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One-pot synthesized functionalized mesoporous silica as a reversed-phase sorbent for solid-phase extraction of endocrine disrupting compounds in milks

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Abstract

A new procedure for the determination of twelve naturally occurring hormones and some related synthetic chemicals in milk, commonly used as growth promoters in cattle, is reported. The method is based on liquid-liquid extraction followed by solid-phase extraction (SPE) using a new one-pot synthesized ordered mesoporous silica (of the SBA-15 type) functionalized with octadecyl groups (denoted as SBA-15-C_{18}-CO) as reversed-phase sorbent. The analytes were eluted with methanol and then submitted to HPLC with diode array detection. Under optimal conditions, the method quantification limit for the analytes ranged from 0.023 µg/mL to 1.36 µg/mL. The sorbent affored the extraction of estrone, 17β-estradiol, estriol, progesterone, hexestrol, diethylstilbestrol, 4-androstene-3,17-dione, ethinylestradiol, 17α-methyltestosterone, nandrolone, prednisolone and testosterone with mean recoveries ranging from 72 to 105% (except for diethylstilbestrol) with RSD < 11%. These results were comparable and, in some cases, even better than those obtained with other extraction methods, therefore SBA-15-C_{18}-CO mesoporous silica possess a high potential as a reversed-phase sorbent for SPE of the twelve mentioned endocrine disrupting compounds in milk samples.

Keywords Solid-phase extraction . SBA-15 . endocrine disrupting compounds estrogens . milk

1. Introduction

Endocrine disruptors are exogenous substances that modify the function of the endocrine system and, consequently, they cause adverse effects in humans' health [1]. Endocrine-disrupting chemicals (EDCs) have been associated with altered reproductive function in males and females, increased incidence of breast cancer, abnormal growth patterns and neurodevelopmental delays, as well as changes in immune function. Several studies have reported that EDCs can adversely affect humans [2, 3]. An increasing broad spectrum of compounds, both natural and synthetic can be considered EDCs, such as pesticides, plasticizers, polycyclic aromatic hydrocarbons and hormones [4]. Steroid hormones are illegally administered to animals as growth promoters in order to gain weight faster and increase milk production. These compounds which can be carcinogenic even at very low levels are listed within Group A in Annex I of the Council Directive 96/22/EC (Group A: substances having anabolic effect and unauthorized substances) [5]. For Group A substances, "zero tolerance" is established by EU, except for melengestrol acetate which maximum residue limit (MRL) has been set at 1 μg/Kg in cow fat. Growth promoters can pass from the blood stream and can be finally excreted in milk by the mammary gland.

As milk and dairy products are major constituents of human diets, the consumption of these products could be considered an important source of these dangerous substances for the humans [6]. For these reasons, it is very important to develop multi-residue methods to determine the levels of these compounds in milks. Most of the methods published in the literature use HPLC-MS [6-10] or GC-MS [11-13] for the determination of steroid hormones in milk. The studies about separation of steroid hormones by HPLC-DAD are quite limited. However, due to its simplicity, this

technique is usually employed as a starting point for the evaluation of new methodologies in sample preparation [14, 15, 16].

Current trends in sample treatment are focused on the synthesis of new materials and their application as sorbents in solid phase extraction (SPE) or other techniques such as matrix solid phase dispersion (MSPD), molecular imprinted solid phase extraction (MISPE), etc. In this sense, ordered mesoporous silicas are promising materials because of their desirable characteristics: (a) highly ordered and size-controlled mesoporous structures, (b) extremely high surface areas and large pore volumes, (c) very good thermal and chemical stability and (d) high flexibility in functionalization to enable the introduction of hydrophilic, hydrophobic, polar as well as charged functional moieties on surface. For all these reasons, mesoporous silicas are presented as a good alternative to classical sorbents, such as amorphous silica and polymeric materials [17, 18]. A variety of hybrid ordered mesoporous silica (MCM-41, SBA-15, MSU, PMOs, etc.) SPE sorbents have been explored for the determination of inorganic (heavy metals) and organic (pesticides, hormones, etc.) contaminants in different samples [16-22]. In general, a common theme of these funtionalization strategies was attachment of the organic moiety by the post-synthesis (or grafting) method. However, organically modified ordered mesoporous silicas can also be prepared by co-condensation (or one-pot) method, in such a way that the organic functionalities project into the pores. In this strategy, since the organic functionalities are direct components of the silica matrix, pore blocking is not a problem. Furthermore, the organic units are generally more homogeneously distributed than in materials synthesized with the grafting process [17].

In any case, hybrid mesoporous silicas remain scarcely used owing to their unknown potential for extracting many emerging contaminants (especially from

complex matrices such as foods). The main objective of this study was therefore to assess the potential of SBA-15 type mesoporous silica, synthesized and functionalized by co-condensation procedure with octadecyl groups (denoted as SBA-15-C_{18}-CO), as an SPE sorbent for preconcentrating the endocrine disrupting compounds estrone (E1), 17β-estradiol (17β-E2), estriol (E3), progesterone (P), hexestrol (HEX), diethylstilbestrol (DES), 4-androstene-3,17-dione (AND), ethinylestradiol (EE2), 17α-methyltestosterone (17α-MT), nandrolone (NAN), prednisolone (PRED) and testosterone (T) from milks prior to their determination by HPLC-DAD. To our knowledge, no application of this type of material to the extraction of twelve steroid hormones as model analytes from complex food matrices has to date been reported.

2. Experimental

2.1 Reagents and materials

Tetraethylorthosilicate (TEOS) 98% (M = 208.33 g/mol, d = 0.934 g/mL), poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) (EO20PO70EO20, Pluronic 123, M_{av} = 5800 g/mol, d = 1.019 g/mL), cetyltrimethylammonium bromide (CTAB) 98%, (M= 364,46 g/mol), octadecylsilane (OTES) 97% (M = 284.61 g/mol, d = 0.795 g/mL), E1, 17β-E2, E3, P, HEX and DES were purchased from Sigma-Aldrich (St. Louis, MO, USA). AND, EE2, 17α-MT, NAN, PRED and T were purchased from Fluka (Busch, Switzerland). Ethanol absolute was purchased from SDS (Peypin, France). Hydrochloride acid 35% (M = 36.45 g/mol, d = 1.19 g/mL) was purchased for Panreac (Castellar del Vallès, Barcelona, Spain).

HPLC-grade solvents acetonitrile (ACN) and methanol (MeOH) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Standard solutions

Stock standard solutions of 4000 mg/L were prepared by diluting in MeOH adequate amounts of each compound and stored at – 20 ºC. Working solutions were prepared at various concentrations by appropriate dilution of the stock solution in MeOH (0.5 – 150 mg/L). All working solutions were filtered through a 0.45 µm pore size nylon filter membrane before analysis. Water (resistance 18.2 MΩ cm) was obtained from a Millipore Milli-Q-System (Billerica, MA, USA).

2.3 Milk samples

Whole and skimmed UHT cow milks have been used. These samples were bought in a commercial market in Madrid (Spain) and frozen in individual fractions at -20 ºC until analysis.

2.4 Synthesis of SBA-15-C_{18}-CO

12 g of poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) was dissolved in 361 g of water and 375 g of 2.0 M HCl solution with stirring at room temperature. After 22 mL of TEOS was added to that homogeneous solution with stirring at room temperature. The resulting mixture was stirred at 40 °C for 3 h for prehydrolysis, and then 4.15 g of OTES was slowly added into the solution. The

resulting mixture was stirred at 40 °C for 20 h and then transferred into a polypropylene bottle and reacted under static condition at 50 ºC for 2 h and 90 °C for 24 h. The solid product was recovered by filtration, washed with water, and dried at room temperature overnight. The template was removed from the synthesized material by refluxing in ethanol: H_2O (95:5, v/v) for 24 h. Finally, the material was dried at 50 °C for 24 h. The synthesized material was characterized by X-ray diffraction (XRD), N_2 gas adsorption-desorption isotherms, transmission electron microscopy (TEM), scanning electron microscopy (SEM) and thermogravimetric analysis (TGA).

2.5 Sample extraction procedure

1 g of spiked milk was mixed with 2 mL of 0.2 M acetate buffer (pH 5.2) and it was shaken before adding 2.5 mL of MeOH. The mixture was vortexed for 1 min and then it was centrifuged at 4000 rpm for 5 min. The supernatant was taken and water was added until a final volume of 25 mL was obtained. This extract was purified by SPE. To prepare the SPE cartridges, 100 mg of SBA-15-C_{18}-CO were packed into a 6 mL syringe type cartridge (65 mm length, 11 mm diameter) plugged with porous PTFE disks at both ends. To prevent the material lost during sample loading, a 0.45 µm pore size nylon filter membrane was also inserted at the bottom of the mesoporous silica bed. In all instances conditioning of the cartridges was accomplished by passing 1 x 3 mL MeOH and 1 x 1 mL Milli-Q water at a flow rate of 1 mL min^{-1}. After sample extract loading (25 mL) cartridges were dried with a Supelco Visiprep™ DL solid phase extraction vacuum manifold 12 port model (Sigma Aldrich, St. Louis, MO, USA) connected to a vacuum pump at 7.6 psi. Once the entire extract was loaded, the stationary phase was washed with 1 x 5 mL Milli-Q water to remove interferences.

Finally, elution of the analytes was performed by passing 1 x 2 mL MeOH at a flow rate of 0.5 mL/min. In all cases, the corresponding extracts were evaporated and re-dissolved with 150 µL of MeOH (preconcentration factor = 6.7) for subsequent analysis in the HPLC-DAD system.

2.6 Chromatographic analysis

HPLC analyses were performed on a Varian ProStar chromatographic system (Varian Ibérica, Madrid, Spain). The system consisted of a 230 ProStar ternary pump, a ProStar 410 autosampler with a six-port injection valve equipped with a 20 µL injection loop (Rheodyne), a photodiode array detector DAD 335 ProStar UV-vis detector and a PC-based data acquisition system Varian Star Workstation.

Separation was achieved on an Ascentis C_{18} (250 x 4.6 mm, 5µm) column (Supelco, St. Louis, MO, USA). As a starting point we selected a separation method previously developed in our laboratory for the analysis of seven steroid hormones [16], but some previous experiments were carried out to develop a proper mobile phase gradient to separate twelve hormones in the current work. The mobile phase gradient employed (mobile phase A: H_2O and mobile phase B: ACN) consisted of: t = 0 min 35% B, t =5 min 40% B (5 min), t =10.5 min 45% B (1 min) and t = 16 min 100% B (4 min). The flow rate was 1.0 mL/min. The detection was recorded at 200 nm for E1, 17β-E2, E3, EE2 and HEX and at 242 nm for PRED, NAN, T, 17α-MT, AND, DES and P in order to obtain the maximum sensitivity for all the compounds (Fig. 1).

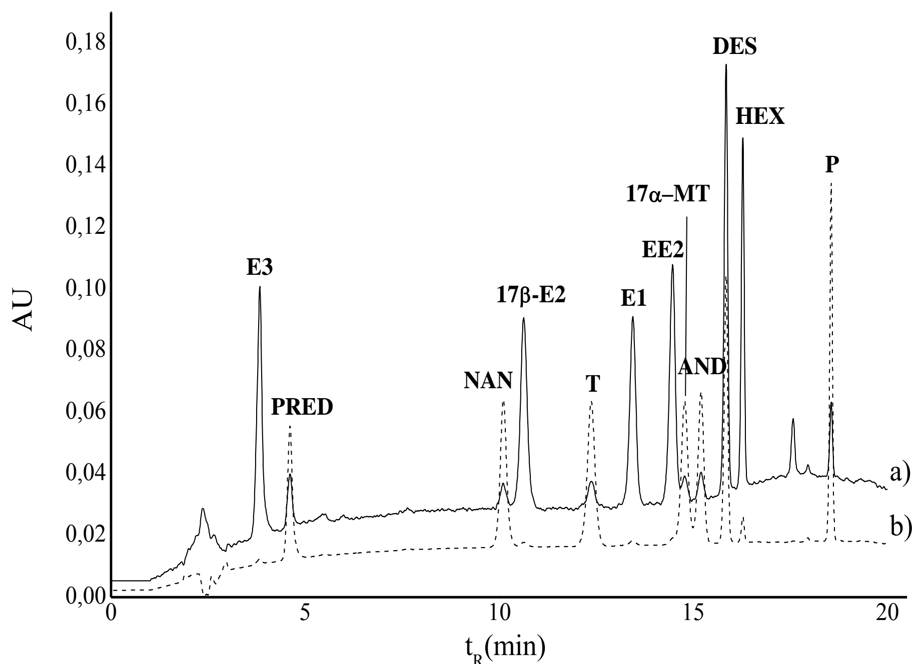

Fig. 1 Chromatographic separation obtained for twelve endocrine disrupting compounds with the optimized gradient elution. Detection was recorded at a) 200 nm for E1, 17β-E2, E3, EE2 and HEX and b) 242 nm for PRED, NAN, T, 17α-MT, AND, DES and P.

3. Results and discussion

3.1 Characterization of SBA-15-C18-CO sorbent

XRD pattern of the SBA-15-C_{18}-CO displayed a well-resolved pattern at low 2θ values with a very sharp (100) diffraction peak at 0.90 and a weak diffraction peak (110) at 1.68. d_{100}-spacing value and unit cell parameter (a_0) were: 98 and 113 Å, respectively (Fig. 2). This pattern suggests that the prepared functionalized silica contains well-ordered hexagonal arrays of one-dimensional channel structure.

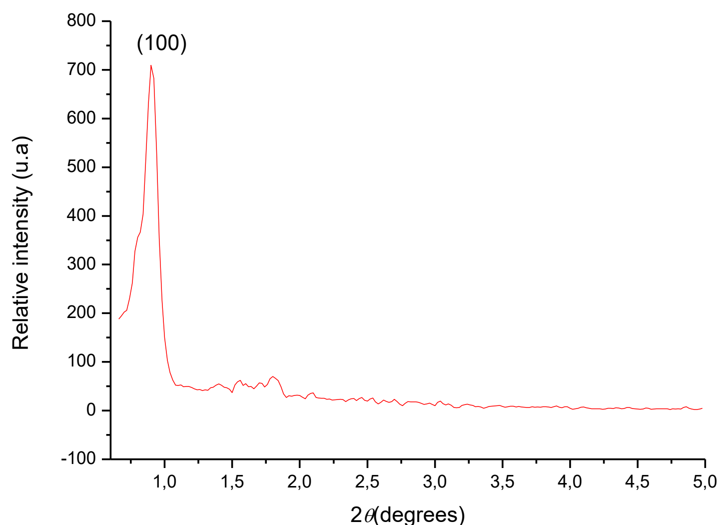

Fig. 2. XRD pattern of SBA-15-C_{18}-CO.

The N_2 adsorption-desorption isotherms for this material were of type IV according to the I.U.P.A.C. classification with an H1 hysteresis loop that is representative of materials with pores of constant cross-section (Fig. 3). The synthesized material possessed very high S_{BET} (796 m^2/g), a pore volume of 0.88 cm^3/g and a BJH pore diameter of 76 Å, typical of surfactant-assembled mesostructures. Scanning electron microscopy (SEM) images showed that SBA-15-C_{18}-CO has cylindrical shape, with an average particle size of 1.4 μm (length) and 750 nm (wide). Transmission electron microscopy (TEM) images demonstrated a clear arrangement of hexagonal pores with uniform size for this material. The amount of attached C_{18} molecules onto the mesoporous silica surface (L_o = 0.69 mmol/g) was estimated from the percentage of carbon in the functionalized mesoporous silica, calculated by elemental analysis (17% C).

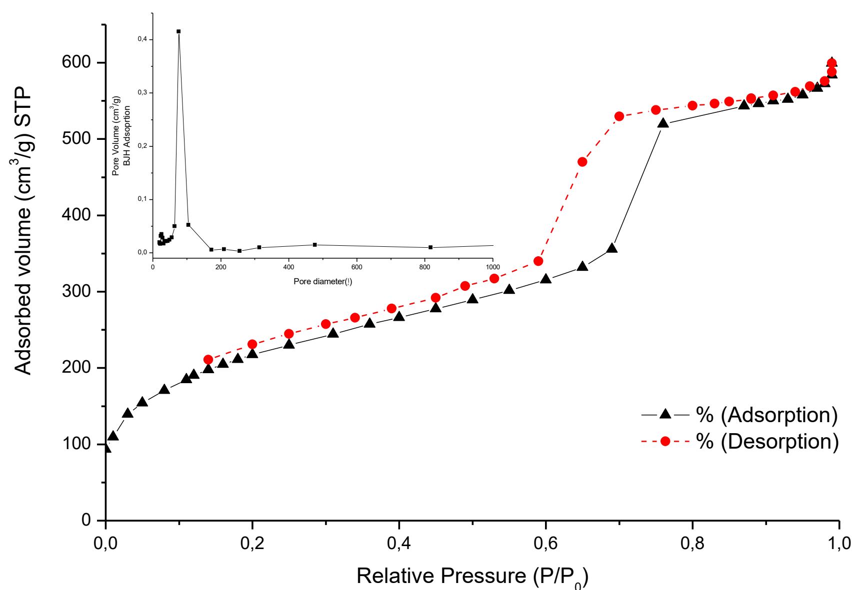

Fig. 3. Nitrogen adsorption-desorption isotherms and ore size distribution (inset) of SBA-15-C_{18}-CO.

Finally, thermogravimetric analysis (TGA) curve of the SBA-15-C_{18}-CO (Fig. 4) showed a degradation process between 200-600 ºC with a weight loss of about 17%, due to the breakage of pendant groups anchored on the silica surface (exothermic degradation process). The mass loss observed in the SBA-15-C_{18}-CO is in agreement with the amount of C_{18} groups covalently bound to the support, calculated by elemental analysis.

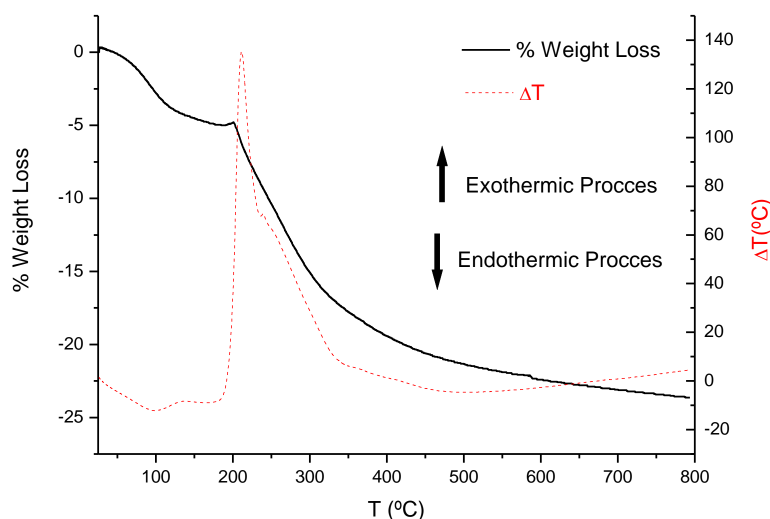

Fig. 4. Thermogravimetric curves and heat flow of SBA-15-C_{18}-CO.

Two main approaches can be used to achieve hybrid mesoporous silicas: (a) the post-synthesis (PS), or "*grafting*", method and (b) the co-condensation (CO), or "*one-pot*", method [17]. In a previous paper of our research group, a PS method was used to modify the surface of previously prepared SBA-15, through silylation with chloro(dimethyl)octadecylsilane in an organic solvent under reflux conditions [16]. One drawback of PS method is the reduction in the porosity of the functionalized material, which depends on the size of organic ligand and the degree of functionalization. Thus, if bulky ligands that react preferentially at the pore openings during the initial stages of the grafting process are used (i.e. C_{18} groups), further diffusion of ligands into the center of pores can be impaired and a pore-blocking effect produced. In this paper, hybrid SBA-15 mesoporous silica has been obtained directly in a "one-step" procedure by hydrolysis and co-condensation of a tetraalkoxysilane (TEOS) with one organoalkoxysilane (OTES) in the presence of a structure-directing agent (Pluronic 123). This procedure overcomes the main drawbacks of the PS method and leads to

hybrid SBA-15 material containing accessible functional groups that are more homogeneously distributed inside the pore channels and without pore blocking. For this reason, the new material SBA-15-C_{18}-CO prepared in the current work has higher S_{BET}, pore volume, pore diameter and amount of attached C_{18} molecules, in comparison with the SBA-15-C_{18} previously prepared by the PS method [16].

3.2 Optimization of the sample treatment

In order to optimize the sample treatment and to evaluate the SBA-15-C_{18}-CO material for the SPE procedure, four different samples were extracted in each set of experiments: three of them were milk samples spiked with the twelve EDCs at a known concentration and another one was a simulated sample prepared in the same way but spiked with the analytes at the end of the treatment process. The recoveries obtained in each experiment were calculated by comparison of the areas of the samples with the areas of the simulated sample.

It is well known that milk is a complex matrix with numerous different compounds, ranging from simple inorganic salts to large proteins, so in order to remove unwanted matrix components from the milk, a previous liquid-liquid extraction (LLE) process is necessary to make this sample suitable for SPE application. In addition, with the aim of developing a more cost effective and environment friendly sample treatment method that would consume lower volumes of organic solvents, a smaller milk sample size (1 g) was selected. Firstly, 1 g of spiked milk was extracted with 2 mL 0.2 M acetate buffer and 2.5, 3.75 or 5 mL of MeOH. The mixture was vortexed during 2 min and after was centrifuged at 4000 rpm for 5 min to separate the precipitate. Finally, the

supernatant was decanted and diluted with water to a final volume of 25 mL (to reduce the MeOH to 10, 15 or 20% by volume, respectively) and, then, the extract was purified by SPE according to the protocol described in previous works [16, 23]. Results obtained indicated that the use of a lower volume of MeOH provides higher recoveries for E3, PRED, NAN, 17β-E2 and T, with an important increase of 60% in the recovery of E3 and of 50% in the recovery of PRED. This fact confirmed that large percentage of MeOH can produce a break-through effect during the loading step for some of the target analytes [8]. On the other hand, for EE2, E1, 17α-MT, AND, DES, HEX and P recoveries were not modified, or suffered a slightly reduction, with the increase in the percentage of MeOH. For this reason, it was concluded that is important than the amount of MeOH remaining from the LLE step was diluted to 10% in the sample extract, in order to achieve the best recoveries for all the target analytes.

The next step to optimize the sample treatment process was the type of elution solvent in the SPE step, since this solvent should have enough elution ability to desorb the analytes and facilitate the further sample treatments. MeOH and ACN were tested for this purpose, setting an elution volume of 2 mL (Fig. 5). Best results were obtained using MeOH as elution solvent, obtaining recoveries over 80% for all analytes, except for DES (54%). The low recovery percentage obtained for DES, somewhat lower than the obtained for the other analytes studied, has been attributed to a phenomenon in which some kind of equilibrium process between two different isomeric forms of this compound could take place [18].

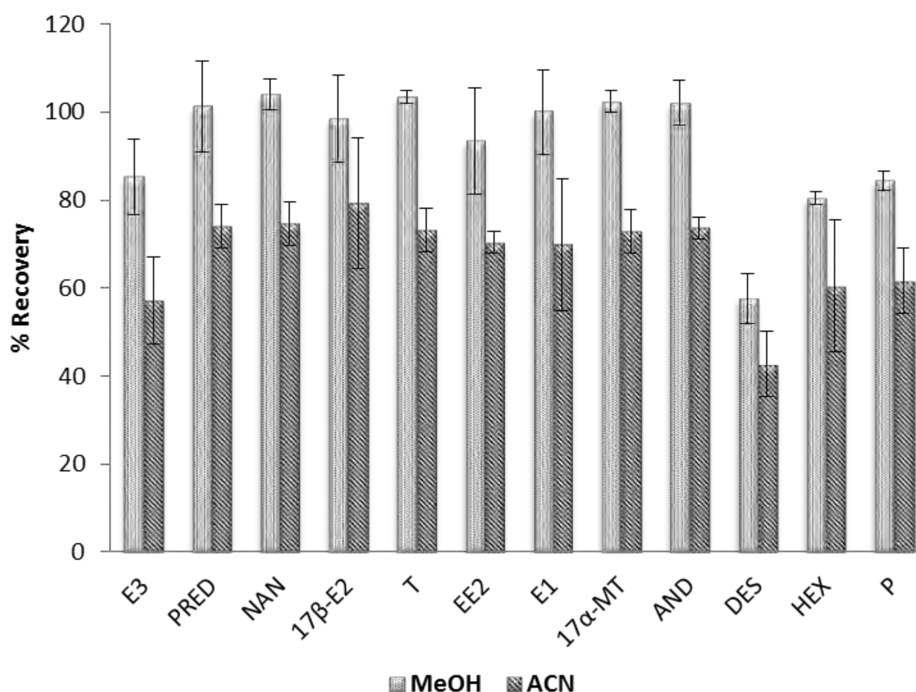

Fig. 5. Effect of different elution solvents on the solid-phase extraction step of the sample treatment procedure.

Finally, the volume of the elution solvent was also investigated as the quantity of MeOH that loaded on the cartridge has great effect on the recovery of analytes. For this purpose, different volumes of MeOH (1 x 2 mL, 1 x 3 mL, and 2 x 2 mL) were tested. Good recoveries and minimal interferences in the detection were observed employing 2 mL as elution volume for the entire target compounds, except for DES, and not significant differences in the recovery values were observed by using higher MeOH volumes (Fig. 6). For this reason, 2 mL of MeOH were found to be the optimum volume, as excessive volume would lead to long time for the next dryness steps.

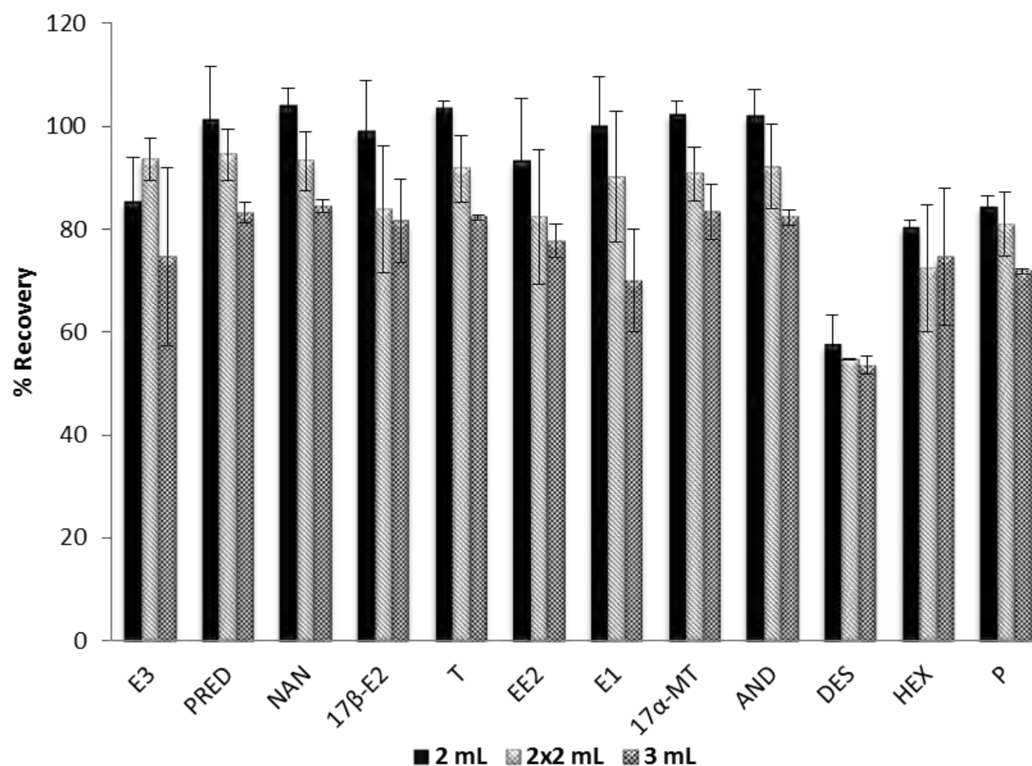

Fig. 6. Effect of different methanol elution volumes on the solid-phase extraction step of the sample treatment procedure.

It is well known that the presence of hydrophobic C_{18} groups onto the silica surface generates advantages to the adsorption of hydrophobic organic compounds, such as the ones studied in this work, and that the capacity of the sorbent to do so improves as the percentage of C_{18} loading increases. In that respect, the good results achieved with the SBA-15-C_{18}-CO sorbent can be attributed not only to its high loading by the C_{18} groups (L_o = 0.69 mmol/g) but also to its uniform surface coverage and good accessibility to these groups. On the other hand, residual fats, proteins and carbohydrates that were not completely removed in the LLE step, which contain numerous hydroxyl, amino and organophosphate groups can interact at multiple sites in the SBA-15-C_{18}-CO sorbent (with C_{18} groups and/or with residual non-modified silanol groups in the silica surface). Hence they are retained in the cartridge and this fact has an

important effect in order to achieve clean extracts to inject in the HPLC system after the SPE step.

3.3 Performance of the method

The instrumental linearity was evaluated using standard mixtures of the twelve steroids in MeOH at seven concentration levels, in the range of instrumental quantitation limit (IQL) to 100 μg/mL for each hormone. The slope and intercept values of the calibration curves were determined using regression analyses. Linear relationship was found between corrected peak areas and the concentration of the analyte in all cases, with regression coefficients (R^2) ≥ 0.990 (Table 1). On the other hand, to evaluate the linearity of the method, external calibration curves were prepared by spiking milk samples (whole and skimmed) with appropriate aliquots of the stock standard solution, to a range of concentration between the method quantification limit (MQL) to 15 μg/mL. A linear relationship was found between peak areas and concentration of the analyte in all cases, with R^2 ≥ 0.990. The results showed that linearity of the method was good for the analytes studied. As Table 1 shows, by comparing the slopes of the matrix-free calibration curves with the matrix-matched calibration curves, a significant difference in the slopes of the linear equations was found in most cases that evidence an important influence of the milk matrix.

Table 1. Calibration data of twelve analytes in Milli-Q water and two types of milk after SPE-HPLC-DAD method.

Analyte	Calibration curve		
	Milli-Q water[a]	Whole milk[b]	Skimmed milk[b]
E3	y= 23.229 x + 173.25 R^2=0.999	y= 40.178 x - 14.909 R^2=0.991	y= 31.418 x + 69.573 R^2=0.997
PRED	y= 36.010 x + 16.19 R^2=0.990	y= 32.534 x + 87.954 R^2=0.995	y= 30.101 x + 67.165 R^2=0.997
NAN	y= 59.599 x + 10.235 R^2=0.998	y= 70.955 x – 31.112 R^2=0.999	y= 77.206 x – 28.686 R^2=0.997
17β-E2	y= 57.103 x + 231.11 R^2=0.995	y= 69.927 x + 45.236 R^2=0.998	y= 62.851 x + 39.563 R^2=0.997
T	y= 53.319 x +279.08 R^2=0.999	y= 65.579 x – 6.2172 R^2=0.999	y= 64.304 x + 21.687 R^2=0.999
EE2	y= 77.115 x – 182.51 R^2=0.996	y= 68.974 x – 36.654 R^2=0.999	y= 65.960 x + 11.215 R^2=0.999
E1	y= 79.455 + 18.848 R^2=0.995	y= 70.817 x + 101.74 R^2=0.9957	y= 77.065 x + 52.394 R^2=0.9971
17α-MT	y= 44.511 x +147.48 R^2=0.999	y= 55.179 x -16.358 R^2=0.999	y= 57.691 x + 5.6886 R^2=0.999
AND	y= 55.746 x – 45.155 R^2=0.996	y= 65.635 x + 3.9965 R^2=0.999	y= 65.965 x – 8.9265 R^2=0.999
DES	y= 30.805 x + 141.66 R^2=0.997	y= 39.554 x – 48.619 R^2=0.998	y= 33.285 x + 117.65 R^2=0.991
HEX	y= 72.181 x – 300.76 R^2=0.991	y= 46.972 x + 133.46 R^2=0.996	y= 48.897 x + 145.63 R^2=0.997
P	y= 43.446 x + 181.56 R^2=0.996	y= 49.116 x – 28.707 R^2=0.998	y= 52.398 x – 5.9716 R^2=0.999

[a] Linear range: IQL-100 µg/mL
[b] Linear range: MQL-15 µg/mL

The instrumental detection (IDL) and quantitation (IQL) limits were calculated at signal-to-noise ratio of 3 and 10, respectively, following IUPAC recommendations. Method sensitivity was estimated by application of the preconcentration factor of 6.7 to the IDL and IQL previously calculated. The method detection limit (MDL) and method quantification limit (MQL) were confirmed by injection of a spiked milk sample (whole and skimmed) extracted following the sample treatment procedure. The MDL and MQL values obtained for each type of milk (whole and skimmed) are shown in Tables 2 and 3. In general, the MQLs obtained in the present work are of the same order of magnitude and in some cases lower, than those obtained in other works for the determination of steroid hormones in this type of matrices by HPLC-DAD [15, 16].

Table 2. Method quantification limit (MQL), accuracy (recovery, %), and precision (RSD, %) for the method developed for the determination of twelve endocrine disrupting compounds in whole milk.

Analyte	MQL	Low level[a]		High level[b]	
	(µg/mL)	Recovery (%) Mean ± SD	RSD (%)	Recovery (%) Mean ± SD	RSD (%)
E3	0.53	84 ± 4	5	76 ± 6	8
PRED	0.06	87 ± 6	8	79 ± 6	11
NAN	0.16	105 ± 5	4	89 ± 5	6
17β-E2	1.10	99 ± 6	6	85 ± 5	6
T	0.10	95 ± 4	4	90 ± 5	5
EE2	0.63	95 ± 4	4	84 ± 5	6
E1	0.36	92 ± 4	4	83 ± 5	6
17α-MT	0.09	95 ± 7	8	90 ± 5	5
AND	0.07	99 ± 7	7	89 ± 5	5
DES	0.10	73 ± 8	11	59 ± 4	7
HEX	0.34	85 ± 4	4	75 ± 4	6
P	0.04	89 ± 7	7	79 ± 6	8

[a] MQL as low level

[b] 15 µg/mL as high level

Table 3. Method quantification limit (MQL), accuracy (recovery, %), and precision (RSD, %) for the method developed for the determination of twelve endocrine disrupting compounds in skimmed milk.

Analyte	MQL (µg/mL)	Low level[a]		High level[b]	
		Recovery (%) Mean ± SD	RSD (%)	Recovery (%) Mean ± SD	RSD (%)
E3	0.99	78 ± 2	1	72 ± 9	11
PRED	0.01	80 ± 6	9	75 ± 7	10
NAN	0.19	91 ± 4	5	88 ± 4	4
17β-E2	1.36	96 ± 2	2	85 ± 5	6
T	0.13	89 ± 6	7	90 ± 5	5
EE2	0.58	80 ± 6	7	84 ± 5	6
E1	0.36	88 ± 6	7	87 ± 7	8
17α-MT	0.08	99 ± 10	10	91 ± 6	7
AND	0.07	92 ± 8	8	89 ± 4	4
DES	0.11	59 ± 6	10	61 ± 3	5
HEX	0.53	76 ± 2	3	84 ± 6	7
P	0.02	88 ± 5	6	84 ± 6	8

[a] MQL as low level

[b] 15 µg/mL as high level

Instrumental precision of the method was studied in terms of repeatability and intermediate precision at two levels concentration (IQL and 100 µg/mL). Results were obtained in terms of relative standard deviations (RSD, %) for retention times (t_R) and peak areas (A). The instrumental repeatability, determined for six consecutive injections of each steroid standard mixture (n = 6), was acceptable at both concentration levels, with RSD < 1.8% and 8.5% for t_R and A, respectively. Intermediate precision was determined for three consecutive injections of each steroid standard mixture, carried out on three different days (n = 9, k = 3). RSD obtained for intermediate precision was between 0.1% and 2.2% for t_R and between 2.3 and 16% for A. Method repeatability was determined for six different assays carried out in the same day, at two concentration levels (MQL and 15 µg/mL) with RSD < 1% and 11% for t_R and A, respectively. These results indicate a good precision of the method.

The accuracy of the method was evaluated spiking the two types of milk (whole and skimmed) at two different concentration levels (MQL and 15 µg/mL) using three individual milk samples for each type. Non spiked samples (blanks) were also processed and demonstrated that the concentration of hormones in the non spiked samples was below the MQL of the method. Tables 2 and 3 summarize the average recoveries obtained for each steroid between 72 – 105%, except for DES that was near 60%, with RSD < 11%. Typical chromatograms of blank whole and skimmed milks and a whole milk sample fortified with each hormone at 5 µg/mL level, extracted following the described procedure are shown in Fig. 7a and Fig. 7b.

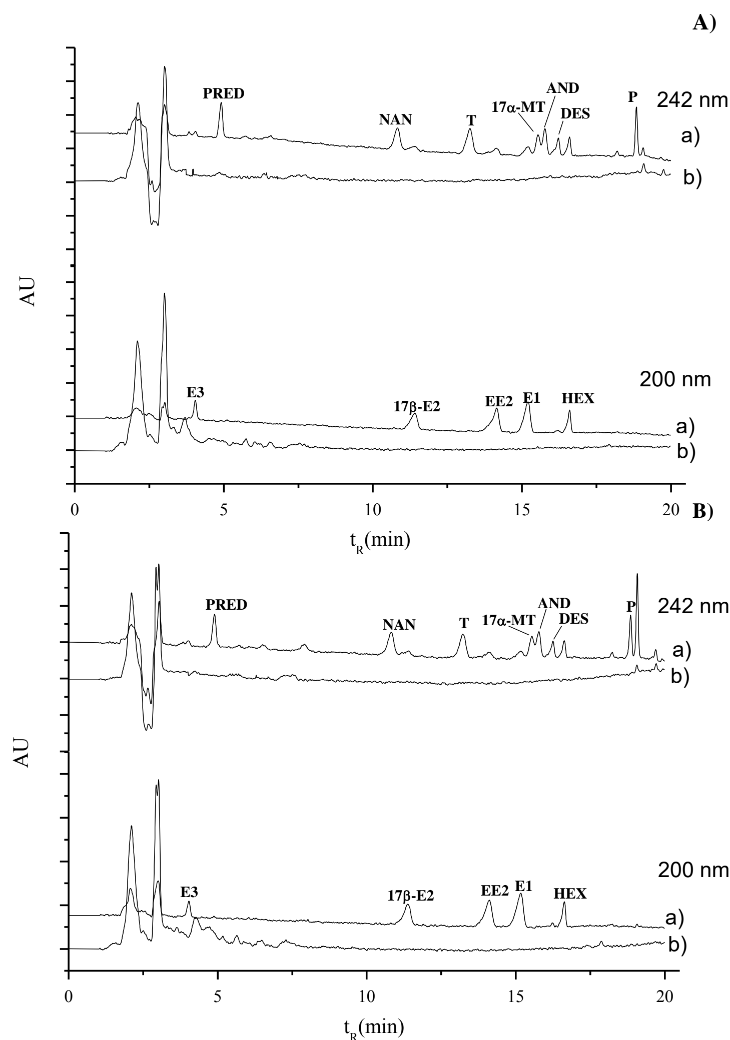

Fig. 7. Chromatograms corresponding to A) whole milk sample and B) skimmed milk sample; a) 5 μg/mL spiked milk sample with twelve endocrine disrupting compounds and b) blank milk sample after the optimized sample treatment method. Experimental conditions as in Fig. 1.

3.4 Comparison with other sample preparation methods

The main difficulty in determining dangerous and/or forbidden substances in complex samples such as milk lies in their extraction from the matrix. In fact, this step is the bottleneck of routine analytical methods, because several sample pre-treatment steps are required in most cases. In the present work, a new sample treatment based LLE and SPE

for the determination of twelve steroids in goat milk has been proposed. The greatest innovation of the developed procedure has been the use of a new one-pot synthesized functionalized SBA-15 mesoporous silica as a reversed-phase sorbent for SPE. Table 4 collects some recent sample preparation methods found for the determinations of the target steroids in milks. As it can be seen, compared with other methods, the sample treatment procedure optimized in this work is simpler and/or faster [6-10, 15, 16]. In addition, recoveries obtained in the present work are in general more satisfactory, between 72 to 105% (except for DES), taken into account that a higher amount of target analytes have been tested. Finally, a comparison of the MQLs obtained in whole milks with a mesoporous silica functionalized by post-synthesis method (0.53 µg/mL for progesterone to 1.30 µg/mL for DES, [16]) and the new SBA-15-C_{18}-CO sorbent (0.035 µg/mL for progesterone, 0.1 µg/mL for DES, this work), indicated that SBA-15-C_{18}-CO achieved the best limits for all compounds, that can be attributed to the better ability of this material not only to remove interferences but also to retain the selected analytes.

Table 4. Comparison of SBA-15-C18-CO sorbent for SPE procedure with other sample preparation methods for extraction of steroids in milk.

Analytes	Sample preparation	Extraction time (min)[a]	Materials (amount)	Recovery (%)	References
E1, 17β-E2, EE2	LLE, HP-LPME	100 min	-	94-118 %	[15]
E1, 17β-E2, EE2, E3	LLE, HLB-SPE + NH_2-SPE	70 min	HLB (500 mg) NH_2 (500 mg)	62-112 %	[8]
17α-MT, DIE, HEX, DES, EE2	LLE, dSPE	56 min	C18 (50 mg)	102.1-104.2 %	[7]
DES, DIE, E1, 17β-E2, E3, HEX, T, 17α-MT, TREM, NAN	LLE, HLB-SPE C18-SPE+NH_2-SPE	75 min	HLB (500 mg) C18 (500 mg) NH_2 (500 mg)	82.2-103.9 %	[9]
E1, 17β-E2, EE2, E3, DIE, HEX, DES, 17α-MT	LLE, HLB-SPE	31 min	HLB (60 mg)	80.7-118.8 %	[6]
E1, 17β-E2, EE2, E3, DES	LLE, C_{30}-SPE on-line	45 min*	-	71.4-97.1 %	[10]
E1, 17β-E2, EE2, E3, DES, T, P	LLE, SBA-15-C18 SPE	30 min	SBA-15-C18 (100 mg)	62-108 %	[16]
E1, 17β-E2, EE2, E3, DES, T, P, AND, NAN, HEX, 17α-MT, PRED	LLE, SBA-15-C18-CO SPE	30 min	SBA-15-C18-CO (100 mg)	72-105 %	This work

[a]Estimated time according to the works; * Total time (sample preparation + analysis)
AND: Androstenodione; DES: Diethylstilbestrol; DIE: Dienestrol; dSPE: dispersive solid phase extraction 17β-E2: 17β-Estradiol; E1: Estrone; E3: Estriol; EE2: Ethinylestradiol; HEX: Hexestrol; HF-LPME: Hollow-Fiber Liquid-phase microextraction; HLB: Hydrophilic Lipophilic balance; LLE: Liquid-liquid extraction; 17α-MT: 17α-Methyltestorone; NAN: Nandrolone; P: Progesterone; PRED: Prednisolone; SPE: solid phase extraction; T: Testosterone; TREM: Trembolone

4. Conclusions

In conclusion, results presented in this work suggest that SBA-15-C_{18}-CO provides satisfactory purification of milk extracts, so this material might be appropriate for simultaneous extraction of a wide variety of synthetic and natural estrogenic hormones in this food.

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