 (7)	1.7
	Human	106 (12)	118 (7)	0.34		Human	82 (14)	77 (18)	1.7
Genistein	Skimmed cow	80 (5)	80 (5)	0.34	DS	Skimmed cow	70 (14)	71 (13)	1.7
	Whole cow	100 (8)	90 (10)	0.34		Whole cow	75 (14)	72 (7)	1.7
	Goat	73 (7)	80 (8)	0.34		Goat	74 (13)	74 (13)	1.7
	Sheep	87 (6)	103 (7)	0.34		Sheep	85 (10)	80 (7)	1.7
	Human	-	-	-		Human	95 (16)	80 (16)	1.7
Formononetin	Skimmed cow	89 (13)	84 (16)	1.7	HEX	Skimmed cow	75 (7)	77 (14)	1.7
	Whole cow	111 (13)	94 (14)	1.7		Whole cow	73 (16)	80 (5)	1.7
	Goat	88 (7)	89 (10)	1.7		Goat	72 (2)	78 (12)	1.7
	Sheep	101 (8)	86 (8)	0.34		Sheep	95 (7)	77 (7)	1.7
	Human	111 (9)	100 (13)	1.7		Human	80 (16)	100 (16)	1.7

Table IV.27.- (Continued).

Analyte	Type of milk	Level 1[a] (n = 5) Recovery % (RSD, %)	Level 2[b] (n = 5) Recovery % (RSD, %)	LOQ_{method}[c] (µg/L)	Analyte	Type of milk	Level 1[a] (n = 5) Recovery % (RSD, %)	Level 2[b] (n = 5) Recovery % (RSD, %)	LOQ_{method}[c] (µg/L)
	Skimmed cow	72 (7)	72 (12)	1.7		Skimmed cow	63 (14)	72 (11)	1.7
	Whole cow	89 (13)	90 (10)	1.7		Whole cow	78 (14)	72 (6)	1.7
Biochanin A	Goat	77 (12)	72 (7)	1.7	E_3	Goat	85 (13)	71 (7)	1.7
	Sheep	87 (6)	70 (8)	0.34		Sheep	78 (10)	89 (9)	1.7
	Human	72 (16)	109 (17)	1.7		Human	86 (15)	72 (18)	1.7
	Skimmed cow	102 (5)	100 (2)	0.34		Skimmed cow	93 (14)	85 (15)	3.3
	Whole cow	93 (5)	95 (3)	0.34		Whole cow	83 (16)	103 (6)	3.3
β-ZAL	Goat	94 (4)	105 (1)	0.34	17β-E_2	Goat	83 (13)	74 (15)	3.3
	Sheep	94 (10)	95 (2)	0.34		Sheep	83 (12)	86 (6)	3.3
	Human	100 (9)	97 (3)	0.34		Human	112 (17)	106 (13)	3.3
	Skimmed cow	105 (5)	109 (6)	0.34		Skimmed cow	74 (11)	77 (12)	1.7
	Whole cow	100 (7)	115 (3)	0.34		Whole cow	75 (9)	90 (5)	1.7
β-ZEL	Goat	98 (5)	116 (1)	0.34	17α-E_2	Goat	71 (12)	91 (10)	1.7
	Sheep	90 (5)	115 (4)	0.34		Sheep	86 (14)	80 (8)	3.3
	Human	101 (12)	110 (3)	0.34		Human	110 (12)	107 (7)	3.3
	Skimmed cow	112 (2)	110 (3)	0.34		Skimmed cow	110 (13)	87 (9)	1.7
	Whole cow	104 (5)	120 (1)	0.34		Whole cow	88 (11)	103 (5)	1.7
α-ZAL	Goat	106 (3)	106 (1)	0.34	E_1	Goat	98 (7)	104 (11)	1.7
	Sheep	104 (8)	112 (2)	0.34		Sheep	102 (12)	97 (4)	1.7
	Human	113 (10)	90 (2)	0.34		Human	72 (7)	99 (12)	1.7
	Skimmed cow	102 (8)	120 (6)	1.7					
	Whole cow	120 (7)	100 (2)	1.7					
α-ZEL	Goat	114 (7)	116 (1)	1.7					
	Sheep	112 (5)	101 (4)	1.7					
	Human	103 (14)	85 (7)	1.7					

[a] Concentrations of the analytes in the samples: 13.4 µg/L. [b] Concentrations of the analytes in the samples: 250 µg/L. [c] Defined as the lowest matrix-matched calibration concentration which provided a S/N higher than 10 for the quantification transition and at least 3 for the confirmation transition (if it was available).

IV.6.5.- Conclusions

From the results obtained in this section the following conclusions can be drawn:

- A fast and environmentally friendly methodology based on Fe_3O_4@pDA NPs μ-dSPE followed by UHPLC-MS/MS has been developed for the analysis of twenty one oestrogenic compounds including seven phytoestrogens (daidzein, enterodiol, glycitein, enterolactone, genistein, formononetin and biochanin A), six mycoestrogens (ZAN, ZEN, α-ZAL, β-ZAL, α-ZEL and β-ZEL), four natural (17α-E_2, 17β-E_2, E_1 and E_3) and four synthetic oestrogens (HEX, EE_2, DES and DS) in different milk samples.

- Matrix effect was evaluated for all matrices finding an important influence of the matrix in almost all cases, especially when natural and synthetic oestrogens were analysed in human breast milk for which matrix effect percentages (based on the Matuszewski method) were in the range 15-22 %, for almost all cases. A particular influence was found for daidzein, glycitein and genistein in human breast milk in which these analytes were not able to be analysed, possibly due to their bond to proteins not removed during the deproteinisation procedure. These results suggest the necessity of the development of a deeper evaluation of the extraction of these three phytoestrogens from human milk in future studies.

- The whole method was validated in terms of linearity by the preparation of matrix-matched calibration curves, taking into account the results obtained from the matrix effect evaluation. R^2 values were higher than 0.9909 and LOQs of the method were in the range 0.34-3.3 μg/L in all studied matrices. The obtained results demonstrated the good linearity of the method in the range of concentration studied as well as the excellent sensitivity that can be achieved by dSPE using Fe_3O_4@pDA NPs and the subsequent analysis by UHPLC-QqQ-MS/MS which is better than the single previous work in which this sorbent was applied for a similar purpose.

- The methodology was also validated by recovery and reproducibility studies obtaining recovery values between 70 and 120 % in all cases with RSDs below 18 % which demonstrated the great extraction capacity of the developed methodology and its excellent reproducibility.

- This method constitutes one the first applications of Fe_3O_4@pDA NPs for the extraction of oestrogenic compounds and the first work in which natural and synthetic oestrogens and

phytoestrogens have been extracted from milk samples using this sorbent. It is also the first time that they have been simultaneously determined with mycoestrogens in such samples.

- To the best of our knowledge, this is also the first time in which these oestrogenic compounds have been determined in sheep milk samples.

CHAPTER V
GENERAL CONCLUSIONS

V.- GENERAL CONCLUSIONS

From the experimental results obtained in the present PhD Thesis and in order to provide a general view of the developed work, the following general conclusions can be drawn:

- New analytical methodologies have been developed for the determination of natural, synthetic, myco- and phytoestrogens in different environmental and food samples including tap, mineral, pond and wastewater, different types of milk such as skimmed, semi-skimmed and whole milk with cow, goat, sheep and human origin as well as cheese, yogurt, probiotic products and kefir samples.
- Different modalities of LC, including HPLC and UHPLC combined with conventional detectors such as diode array and fluorescence detectors as well as MS/MS have been successfully applied for the separation and quantification of the studied oestrogenic compounds obtaining low LODs and LOQs in the range of µg/L.
- LPME techniques, including HF-LPME and DLLME, have been applied for the selective extraction and preconcentration of the target oestrogenic compounds in food and water samples. Additionally, a laboratory synthesised IL has been evaluated as an alternative extraction solvent in DLLME with good results.
- The QuEChERS method has been successfully applied as a selective extraction and clean up procedure for the analysis of milk and diverse dairy products providing very clean extracts with a reduced amount of coextracted materials.
- Miniaturised methods, based on the use of solid sorbents, such as µ-dSPE and m-µ-dSPE, have been applied for the extraction of the selected oestrogenic compounds from water, milk and different dairy products achieving a high extraction capacity and preconcentration as well as a selective extraction of the target analytes.
- The suitability of pDA coated m-NPs, prepared and characterised in our laboratory, as well as pristine MWCNTs as extraction sorbents was evaluated for the analysis of the target analytes in environmental and food complex samples, demonstrating great selectivity and extraction efficiency.
- The developed methodologies were validated in terms of repeatability, calibration curves, recovery, precision, accuracy, LODs and LOQs in each of the studied matrices demonstrating their effectiveness in the determination of the selected oestrogenic

compounds at the low levels at which they may appear in samples as well as their capacity to obtain reliable and useful analytical information.

- Based on the excellent results obtained, the developed methodologies may be applied for the evaluation of the content of oestrogenic compounds of interest in real samples. In fact, the proposed QuEChERS method was applied for the analysis of a great variety of samples from the Czech and Spanish markets finding the presence of some phytoestrogens and one natural oestrogen at levels above the LOQs of the method.
- The proposed methodologies can be defined as environmentally friendly techniques since they involve a low consumption of organic solvents and the use of small amounts of solid sorbents.
- Taking into account the good results obtained as well as the simplicity and rapidity of the developed methods proposed in the present PhD Thesis, they could be applied as routine methods for the analysis of the selected compounds in the evaluated matrices and, in addition, their applicability could be extended to the analysis of other types of compounds and matrices after the development of a suitable validation study.

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GLOSSARY

GLOSSARY

$^{13}C_{18}$-ZEN	$^{13}C_{18}$-zearalenone
17α-E_2	17α-estradiol
17β-E_2	17β-estradiol
17β-E_2-D_5	17β-estradiol-2,4,16,16,17-d_5
2-MeOE_2	2-methoxyestradiol
2-OHE_2	2-hydroxyestradiol
ACN	Acetonitrile
α-ZAL	α-zearalanol
α-ZEL	α-zearalenol
APCI	Atmospheric pressure chemical ionisation
APFO	Ammonium perfluorooctanoate
API	Atmospheric pressure ionisation
APPI	Atmospheric pressure photoionisation
[BBIm][PF_6]	1,3-dibutylimidazolium hexafluorophosphate
BET	Estimation of the Brunauer-Emmett-Teller
β-ZAL	β-zearalanol
β-ZAL-D_5	β-zeralanol-10,10,11,12,12-d_5
β-ZEL	β-zearalenol
BGE	Background electrolyte
BME	2-mercaptoethanol
[BMIm][PF_6]	1-butyl-3-methylimidazolium hexafluorophosphate
BPC	Base peak chromatogram
BSTFA	N-O-bis-(trimethylsilyl)-trifluoroacetamide

C_{18}	Octadecylsilane
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
CFME	Continuous flow microextraction
CLC	Capillary-liquid chromatography
CNT	Carbon nanotube
CTAB	Cetyltrimethylammonium bromide
CVD	Chemical vapour deposition
CZE	Capillary zone electrophoresis
DA	Dopamine
DAD	Diode array detection
DCM	Dichloromethane
DDME	Drop-to-drop microextraction
DES	Diethylstilbestrol
DI-SDME	Direct immersion-single drop microextraction
DLLME	Dispersive liquid-liquid microextraction
DS	Dienestrol
DSDME	Directly suspended droplet microextraction
dSPE	Dispersive solid-phase extraction
DTE	Dithioerythritol
E_1	Estrone
E_3	Estriol
EC	European Commission
ECD	Electron capture detector

ED	Electrochemical detector
EDC	Endocrine disrupting compound
EE_2	Ethynylestradiol
EFSA	European Food Safety Authority
EI	Electron ionisation
EKC	Electrokinetic chromatography
ELISA	Enzyme-linked immunosorbent assay
EME	Electromembrane microextraction
ER	Oestrogenic receptor
ESI	Electrospray ionisation
EU	European Union
FD	Fluorescence detection
FID	Flame ionisation detector
FO-DLLME	Floating organic-dispersive liquid-liquid microextraction
FTN	Flow-through needle
FWHM	Full-width at half diffraction
GC	Gas chromatography
GCB	Graphitised carbon black
GCxGC	Two dimensional gas chromatography
HEX	Hexestrol
HF	Hollow fibre
HF-LPME	Hollow fibre-liquid-phase microextraction
HLB	Hydrophilic-lipophilic-balanced
[HMIm][PF_6]	1-hexyl-3-methylimidazolium hexafluorophosphate

HPLC	High-performance liquid chromatography
HP-β-CD	Hydroxypropyl-β-cyclodextrin
HS-SDME	Headspace-single drop microextraction
i.d.	Internal diameter
ICC	Ion charge control
IL	Ionic liquid
IP	Isoelectric point
IR	Infrared
IS	Internal standard
IT	Ion trap
IUPAC	International Union of Pure and Applied Chemistry
JCPDS	Joint Committee on Powder Diffraction Standards
K_a	Acidity constant
K_{ow}	Octanol/water partition-coefficient
LC	Liquid chromatography
LCxLC	Two dimensional liquid chromatography
LLE	Liquid-liquid extraction
LLLME	Liquid-liquid-liquid microextraction
LOD	Limit of detection
LOQ	Limit of quantification
LPME	Liquid-phase microextraction
MAE	Microwave-assisted extraction
MALDI	Matrix-assisted laser desorption/ionisation
m-dSPE	Magnetic-dispersive solid-phase extraction

MEEKC	Micro-emulsion electrokinetic chromatography
MEKC	Micellar electrokinetic chromatography
MeOH	Methanol
MIP	Molecularly imprinted polymer
m-µ-dSPE	Magnetic-micro-dispersive solid-phase extraction
MOF	Metal-organic framework
MRL	Maximum residue limit
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MSPD	Matrix-solid phase dispersion
MSTFA	N-methyl-N-trimethylsilyltrifluoroacetamide
MTBSTFA	N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide
µ-dSPE	Micro-dispersive solid-phase extraction
µ-SPE	Micro-solid-phase extraction
MWCNT	Multi-walled carbon nanotube
nano-LC	Nano-liquid chromatography
NMR	Nuclear magnetic resonance
NP	Nanoparticle
o.d	Outer diameter
[OMIm][PF_6]	1-octyl-3-methylimidazolium hexafluorophosphate
PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate buffered saline
PCB	Polychlorinated biphenyl

pDA	Polydopamine
PDMS	Polydimethylsiloxane
PE	Polyethylene
PET	Polyethylene terephthalate
PFBBr	Pentafluorobenzyl bromide
PFPA	Pentafluoropropionic anhydride
PLE	Pressurized liquid extraction
PP	Polypropylene
[PPIm][PF_6]	1,3-dipenthylimidazolium hexafluorophosphate
PTFE	Polytetrafluoroethylene
PVFD	Polyvinylidene fluoride
Pz	Z potential
Q	Simple quadrupole
QqQ	Triple quadrupole
QTOF	Quadrupole-time of flight
Qtrap	Triple quadrupole/linear ion trap
R^2	Determination coefficient
RIA	Radioimmunoassay
RSD	Relative standard deviation
SBSE	Stir bar sorptive extraction
SCSE	Stir cake sorptive extraction
SDME	Single-drop microextraction
SDS	Dodecyl sodium sulfate
SEM	Scanning electron microscopy

SFE	Supercritical fluid extraction
SFO-DLLME	Solidified organic drop-dispersive liquid-liquid microextraction
SLE	Solid-liquid extraction
SLM	Supported liquid membrane
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
SWCNT	Single-walled carbon nanotube
TEA	Triethylamine
TEM	Transmission electron microscopy
THF	Tetrahydrofuran
TMCS	Trimethylchlorosilane
TMIS	Trimethyliodosilane
TOF	Time of flight
UHPLC	Ultra-high-performance liquid chromatography
UV	Ultraviolet
VSM	Vibrating sample magnetometer
XPS	X-ray photoelectron spectroscopy
XRD	X-ray diffraction
ZAN	Zearalanone
ZEN	Zearalenone