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Validation of an ultra high performance liquid chromatography–tandem mass spectrometry method for detection and quantitation of 19 endocrine disruptors in milk

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ABSTRACT

An endocrine disruptor (ED) is an exogenous compound that interferes with the body's endocrine system. Exposure to EDs may result in adverse health effects such as infertility and cancer. EDs are composed of a vast group of chemicals including compounds of natural origin such as phytoestrogens or mycotoxins and a wide range of man-made chemicals such as pesticides. Synthetic compounds may find their way into the food chain where a number of them can biomagnify. Additionally, processing activities and food contact materials may add further to the already existing pool of food contaminants. Thus, our diet is considered to be one of the main exposure routes to EDs. Some precautionary legislation has already been introduced to control production and/or application of some persistent organic pollutants with ED characteristics. However, newly emerging EDs with bioaccumulative properties have recently been reported to appear at lower tiers of the food chain but have not been monitored at the grander scale.

Milk and dairy products are a major component of our diet, thus it is important to monitor them for EDs. However, most methods developed to date are devoted to one group of compounds at a time. The UHPLC–MS/MS method described here has been validated according to EC decision 2002/657/EC and allows simultaneous extraction, detection, quantitation and confirmation of 19 EDs in milk. The method calibration range is between 0.50 and 20.0 μg kg^{-1} with coefficients of determination above 0.99 for all analytes. Precision varied from 4.7% to 23.4% in repeatability and reproducibility studies. Established $CC\alpha$ and $CC\beta$ values (0.11–0.67 μg kg^{-1}) facilitate fast, reliable, quantitative and confirmatory analysis of sub μg kg^{-1} levels of a range of EDs in milk.

1. Introduction

An endocrine disruptor (ED) has been defined as ' … an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)population' (IPCS, 2002). The adverse effects of exposure to EDs have been extensively described and depending on the time of the exposure include; disrupted reproductive function through reduced semen quality, poor pregnancy outcomes, infertility, urogenital tract abnormalities and cancer (Diamanti-Kandarakis et al., 2009). EDs have also been associated with thyroid, adrenal, metabolic neurodevelopmental and –behavioural disorders (WHO & UNEP, 2012). EDs encompass a vast group of chemicals consisting of natural compounds such as phytoestrogens or mycotoxins but also a wide range of man-made chemicals. Synthetic EDs can be found in an infinite range of consumer products from furniture and clothing (perfluoroalkylated compounds (PFCs)) to electrical appliances, car interiors (brominated flame retardants) and cleaning products (antimicrobials). A number of them are used as pesticides, in food packaging and plastics (phthalates), personal care products (UV-filters) or as food preservatives (parabens) (WWF, 2006). Inappropriate disposal of waste and 'leaching' allows such compounds to find their way into the environment and the food chain where a number of them have been found to biomagnify. Additionally, food processing activities and food contact materials may add to the already existing pool of

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food contaminants. Our diet is considered to be one of the main exposure routes to EDs (Connolly, 2009). Since milk and dairy products are a major component of our diets (IUNA, 2011) screening as well as quantitative analysis of ED contamination is important in these food types.

Chemical production has reached 400 million tons globally (Casals-Casas & Desvergne, 2011). This is a matter of concern for further contamination of the food chain. Precautionary legislation (EU., 2007; UN., 2001) has already been introduced to control production and/or application of compounds such as certain chlorinated pesticides, polybrominated biphenyl ethers (PBDEs) and phthalates, which have been confirmed to have a detrimental effect on human health and detected in various food products (WWF, 2006). Nevertheless, there is a number of newly emerging environmental contaminants, such as antimicrobials or UV-filters, which are not currently monitored but have been reported at the lower levels of the food chain and possess bioaccumulative properties (Diaz-Cruz et al., 2009; Ramaswamy et al., 2011). The contamination routes for these emerging contaminants should be investigated but may include practices such as the land application of biosolids, one which is favoured by the European Commission in the management of sludge (Clarke & Smith, 2011). The transfer of classic persistent organic pollutants (POPs) to milk and effects exerted on animals grazing on soil previously treated with sewage sludge have been described (Lind et al., 2010; Rychen, Jurjanz, Toussaint, & Feidt, 2008; Young, South, Begley, Diachenko, & Noonan, 2012). Thus, there is a growing concern about emerging pollutants which have been detected in elevated concentrations in sludge all over the world such as PFCs, UV filters, synthetic musks and antimicrobials (Clarke & Smith, 2011; Diaz-Cruz et al., 2009; Giokas, Salvador, & Chisvert, 2007; Kupper et al., 2006; Liu, Ying, Shareef, & Kookana, 2011; Zhang et al., 2011). Taking into consideration the physiochemical properties of those compounds (similar to PFCs and PBDEs) and confirmed world-wide contamination at lower levels of the food chain (Diaz-Cruz et al., 2009; Ramaswamy et al., 2011), concerns over contamination of higher levels of the food chain seems to be justified.

Many classes of EDs may be present in milk. A number of studies report the presence of POPs such as PBDEs, dioxins, chlorinated pesticides and their metabolites in milk (Table 1), alongside a decrease in their concentrations due to introduced legislation (Abad, Llerena, Saulo, Caixach, & Rivera, 2002; Bocio et al., 2003; Kaushik, Sharma, Gulati, & Kaushik, 2011; Petro et al., 2010; Schecter et al., 2006; Schmid, Gujer, Zennegg, & Studer, 2003). Contaminants such as PFCs (Haug et al., 2010; Noorlander, van Leeuwen, Biesebeek, Mengelers, & Zeilmaker, 2011), phthalates (Casajuana & Lacorte, 2004; Sorensen, 2006) and 4-nonylphenol have also been detected in milk at low $\mu g\ kg^{-1}$ levels (Guenther et al., 2002; Shao, Han, Tu, & Huang, 2007). In the case of pyrethroid pesticides (and their metabolites), their transfer to milk has been confirmed (Chen, Fink, Letinski, Barrett, & Pearsall, 1997). However, no residues (Guvenc & Aksoy, 2010) or non-compliant samples (EU, 2008–2011) have been reported. Additionally, there is no data available on the presence of pyrethroid metabolites in milk samples as legislation only requires monitoring of parent compounds. Finally, there is also a lack of data on the possible transfer of personal care products to milk.

A variety of chemical assays, employing both LC and GC with different detectors have been developed for screening of single contaminant groups (Table 1). However, no methods have been described which screen a broad range of EDs in milk. This may be due to the varied physicochemical properties within ED groups, making them challenging to analyse in one method but also due to background contamination as in the case of phthalates, phenols and PFCs.

Table 1
Reported levels of contaminants in milk (values in brackets quoted next to natural hormones concentrations represent the level of conjugation of the hormone in milk).

Class	Compound	Reported concentrations		References
		ng/L		
Natural hormones	17α-estradiol	31.7	(69%)	(Courant et al., 2008)
	17β-estradiol	13.5	(81%)	
	Estrone	187	(93%)	
	Epiandrosterone	99	(13%)	
	α-testosterone	9.0	(60%)	
	Androsenedione	876		
	Progesterone	2.1	skim milk	(Hartmann, Lacorn & Steinhart, 1998)
		5.8	low fat milk	
		9.5	whole milk	
		11.3	raw milk	
		μg/L		
Mycotoxins	Zearalenone	N.D		(Xia et al., 2009)
	α-zearalanol	3.0		
		μg/L		
Phytoestrogens	Genistein	1.9–3.0		(Nielsen, Norgaard, Purup, Frette, & Bonefeld-Jorgensen, 2009; Steinshamn, Purup, Thuen, & Hansen-Moller, 2008)
	Daidzein	0.3–5.7; 0.2–7.7		
	Equol	52–364; 57–1003		
	Enterolactone	21.6–38.8; 11.4–24.6		
		pg/g		
BfRs	Sum of PBDEs	24; 7.9		(Bocio et al., 2003; Schecter et al., 2006)
		pg I-TEQ/g		
Dioxins	PCDD/Fs	0.36; 0.63		(Abad et al., 2002; Schmid et al., 2003)
	PCDD/Fs + dl-PCBs	1.0		
		ng/g lipid weight		
Organochlorine compounds	Sum of PCBs	15.5		(Petro et al., 2010)
	HCB	4.7		
	p,p′-DDE	19.0		
	Sum of HCH	1.9		
		pg/g		
PFCs	PFOA	4.7		(Haug et al., 2010; Noorlander et al., 2011)
	PFOS	10; 7		
		ng/g		
Phthalates	DEHP	7–30		(Sorensen, 2006; Casajuana & Lacorte, 2004)
	DBP	<9; 7.3–50		
	BBP	<4; 1.1–2.9		
Phenols	4-NP	4.2–17.6; 16.5–34.8		(Casajuana & Lacorte, 2004; Shao et al., 2007)
	BPA	0.28–2.6; 0.49		

This study aimed to develop a multi-residue method for the determination of 19 EDs in milk, including emerging EDs which have not previously been assessed. To our knowledge, this is the most extensive analytical method that has been developed for the determination of such a wide range of EDs in milk to date.

2. Experimental

2.1. Reagents and apparatus

Ultra-pure water (18.2 MΩ cm^{-1}) was generated in-house using a Millipore (Merk Millipore, Billerica, MA, USA) water purification system. Fisher Scientific UK Ltd (Loughborough, UK) provided LC–MS grade acetonitrile (MeCN) and methanol (MeOH). Acetone, isopropanol (IPA), ethyl acetate, ammonium hydroxide solution (32%) puriss p.a. (NH_4OH), acetic acid (AcOH), formic acid (HCOOH), sodium acetate (NaOAc), ammonium acetate (NH_4OAc), sodium chloride puriss p.a. (NaCl) were sourced from Sigma–Aldrich (St Louis, MO, USA). Magnesium sulphate puriss p.a. anhydrous ($MgSO_4$) was ordered for VWR International Ltd (Lutterworth, UK).

Table 2
UHPLC–MS/MS conditions for milk where: MW = Molecular weight tr = Retention Time CE = Collision Energy and MRM widows: MRM 1 (1.75–2.35 min); MRM 2 (2.21–2.47 min); MRM 3 (2.44–3.02 min); MRM 4 (2.9–3.2 min); MRM 5 (3.05–3.30 min); MRM 6 (3.4–4.35 min); MRM 7 (4.35–4.67 min); MRM 8 (4.62–4.88 min); MRM 9 (4.92–5.25 min); MRM 10 (5.50–5.7 min); MRM 11 (6.0–6.24 min); MRM 12 (6.22–6.45 min); MRM 13 (6.38–6.54 min).

Group	No	Compound	Formula	MW [g/mol]	tr [min]	Molecular ions [M ± H]	Cone [V]	Fragment ions	CE [V]	MRM window	ESI
Hormones	1	Testosterone	$C_{19}H_{28}O_2$	288.2	4.8	289.3	30	97.1 109.0	20 25	8	+
	2	Progesterone	$C_{21}H_{30}O_2$	314.2	6.1	315.2	30	96.8 106.9	20 25	11	+
Isoflavones	3	Daidzein	$C_{15}H_{10}O_4$	254.1	2.4	254.8	50	136.9 199.0	27 22	2	+
	4	Genistein	$C_{15}H_{10}O_5$	270.1	3.0	271.1	40	91.0 152.9	37 27	4	+
Mycotoxins	5	Zearalenone	$C_{18}H_{22}O_5$	318.2	5.0	317.0	45	174.9 273.1	25 26	9	−
	6	α-Zearalenol	$C_{18}H_{24}O_5$	320.2	4.5	319.0	46	91.9 136.8	30 18	7	−
Perfluoroalkylated compounds	7	Perfluorooctanoic acid	$C_8HF_{15}O_2$	414.0	4.2	412.9	13	168.9 368.9	17 10	6	−
	8	Perfluoroheptanoic acid	$C_7HF_{13}O_2$	364.0	3.6	363.1	10	169.0 319.1	15 10	6	−
	9	Perfluorooctane sulfonic acid	$C_8HF_{17}O_3S$	499.9	5.1	498.9	55	80.0 98.9	45 35	9	−
Preservatives and antimicrobials	10	Paraben methyl	$C_8H_8O_3$	152.1	2.1	150.9	25	91.9 135.9	20 14	1	−
	11	Paraben ethyl	$C_9H_{10}O_3$	166.1	2.9	165.1	25	92.1 137.1	22 14	3	−
	12	Paraben propyl	$C_{10}H_{12}O_3$	180.1	3.8	178.9	28	91.9 135.9	20 14	6	−
	13	Paraben butyl	$C_{11}H_{14}O_3$	194.1	4.6	193.0	35	91.9 136.8	24 16	7	−
	14	Trichlorocarban	$C_{13}H_9Cl_3N_2O$	314.0	6.3	313.0 315.0	25	125.8 127.8	22 22	12	−
UV filter	15	Benzophenone-3	$C_{14}H_{12}O_3$	228.1	5.6	229.2	25	105.1 151.1	20 20	10	+
Phthalate	16	Benzyl butyl phthalate	$C_{19}H_{20}O_4$	312.1	6.5	313.1	18	90.9 204.9	21 8	13	+
Pyrethroid metabolites	17	4-Fluoro-3-phenoxybenzoic acid	$C_{13}H_9FO_3$	232.1	2.2	230.9	25	91.9 135.9	29 13	1	−
	18	3-Phenoxybenzoic acid	$C_{13}H_{10}O_3$	214.1	2.1	212.9	22	92.9 169.0	22 15	1	−
	19	Cyhalothric acid	$C_9H_{10}ClF_3O_2$	242.0	3.2	240.9	12	134.9 204.9	15 9	5	−

Polypropylene tubes (50 and 15 mL) with screw caps were obtained from Sigma–Aldrich (St Louis, MO, USA). Sorvall Legend RT centrifuge (Thermo Scientific, Waltham, MA, USA), vacuum manifold IST VacMaster (Biotage, Uppsala, Sweden) and Turbovap LV evaporator (Caliper Life Sciences, Mountain View, USA) were used during sample preparation.

Standards were purchased from Sigma–Aldrich : testosterone, progesterone, progesterone-d$_9$, genistein, daidzein, 3,4,4′-trichlorocarbanilide (TCC), methyl 4-hydroxybenzoate (PB–Me), ethyl 4-hydroxybenzoate (PB–Et), propyl 4-hydroxybenzoate (PB–Pr), butyl 4-hydroxybenzoate (PB–Bu), benzyl butyl phthalate (BBP), 2-hydroxy-4-methoxybenzophenone (Bp-3), heptadecafluorooctane sulfonic acid potassium salt (PFOS K-salt), perfluoroheptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), zearalenone (ZON), alpha zearalenol (α-ZOL). 3-phenoxybenzoic acid (3-PBA), 4-fluoro-3-phenoxybenzoic acid (4-F-3-PBA) were purchased from Dr. Ehrenstorfer GmbH (Augsbusrg, Germany) while cis-3-(2-chloro-3,3,3-trifluoro-1-propenyl)-2,2-dimethyl cyclopropanecarboxylic acid (λ-cyhalothric acid) was obtained from TRC (Toronto Research Chemicals, North York, Ontario, Canada).

Molecular formulae and weight of individual compounds are included in Table 2. All of the standard solutions were prepared in MeOH at 4 mg mL^{-1} with the exception of genistein, daidzein, ZON, a-ZOL, 3-PBA, λ-cyhalothric acid and progesterone-d$_9$ which were prepared at 1 mg mL^{-1} 4-F-3-PBA was supplied as a solution of 100 μg mL^{-1} in MeCN. An intermediate mixed standard solution was prepared at 40 μg mL^{-1} for all other compounds. Primary and intermediate stock standard solutions were found to be stable for at least 12 months when stored at −20 °C during ‘in-house’ stability studies.

2.2. *Choice of target compounds*

This method has been developed as a complimentary method to an oestrogen reporter gene assay (RGA) screening method (Wielogorska, Elliott, Danaher, & Connolly, 2014) with the aim to create a holistic screening approach enabling the association of detected biological activity with the targeted EDs. This will provide a valuable tool for further research into EDCs, biomonitoring and risk assessment. The analytes of interest were chosen through a literature review focussing on compounds which are already known to be EDs (isoflavones, mycotoxins, phthalates) or are suspected to have endocrine disrupting properties (pyrethroids metabolites). The targeted compounds were further narrowed down on the basis of their hormonal activity in a biological oestrogenic RGA during primary screening (data not published). The method also aimed to include a variety of substances which had already been reported in milk (Table 1) but through separate LC–MS/MS methods. What is more, the reported contribution of certain EDs to the oestrogenic exposure in an average western diet (Thomson, 2009) as well as possible transfer to milk of novel EDs have been taken into consideration. Finally, available equipment and its sensitivity had a major impact on the choice of analytes (Antignac, Courant, & Le Bizec, 2009a). Also due to variable physicochemical properties of analytes and omnipresent contamination with phthalates (Fankhauser-Noti & Grob, 2007) and phenols, only analytes retained by the extraction method and yielding reasonable levels of background contamination, translating to reliability of the analysis could be included in the final method.

2.3. Preparation of extracted matrix calibrants and recovery control checks

A working mixed standard solution was prepared from the intermediate mixed standard solution at the following concentrations of 4 (std 6), 2 (std 5), 1 (std 4), 0.4 (std 3), 0.2 (std 2), 0.1 (std 1) $\mu g\ mL^{-1}$. 4-F-3-PBA was added to std 6 and 5 solutions and from there diluted to other four. Due to high levels of progesterone in milk (Hartmann et al., 1998) deuterated progesterone-d_9 standard was used for quantitation purposes. Extracted milk matrix calibrants were prepared by spiking negative milk samples (10 g) with 50 μL of the working standard solutions prior to extraction. This gave six point calibration curves in the range 0.50–20 $\mu g\ kg^{-1}$. Recovery controls were prepared by spiking two blank samples post-extraction with std 5 (50 μL) to monitor for loss of analytes during extraction for quality control purposes.

2.4. Sample preparation

Milk samples used in this study included a wide range of matrices i.e. raw milk collected from farms' bulk tanks, commercially available bovine milk of different fat content, butter milk, goat milk, baby formulae, soya and almond milk. Studies of analytes stability in the matrix revealed that some analytes (like isoflavones, PFOS and TCC) were stable in the matrix for only up to two weeks. Thus, all samples were either analysed immediately after collection or stored in their original packaging at −20 °C for no more than two weeks, before being thawed for analysis purposes.

Milk samples (10 g ± 0.01 g) were weighed into 50 mL polypropylene tubes. Samples were fortified with working standard solution and left to stand for 15 min. 1% (v/v) acetic acid in MeCN (12 mL), NaCl (1.0 g) and $MgSO_4$ (2.5 g) were added to each milk sample to form slurry. The slurry was shaken (1 min) by hand, centrifuged (4500 rpm, 10 min) and supernatant transferred to a 15 mL polypropylene tube. The supernatant was evaporated under nitrogen in a Turbovap (60 °C for 60 min). Samples were reconstituted in 5 mL of water and vortexed. HLB Oasis columns were pre-conditioned with 3 mL of TBME, MeOH, and water. Samples were loaded onto the cartridges, which were subsequently washed with 3 mL of water. Analytes were eluted with 6 mL of 1% acetic acid (v/v) in MeOH/TMBE (1:9, v/v). 100 μL of DMSO was used as a keeper while the solvent was evaporated under nitrogen in a Turbovap (60 °C for 40 min) and analytes were diluted with 200 μL of H_2O/MeOH (3:1 v/v), filtered through 0.2 μm PTFE 13 mm Millex syringe filters (Merck Millipore, Billerica, MA, USA) and 10 μL was injected onto the UHPLC–MS/MS system.

2.5. UHPLC–MS/MS conditions

Separations were performed using a Waters (Milford MA, USA) Acquity UPLC system comprising of a stainless steel Acquity HSS T3 analytical column (2.1 × 100 mm, particle size 1.8 μm) equipped with an inline filter and maintained at a temperature of 45 °C while the pump was operated at a flow rate of 0.4 $mL\ min^{-1}$. A binary gradient system was used to separate analytes comprising of mobile phase A, 5 mM ammonium acetate in H_2O/MeCN (9:1, v/v) and mobile phase B, MeOH:MeCN (1:1, v/v).

The gradient profile was linear from 80% A to 30% over 5 min and 0% at 6.5 min then 2 min at 0%A followed by 2 min for re-equilibration at 80%A. After each injection, in the full loop mode, the UHPLC autosampler was sequentially rinsed using strong (0.5 mL) and weak washes (1.5 mL) that consisted of H_2O:MeOH:MeCN:IPA (1:1:1:1 v/v) and H_2O:MeCN (1:1 v/v), respectively. These washes were adjusted to reduce carryover between injections but exert no negative impact on the chromatographic peak shape.

EDs were detected using a Waters Quattro Premier XE triple quadrupole instrument operating in electrospray ionisation (ESI) mode (Milford, MA, USA). The UHPLC–MS/MS system was controlled by MassLynx™ software and data was processed using TargetLynx™ software (both from Waters). The electrospray voltage was set at 3.0 and 1.0 kV for positive and negative modes, respectively. The desolvation and source temperatures were set at 400 and 150 °C, respectively. Nitrogen was employed as the desolvation and cone gas and its flow rate was set to 1000 $L\ h^{-1}$ and 50 $L\ h^{-1}$, respectively. Argon was used as the collision gas, at a flow rate of 0.3 $mL\ min^{-1}$, which typically gave pressures of 3.3×10^{-3} mBar.

The MS conditions were optimised by teed infusion of 1 $\mu g\ mL^{-1}$ standard solutions with syringe flow rate of 10 $\mu L\ min^{-1}$ and 50% mobile phases A and B at flow rate of 0.3 $mL\ min^{-1}$. The cone voltage was optimised for each precursor ion and the two most appropriate product fragment ions produced were selected (Table 2). The MRM windows were time-sectored, dwell time, inter-scan delay and inter-channel delays were set to get maximum response from the instrument i.e. inter-scan delay was set to 2 ms between successive MRM windows while inter-channel delay was set to 5 ms. Dwell times ranged from 5 ms to 250 ms.

2.6. Method validation

The method was validated according to EU directive 2002/657/EC (EU, 2002). The following performance studies were carried out: specificity, linearity, recovery, within laboratory repeatability (WLr) and reproducibility (WLR), decision limit (CCα) and detection limit (CCβ). Validation was carried out at 1.0, 1.5, and 2 times the lowest validation level which was selected based on sensitivity and quantification ability so 1 $\mu g\ kg^{-1}$. Thus blank samples were fortified at 1, 1.5 and 2 $\mu g\ kg^{-1}$ ($n = 6$ for each level).

CCα is defined as the limit above which it can be concluded with an error probability of α, that a sample contains the analyte of interest. *CCβ* is defined as the lowest concentration of analyte at which the method is able to detect, identify and/or quantify the analyte in the truly contaminated samples with a statistical certainty of 1-β. *CCα* and *CCβ* were calculated as defined in 2002/657/EC.

Two different validations were carried out, namely within laboratory repeatability (WLr) and within laboratory reproducibility (WLR), which are described below. The WLr study was carried out by a single analyst, and the method was repeated on three separate days. To evaluate WLR, the method was carried out on three separate days by three different analysts. In total five runs were executed; three by one analyst, and two further runs by two other analysts. The same negative control sample was employed in both WLr and WLR studies.

To evaluate matrix effects in milk matrices, 8 milk samples from different origins, were extracted and then spiked at 5 $\mu g\ kg^{-1}$. The samples tested included milk of bovine origin with different fat content, goat milk, butter milk, almond milk, baby formulae and powdered milk. Matrix effect was evaluated as a ratio of the spiked milk response and response of standard solution of equivalent concentration.

3. Results and discussion

3.1. Method development

3.1.1. UPLC–MS/MS conditions

A range of different mobile phase additives were investigated including buffers (ammonium acetate and formate) and acids (acetic and formic). The most satisfactory results were achieved using mobile phases A and B composed of H_2O:MeCN containing 5 mM ammonium acetate and MeOH:MeCN, respectively. Substances were analysed by electrospray ionization mass spectrometry (ESI–MS) using positive and negative ionisation modes. The conventional single-stage ESI–MS allowed for the detection of protonated molecules $[M + H]^+$ and the deprotonated molecules $[M - H]^-$. In the cases of trichlorocarban, two deprotonated molecular ions resulting from chlorine isotopic pattern of Cl^{35} and Cl^{37} were selected as precursors i.e. 313 and 315 *m/z*. The MS/MS fragmentation pathways for the compounds vary, according to the structural properties of the compounds. For some of the EDs like parabens or PFCs it is a straightforward process (Arsenault, McAlees, McCrindle, & Riddell, 2007; Gonzalez-Marino, Quintana, Rodriguez, & Cela, 2009). For the isoflavones or mycotoxins, however, a loss of water or cleavage of the carbonyl groups with loss of carbon monoxide can be observed. Also more complex rearrangements, like retro-Diels-Alder reaction, can take place (Antignac, Courant, et al., 2009; Antignac, Gaudin-Hirret, et al., 2009; Hildebrand, Pfeiffer, Damm, & Metzler, 2012).

The method being described was developed in accordance to EU Commission Decision 2002/657/EC (EU, 2002) concerning the performance of analytical methods and interpretation of results. Thus at least 4 identification points for every compound tested was required earned by 1 precursor and 2 daughter ions. Alternatively, 2 precursor ions, each with 1 daughter ion earning 5 identification points is also satisfactory. This requirement was achieved for all of the tested compounds (Table 2).

A simple binary gradient was developed for the separation of the EDs on a HSS T3 column. The T3 column was selected due to its versatility (Whelan et al., 2010) which is pivotal in case of analysis of multi-class compounds such as EDs. The MRM windows were time sectored to accommodate ESI positive and ESI negative modes in the same injection. A total of 10–15 data points were typically obtained across a peak to obtain reliable integration and thus reproducible quantitative and qualitative analysis. MRM conditions were established through effective set-up of dwell-times, inter-scan delay and inter-channel delay.

3.1.2. Sample preparation

The aim was to develop a fast, generic extraction method that would be suitable for all 19 analytes. Initial experiments investigated different extraction solvents (MeCN, acetone, ethyl acetate) and their binary mixtures as well as the effect of different volumes on the extraction efficiency. Also the addition of different amounts of acetic acid and salts like magnesium sulphate desiccant and NaCl on extraction efficiency was investigated.

The best extraction solvent turned out to be acidified MeCN due to the best phases separation, protein precipitation and recovery values. Also 12 mL of the solvent proved to improve analytes extraction over lower volumes. Magnesium sulphate improves drying time, however amounts bigger than 2.5 g lowered the analytes recovery. 1.0 g of NaCl was sufficient to induce satisfactory separation of phases.

A number of different sample clean-up procedures were investigated including dispersive solid phase extraction (dSPE) on C_{18} based sorbent, HybridSPE, Oasis HLB and ion exchange columns (WCX, MCX, WAX, MAX). Best recoveries were achieved for both dSPE and HLB column however the latter was chosen due to the glass cartridge, limiting background contamination with phthalates. Evaporation in the Turbovap apparatus was also investigated and temperature of 60 °C was chosen due to significantly improved drying time without loss in recovery. Also a range of different reconstitution solvents were investigated to limit variation due to remaining fat globules in the final extract. DMSO was used as a keeper during final drying stage to improve recoveries and its dilution with MeOH:H_2O (4:1, v/v) was found to provide fat solubilisation but still with satisfactory sensitivity and precision, as reported previously (Whelan et al., 2010).

3.2. Method validation

3.2.1. Selectivity, specificity, linearity and matrix studies

The specificity of the method was established by investigating possible interferences in the UHPLC–MS/MS traces between analytes, compounds similar in structure and deuterated progesterone standard. Typical LC–MS/MS traces for the analytes are shown in Fig. 1.

The selectivity of the method was assessed by testing over 100 different milk samples of different origin and sources without observed interferences. The linearity of the method was evaluated over the ranges specified in Section 2.2 during validation studies. The curves were fitted using a linear fit and their linearity, determined by coefficient of determination (R^2), was greater than 0.99. Suppression effects were also evaluated (Table 3); the greatest enhancement was observed for perfluorooctanoic acid (148%), while the greatest amount of suppression was observed for analytes in positive ionisation mode with the worst being daidzein (57%).

3.2.2. Precision

The results of the repeatability and reproducibility studies (Table 4) indicate that the precision of the method was satisfactory for all analytes. The EC decision 2002/657/EC specifies that ‘for mass fractions lower than 100 $\mu g\ kg^{-1}$, the application of the Horowitz equation gives unacceptably high values. Therefore, the CVs for concentrations lower than 100 $\mu g\ kg^{-1}$ shall be as low as possible’. Obtained CV values are below 20% for majority of compounds and are comparable to those available for other published methods for isoflavones, mycotoxins and the phthalate (Antignac, Gaudin-Hirret, et al., 2009b; Casajuana & Lacorte, 2004; Chen et al., 2013).

3.2.3. Decision limit (CCα) and detection capability (CCβ)

In the case of substances with no permitted limit, samples were to be fortified at 1, 1.5 and 2 × minimum performance limit i.e. 1, 1.5 and 2 $\mu g\ kg^{-1}$. Due to the fact that none of the tested compounds have an established MRL in milk the aim was to achieve lowest possible values for *CCα* and *CCβ*. Based on reported levels of contaminants (Table 1) achieved detection limits seem to provide enough sensitivity to provide reliable analysis of analytes at expected concentrations. Obtained limits for mycotoxins, isoflavones and the phthalate are comparable or better than those in the literature (Antignac, Gaudin-Hirret, et al., 2009b; Casajuana & Lacorte, 2004; Chen et al., 2013; Sorensen, 2006). There are no methods for preservatives, antimicrobials and the UV-filters in milk available in the literature. However, the detection limit for PFCs analysis is rather high when compared to other studies (Karrman et al., 2007; Tao, Kannan, Wong, Arcaro, & Butenhoff, 2008). Nevertheless the methods developed were devoted to PFCs only and thus could be far better tailored to achieve the best performance for this group of

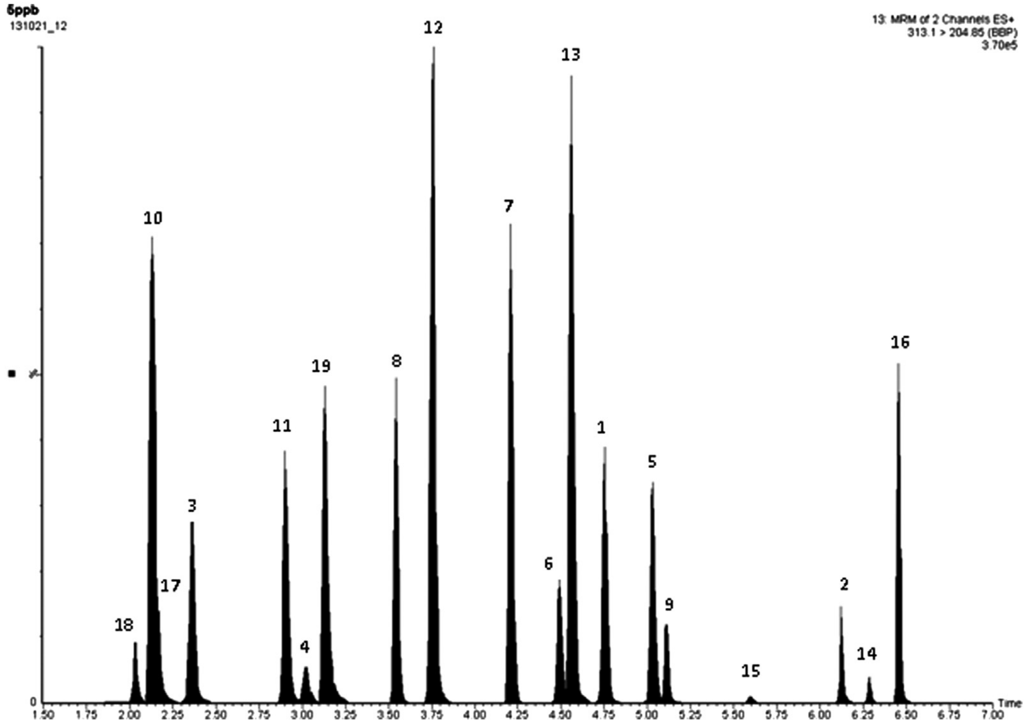

Fig. 1. Overlay of LC–MS/MS chromatograms for all 19 EDs at concentrations of 5 μg kg^{-1}, (numbers represent analytes presented in Table 2).

compounds. What is more, the limits reported in these studies were calculated on a signal to noise basis. The same comment has to be made for testosterone which has been analysed by a steroid hormone devoted method but also using GC–MS/MS (Courant et al., 2008) which allows better sensitivity (Antignac, Courant, et al., 2009a).

Table 3
Recovery and matrix effect study results where Rec = recovery ME = matrix effect CV = covariance and R^2 = coefficient of determination.

Analyte	Rec[a] [%]	CV [%]	ME[b] [%]	CV [%]	Calibration range [μg kg^{-1}]	R^2
Testosterone	105	9	80	10	0.5–20	0.996
Progesterone	100	4	76	24	0.5–20	0.990
Daidzein	120	15	57	48	0.5–20	0.991
Genistein	102	11	68	32	0.5–20	0.999
Zearalenone	105	6	101	10	0.5–20	0.996
α-Zearalenol	107	6	94	11	0.5–20	0.997
Perfluorooctanoic acid	108	8	148	14	0.5–20	0.999
Perfluoroheptanoic acid	108	14	142	9	0.5–20	0.998
Perfluorooctane sulfonic acid	106	8	125	10	0.5–20	0.997
Paraben methyl	99	9	104	21	0.5–20	0.991
Paraben ethyl	105	9	107	16	0.5–20	0.999
Paraben propyl	99	8	109	5	0.5–20	0.995
Paraben butyl	95	8	123	6	0.5–20	0.991
Trichlorocarban	107	4	114	6	0.5–20	0.997
Benzophenone-3	108	4	65	30	0.5–20	0.996
Benzyl butyl phthalate	104	10	87	18	0.5–20	0.995
4-Fluoro-3-phenoxybenzoic acid	102	6	141	10	0.5–20	0.996
3-Phenoxybenzoic acid	100	5	108	9	0.5–20	0.998
Cyhalothric acid	106	4	108	7	0.5–20	0.995

[a] Recovery was determined by comparing spiking level and calculated concentration in fortified samples during validation study. It is represented as a mean of the three levels tested.

[b] Ion suppression results are based on the analysis of 8 samples, from different source and/or origin.

4. Application to real samples

The first set of samples screened was performed on 28 milk samples including raw milk, commercially available bovine milk with various fat content and packaging, baby formulae, goat, soy and almond milk (Table 5). Progesterone was detected in every milk sample of bovine origin with only sub μk kg^{-1} levels in baby formulae and goat milk. Even in this small number of commercial milk samples there was a positive correlation between fat content and progesterone levels ($R^2 = 0.62$), which corresponds to the data presented in the literature (EMEA., 2004; Hartmann et al., 1998). High levels of isoflavones were detected in soy milks and lower levels in one baby milk sample, which corresponds to levels cited (Nielsen et al., 2009; Steinshamn et al., 2008). No mycotoxins were detected which is in agreement with available data (Xia et al., 2009). BBP was detected in one raw milk sample and almond milk sample (and two others below CCα) while paraben methyl was present in a low fat milk sample. What is more, paraben methyl was detected in 1 samples, paraben ethyl in 18 samples and paraben propyl in 4 samples but below the CCα. No traces of parabens were detected in milk samples of plant origin, which calls for further investigations.

5. Conclusions

A comprehensive and sensitive analytical LC–MS/MS method has been developed for the quantitative confirmatory analysis of 19 EDs in milk.

The method is advantageous compared to existing analytical methods because it allows for analysis of a wider range of EDs in milk than other published methods, uses a simple sample preparation procedure and can analyse both positively and negatively ionised substances in a single injection. These advantages provide cost effective, rapid and high throughput analysis of EDs. In a typical analytical run, one analyst using a single LC–MS/MS

Table 4
Within laboratory repeatability and reproducibility validation results for fortified milk samples where VL = validation level CV = covariance.

Analyte	Repeatability($n = 7$)			Reproducibility ($n = 7$)			CCα [μg g^{-1}]	CCβ [μg g^{-1}]
	1 × VL[a]	1.5 × VL	2 × VL	1 × VL	1.5 × VL	2 × VL		
	CV [%]			CV [%]				
Testosterone	11.9	17.9	11.0	11.7	11.3	6.6	0.17	0.29
Progesterone	13.5	15.0	16.6	11.6	11.7	9.8	0.24	0.4
Daidzein	13.5	13.5	12.8	8.7	13.5	13.0	0.20	0.35
Genistein	15.4	15.3	12.4	11.6	12.2	12.1	0.21	0.36
Zearalenone	9.8	8.4	12.7	6.2	4.7	8.1	0.14	0.23
α-Zearalenol	6.3	7.0	9.9	6.4	5.4	7.0	0.13	0.22
Perfluorooctanoic acid	15.1	22.0	14.1	15.1	15.9	12.0	0.26	0.45
Perfluoroheptanoic acid	18.3	23.4	15.0	12.7	14.3	12.2	0.24	0.41
Perfluorooctane sulfonic acid	11.6	27.1	16.5	18.6	19.3	20.8	0.39	0.67
Paraben methyl	15.3	10.5	12.8	7.3	9.2	8.1	0.16	0.27
Paraben ethyl	20.1	20.9	15.2	8.4	7.1	9.0	0.17	0.29
Paraben propyl	16.2	8.9	9.4	7.3	6.8	7.8	0.14	0.24
Paraben butyl	10.8	5.8	9.6	9.7	5.4	7.7	0.14	0.27
Trichlorocarban	13.2	22.1	16.5	12.5	11.5	11.8	0.21	0.36
Benzophenone-3	15.7	16.7	16.6	12.2	10.7	15.5	0.32	0.54
Benzyl butyl phthalate	16.3	20.0	19.1	14.4	11.2	15.1	0.25	0.42
4-Fluoro-3-phenoxybenzoic acid	8.2	7.2	11.5	6.0	10.3	9.3	0.11	0.18
3-Phenoxybenzoic acid	7.4	9.5	11.3	5.4	8.2	6.8	0.15	0.25
Cyhalothric acid	10.4	10.2	12.5	10.5	13.3	10.6	0.24	0.42

[a] Samples were fortified as described in Section 3.2.3.

Table 5
Detected levels of EDs in real milk samples.

Sample type	Analyte						
	Progesterone	PB–Me	PB–Et	PB–Pr	Daidzein	Genistein	BBP
	[μg kg^{-1}]						
Skimmed milk	1.4	N.D	<CCα	<CCα	N.D	N.D	N.D
Skimmed milk	1.1	<CCα	<CCα	N.D	N.D	N.D	N.D
Fat free organic milk	0.51	N.D	<CCα	N.D	N.D	N.D	N.D
Butter milk	2.4	N.D	<CCα	N.D	N.D	N.D	N.D
Butter milk	3.2	N.D	N.D	N.D	N.D	N.D	N.D
Low fat milk	7.0	N.D	<CCα	N.D	N.D	N.D	N.D
Low fat milk	5.9	N.D	<CCα	N.D	N.D	N.D	N.D
Low fat milk	4.4	0.3	<CCα	N.D	N.D	N.D	N.D
Whole organic milk	12	N.D	N.D	N.D	N.D	N.D	N.D
Whole organic milk	5.9	N.D	<CCα	N.D	N.D	N.D	N.D
Whole organic milk	14.0	N.D	<CCα	N.D	N.D	N.D	N.D
Fresh milk	5.3	N.D	<CCα	N.D	N.D	N.D	N.D
Fresh milk	9.5	N.D	<CCα	<CCα	N.D	N.D	N.D
Fresh milk	15.2	N.D	N.D	<CCα	N.D	N.D	N.D
Fresh milk	9.8	N.D	<CCα	N.D	N.D	N.D	N.D
Raw milk	15.5	N.D	N.D	N.D	N.D	N.D	N.D
Raw milk	14.2	N.D	N.D	N.D	N.D	N.D	N.D
Raw milk	8.4	N.D	<CCα	N.D	N.D	N.D	N.D
Raw milk	4.1	N.D	<CCα	N.D	N.D	N.D	<CCα
Raw milk	1.3	N.D	<CCα	<CCα	N.D	N.D	2.34
Baby formulae	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Baby formulae	0.56	N.D	<CCα	N.D	N.D	N.D	N.D
Baby formulae	0.31	N.D	<CCα	N.D	N.D	N.D	N.D
Baby formulae	0.78	N.D	N.D	N.D	2.7	2.9	N.D
Soya milk	N.D	N.D	N.D	N.D	684	977	N.D
Soya milk	N.D	N.D	N.D	N.D	292	693	N.D
Almond milk	N.D	N.D	N.D	N.D	N.D	N.D	0.43
Goats Milk	0.76	N.D	<CCα	N.D	N.D	N.D	<CCα

instrument can test 30 samples plus 10 controls. The method presented is a comprehensive, screening bioassay compatible and 'fit-for-purpose' analytical application for the analysis of 19 EDs in milk.

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