Accepted Manuscript

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

Author: Judith Gañán Sonia Morante-Zarcero D. Pérez-Quintanilla María Luisa Marina Isabel Sierra

PII: S0021-9673(15)01260-1
DOI: http://dx.doi.org/doi:10.1016/j.chroma.2015.08.063
Reference: CHROMA 356814

To appear in: *Journal of Chromatography A*

Received date: 7-5-2015
Revised date: 25-8-2015
Accepted date: 27-8-2015

Please cite this article as: J. Gañán, S. Morante-Zarcero, D. Pérez-Quintanilla, M.L. Marina, I. Sierra, One-pot synthesized functionalized mesoporous silica as a reversed-phase sorbent for solid-phase extraction of endocrine disrupting compounds in milks, *Journal of Chromatography A* (2015), http://dx.doi.org/10.1016/j.chroma.2015.08.063

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

ACCEPTED MANUSCRIPT

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

Judith Gañán[a] · Sonia Morante-Zarcero[a] · D. Pérez-Quintanilla[a]

María Luisa Marina[b] · Isabel Sierra[a]*

[a]*Departamento de Tecnología Química y Energética, Tecnología Química y Ambiental, Tecnología Mecánica y Química Analítica, E.S.C.E.T, Universidad Rey Juan Carlos, C/ Tulipán s/n, 28933 Móstoles, Madrid, Spain*

[b] *Departamento de Química Analítica, Química Física e Ingeniería Química, Universidad de Alcalá, Alcalá de Henares, Madrid, Spain*

* Corresponding author: Tel.: (+34) 914887018; fax: (+34) 914888143.

E-mail address: isabel.sierra@urjc.es

Accepted Manuscript

ACCEPTED MANUSCRIPT

20 **Abstract**

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

36

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

39

Accepted Manuscript

ACCEPTED MANUSCRIPT

39 **1. Introduction**

40

41 Endocrine disruptors are exogenous substances that modify the function of the
42 endocrine system and, consequently, they cause adverse effects in humans' health [1].
43 Endocrine-disrupting chemicals (EDCs) have been associated with altered reproductive
44 function in males and females, increased incidence of breast cancer, abnormal growth
45 patterns and neurodevelopmental delays, as well as changes in immune function.
46 Several studies have reported that EDCs can adversely affect humans [2, 3]. An
47 increasing broad spectrum of compounds, both natural and synthetic can be considered
48 EDCs, such as pesticides, plasticizers, polycyclic aromatic hydrocarbons and hormones
49 [4]. Steroid hormones are illegally administered to animals as growth promoters in order
50 to gain weight faster and increase milk production. These compounds which can be
51 carcinogenic even at very low levels are listed within Group A in Annex I of the
52 Council Directive 96/22/EC (Group A: substances having anabolic effect and
53 unauthorized substances) [5]. For Group A substances, "zero tolerance" is established
54 by EU, except for melengestrol acetate which maximum residue limit (MRL) has been
55 set at 1 μg/Kg in cow fat. Growth promoters can pass from the blood stream and can be
56 finally excreted in milk by the mammary gland.

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

Accepted Manuscript

ACCEPTED MANUSCRIPT

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

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

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

Accepted Manuscript

ACCEPTED MANUSCRIPT

89 complex matrices such as foods). The main objective of this study was therefore to
90 assess the potential of SBA-15 type mesoporous silica, synthesized and functionalized
91 by co-condensation procedure with octadecyl groups (denoted as SBA-15-C_{18}-CO), as
92 an SPE sorbent for preconcentrating the endocrine disrupting compounds estrone (E1),
93 17β-estradiol (17β-E2), estriol (E3), progesterone (P), hexestrol (HEX),
94 diethylstilbestrol (DES), 4-androstene-3,17-dione (AND), ethinylestradiol (EE2), 17α-
95 methyltestosterone (17α-MT), nandrolone (NAN), prednisolone (PRED) and
96 testosterone (T) from milks prior to their determination by HPLC-DAD. To our
97 knowledge, no application of this type of material to the extraction of twelve steroid
98 hormones as model analytes from complex food matrices has to date been reported.
99
100 **2. Experimental**
101
102 *2.1 Reagents and materials*
103
104 Tetraethylorthosilicate (TEOS) 98% (M = 208.33 g/mol, d = 0.934 g/mL),
105 poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol)
106 (EO20PO70EO20, Pluronic 123, M_{av} = 5800 g/mol, d = 1.019 g/mL),
107 cetyltrimethylammonium bromide (CTAB) 98%, (M= 364,46 g/mol), octadecylsilane
108 (OTES) 97% (M = 284.61 g/mol, d = 0.795 g/mL), E1, 17β-E2, E3, P, HEX and DES
109 were purchased from Sigma-Aldrich (St. Louis, MO, USA). AND, EE2, 17α-MT,
110 NAN, PRED and T were purchased from Fluka (Busch, Switzerland). Ethanol absolute
111 was purchased from SDS (Peypin, France). Hydrochloride acid 35% (M = 36.45 g/mol,
112 d = 1.19 g/mL) was purchased for Panreac (Castellar del Vallès, Barcelona, Spain).

Accepted Manuscript

ACCEPTED MANUSCRIPT

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

115

116 *2.2 Standard solutions*

117

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

124

125 *2.3 Milk samples*

126

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

130

131 *2.4 Synthesis of SBA-15-C_{18}-CO*

132

133 12 g of poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol)
134 was dissolved in 361 g of water and 375 g of 2.0 M HCl solution with stirring at room
135 temperature. After 22 mL of TEOS was added to that homogeneous solution with
136 stirring at room temperature. The resulting mixture was stirred at 40 °C for 3 h for
137 prehydrolysis, and then 4.15 g of OTES was slowly added into the solution. The

Accepted Manuscript

ACCEPTED MANUSCRIPT

138 resulting mixture was stirred at 40 °C for 20 h and then transferred into a polypropylene
139 bottle and reacted under static condition at 50 ºC for 2 h and 90 °C for 24 h. The solid
140 product was recovered by filtration, washed with water, and dried at room temperature
141 overnight. The template was removed from the synthesized material by refluxing in
142 ethanol: H_2O (95:5, v/v) for 24 h. Finally, the material was dried at 50 °C for 24 h. The
143 synthesized material was characterized by X-ray diffraction (XRD), N_2 gas adsorption-
144 desorption isotherms, transmission electron microscopy (TEM), scanning electron
145 microscopy (SEM) and thermogravimetric analysis (TGA).
146
147 *2.5 Sample extraction procedure*
148
149 1 g of spiked milk was mixed with 2 mL of 0.2 M acetate buffer (pH 5.2) and it was
150 shaken before adding 2.5 mL of MeOH. The mixture was vortexed for 1 min and then it
151 was centrifuged at 4000 rpm for 5 min. The supernatant was taken and water was added
152 until a final volume of 25 mL was obtained. This extract was purified by SPE. To
153 prepare the SPE cartridges, 100 mg of SBA-15-C_{18}-CO were packed into a 6 mL
154 syringe type cartridge (65 mm length, 11 mm diameter) plugged with porous PTFE
155 disks at both ends. To prevent the material lost during sample loading, a 0.45 µm pore
156 size nylon filter membrane was also inserted at the bottom of the mesoporous silica bed.
157 In all instances conditioning of the cartridges was accomplished by passing 1 x 3 mL
158 MeOH and 1 x 1 mL Milli-Q water at a flow rate of 1 mL min^{-1}. After sample extract
159 loading (25 mL) cartridges were dried with a Supelco Visiprep™ DL solid phase
160 extraction vacuum manifold 12 port model (Sigma Aldrich, St. Louis, MO, USA)
161 connected to a vacuum pump at 7.6 psi. Once the entire extract was loaded, the
162 stationary phase was washed with 1 x 5 mL Milli-Q water to remove interferences.

Accepted Manuscript

ACCEPTED MANUSCRIPT

163 Finally, elution of the analytes was performed by passing 1 x 2 mL MeOH at a flow rate
164 of 0.5 mL/min. In all cases, the corresponding extracts were evaporated and re-
165 dissolved with 150 µL of MeOH (preconcentration factor = 6.7) for subsequent analysis
166 in the HPLC-DAD system.
167
168
169 *2.6 Chromatographic analysis*
170
171 HPLC analyses were performed on a Varian ProStar chromatographic system (Varian
172 Ibérica, Madrid, Spain). The system consisted of a 230 ProStar ternary pump, a ProStar
173 410 autosampler with a six-port injection valve equipped with a 20 µL injection loop
174 (Rheodyne), a photodiode array detector DAD 335 ProStar UV-vis detector and a PC-
175 based data acquisition system Varian Star Workstation.
176 Separation was achieved on an Ascentis C_{18} (250 x 4.6 mm, 5µm) column
177 (Supelco, St. Louis, MO, USA). As a starting point we selected a separation method
178 previously developed in our laboratory for the analysis of seven steroid hormones [16],
179 but some previous experiments were carried out to develop a proper mobile phase
180 gradient to separate twelve hormones in the current work. The mobile phase gradient
181 employed (mobile phase A: H_2O and mobile phase B: ACN) consisted of: t = 0 min
182 35% B, t =5 min 40% B (5 min), t =10.5 min 45% B (1 min) and t = 16 min 100% B (4
183 min). The flow rate was 1.0 mL/min. The detection was recorded at 200 nm for E1, 17β-
184 E2, E3, EE2 and HEX and at 242 nm for PRED, NAN, T, 17α-MT, AND, DES and P in
185 order to obtain the maximum sensitivity for all the compounds (Fig. 1).
186
187

Accepted Manuscript

ACCEPTED MANUSCRIPT

188

189 3. Results and discussion

190

191 *3.1 Characterization of SBA-15-C18-CO sorbent*

192

193 XRD pattern of the SBA-15-C_{18}-CO displayed a well-resolved pattern at low 2θ values
194 with a very sharp (100) diffraction peak at 0.90 and a weak diffraction peak (110) at
195 1.68. d_{100}-spacing value and unit cell parameter (a_0) were: 98 and 113 Å, respectively
196 (Fig. 2). This pattern suggests that the prepared functionalized silica contains well-
197 ordered hexagonal arrays of one-dimensional channel structure. The N_2 adsorption-
198 desorption isotherms for this material were of type IV according to the I.U.P.A.C.
199 classification with an H1 hysteresis loop that is representative of materials with pores of
200 constant cross-section (Fig. 3). The synthesized material possessed very high S_{BET} (796
201 m^2/g), a pore volume of 0.88 cm^3/g and a BJH pore diameter of 76 Å, typical of
202 surfactant-assembled mesostructures. Scanning electron microscopy (SEM) images
203 showed that SBA-15-C_{18}-CO has cylindrical shape, with an average particle size of 1.4
204 µm (length) and 750 nm (wide). Transmission electron microscopy (TEM) images
205 demonstrated a clear arrangement of hexagonal pores with uniform size for this
206 material. The amount of attached C_{18} molecules onto the mesoporous silica surface (L_o
207 = 0.69 mmol/g) was estimated from the percentage of carbon in the functionalized
208 mesoporous silica, calculated by elemental analysis (17% C). Finally,
209 thermogravimetric analysis (TGA) curve of the SBA-15-C_{18}-CO (Fig. 4) showed a
210 degradation process between 200-600 ºC with a weight loss of about 17%, due to the
211 breakage of pendant groups anchored on the silica surface (exothermic degradation

Accepted Manuscript

ACCEPTED MANUSCRIPT

212 process). The mass loss observed in the SBA-15-C_{18}-CO is in agreement with the
213 amount of C_{18} groups covalently bound to the support, calculated by elemental analysis.
214 Two main approaches can be used to achieve hybrid mesoporous silicas: (a) the
215 post-synthesis (PS), or "*grafting*", method and (b) the co-condensation (CO), or "*one-*
216 *pot*", method [17]. In a previous paper of our research group, a PS method was used to
217 modify the surface of previously prepared SBA-15, through silylation with
218 chloro(dimethyl)octadecylsilane in an organic solvent under reflux conditions [16]. One
219 drawback of PS method is the reduction in the porosity of the functionalized material,
220 which depends on the size of organic ligand and the degree of functionalization. Thus, if
221 bulky ligands that react preferentially at the pore openings during the initial stages of
222 the grafting process are used (i.e. C_{18} groups), further diffusion of ligands into the center
223 of pores can be impaired and a pore-blocking effect produced. In this paper, hybrid
224 SBA-15 mesoporous silica has been obtained directly in a "one-step" procedure by
225 hydrolysis and co-condensation of a tetraalkoxysilane (TEOS) with one
226 organoalkoxysilane (OTES) in the presence of a structure-directing agent (Pluronic
227 123). This procedure overcomes the main drawbacks of the PS method and leads to
228 hybrid SBA-15 material containing accessible functional groups that are more
229 homogeneously distributed inside the pore channels and without pore blocking. For this
230 reason, the new material SBA-15-C_{18}-CO prepared in the current work has higher S_{BET},
231 pore volume, pore diameter and amount of attached C_{18} molecules, in comparison with
232 the SBA-15-C_{18} previously prepared by the PS method [16].
233
234
235
236

Accepted Manuscript

ACCEPTED MANUSCRIPT

237 *3.2 Optimization of the sample treatment*

238

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

246 It is well known that milk is a complex matrix with numerous different
247 compounds, ranging from simple inorganic salts to large proteins, so in order to remove
248 unwanted matrix components from the milk, a previous liquid-liquid extraction (LLE)
249 process is necessary to make this sample suitable for SPE application. In addition, with
250 the aim of developing a more cost effective and environment friendly sample treatment
251 method that would consume lower volumes of organic solvents, a smaller milk sample
252 size (1 g) was selected. Firstly, 1 g of spiked milk was extracted with 2 mL 0.2 M
253 acetate buffer and 2.5, 3.75 or 5 mL of MeOH. The mixture was vortexed during 2 min
254 and after was centrifuged at 4000 rpm for 5 min to separate the precipitate. Finally, the
255 supernatant was decanted and diluted with water to a final volume of 25 mL (to reduce
256 the MeOH to 10, 15 or 20% by volume, respectively) and, then, the extract was purified
257 by SPE according to the protocol described in previous works [16, 23]. Results obtained
258 indicated that the use of a lower volume of MeOH provides higher recoveries for E3,
259 PRED, NAN, 17β-E2 and T, with an important increase of 60% in the recovery of E3
260 and of 50% in the recovery of PRED. This fact confirmed that large percentage of
261 MeOH can produce a break-through effect during the loading step for some of the target

Accepted Manuscript

ACCEPTED MANUSCRIPT

262 analytes [8]. On the other hand, for EE2, E1, 17α-MT, AND, DES, HEX and P
263 recoveries were not modified, or suffered a slightly reduction, with the increase in the
264 percentage of MeOH. For this reason, it was concluded that is important than the
265 amount of MeOH remaining from the LLE step was diluted to 10% in the sample
266 extract, in order to achieve the best recoveries for all the target analytes.

267 The next step to optimize the sample treatment process was the type of elution
268 solvent in the SPE step, since this solvent should have enough elution ability to desorb
269 the analytes and facilitate the further sample treatments. MeOH and ACN were tested
270 for this purpose, setting an elution volume of 2 mL (Fig. 5). Best results were obtained
271 using MeOH as elution solvent, obtaining recoveries over 80% for all analytes, except
272 for DES (54%). The low recovery percentage obtained for DES, somewhat lower than
273 the obtained for the other analytes studied, has been attributed to a phenomenon in
274 which some kind of equilibrium process between two different isomeric forms of this
275 compound could take place [18]. Finally, the volume of the elution solvent was also
276 investigated as the quantity of MeOH that loaded on the cartridge has great effect on the
277 recovery of analytes. For this purpose, different volumes of MeOH (1 x 2 mL, 1 x 3 mL,
278 and 2 x 2 mL) were tested. Good recoveries and minimal interferences in the detection
279 were observed employing 2 mL as elution volume for the entire target compounds,
280 except for DES, and not significant differences in the recovery values were observed by
281 using higher MeOH volumes (Fig. 6). For this reason, 2 mL of MeOH were found to be
282 the optimum volume, as excessive volume would lead to long time for the next dryness
283 steps.

284 It is well known that the presence of hydrophobic C_{18} groups onto the silica
285 surface generates advantages to the adsorption of hydrophobic organic compounds, such
286 as the ones studied in this work, and that the capacity of the sorbent to do so improves

Accepted Manuscript

ACCEPTED MANUSCRIPT

287 as the percentage of C_{18} loading increases. In that respect, the good results achieved
288 with the SBA-15-C_{18}-CO sorbent can be attributed not only to its high loading by the
289 C_{18} groups (L_o = 0.69 mmol/g) but also to its uniform surface coverage and good
290 accessibility to these groups. On the other hand, residual fats, proteins and
291 carbohydrates that were not completely removed in the LLE step, which contain
292 numerous hydroxyl, amino and organophosphate groups can interact at multiple sites in
293 the SBA-15-C_{18}-CO sorbent (with C_{18} groups and/or with residual non-modified silanol
294 groups in the silica surface). Hence they are retained in the cartridge and this fact has an
295 important effect in order to achieve clean extracts to inject in the HPLC system after the
296 SPE step.
297
298 *3.3 Performance of the method*
299
300 The instrumental linearity was evaluated using standard mixtures of the twelve steroids
301 in MeOH at seven concentration levels, in the range of instrumental quantitation limit
302 (IQL) to 100 µg/mL for each hormone. The slope and intercept values of the calibration
303 curves were determined using regression analyses. Linear relationship was found
304 between corrected peak areas and the concentration of the analyte in all cases, with
305 regression coefficients (R^2) ≥ 0.990 (Table 1). On the other hand, to evaluate the
306 linearity of the method, external calibration curves were prepared by spiking milk
307 samples (whole and skimmed) with appropriate aliquots of the stock standard solution,
308 to a range of concentration between the method quantification limit (MQL) to 15
309 µg/mL. A linear relationship was found between peak areas and concentration of the
310 analyte in all cases, with R^2 ≥ 0.990. The results showed that linearity of the method
311 was good for the analytes studied. As Table 1 shows, by comparing the slopes of the

Accepted Manuscript

ACCEPTED MANUSCRIPT

312 matrix-free calibration curves with the matrix-matched calibration curves, a significant
313 difference in the slopes of the linear equations was found in most cases that evidence an
314 important influence of the milk matrix.

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

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

Accepted Manuscript

ACCEPTED MANUSCRIPT

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

346

347 *3.4 Comparison with other sample preparation methods*

348

349 The main difficulty in determining dangerous and/or forbidden substances in complex
350 samples such as milk lies in their extraction from the matrix. In fact, this step is the
351 bottleneck of routine analytical methods, because several sample pre-treatment steps are
352 required in most cases. In the present work, a new sample treatment based LLE and SPE
353 for the determination of twelve steroids in goat milk has been proposed. The greatest
354 innovation of the developed procedure has been the use of a new one-pot synthesized
355 functionalized SBA-15 mesoporous silica as a reversed-phase sorbent for SPE. Table 4
356 collects some recent sample preparation methods found for the determinations of the
357 target steroids in milks. As it can be seen, compared with other methods, the sample
358 treatment procedure optimized in this work is simpler and/or faster [6-10, 15, 16]. In
359 addition, recoveries obtained in the present work are in general more satisfactory,
360 between 72 to 105% (except for DES), taken into account that a higher amount of target
361 analytes have been tested. Finally, a comparison of the MQLs obtained in whole milks

Accepted Manuscript

ACCEPTED MANUSCRIPT

362 with a mesoporous silica functionalized by post-synthesis method (0.53 µg/mL for
363 progesterone to 1.30 µg/mL for DES, [16]) and the new SBA-15-C_{18}-CO sorbent
364 (0.035 µg/mL for progesterone, 0.1 µg/mL for DES, this work), indicated that SBA-15-
365 C_{18}-CO achieved the best limits for all compounds, that can be attributed to the better
366 ability of this material not only to remove interferences but also to retain the selected
367 analytes.

368

369 **4. Conclusions**

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

374

375 **Acknowledgements** Authors thank financial support from the Comunidad of Madrid
376 and European funding from FEDER program (project S2013/ABI-3028,
377 AVANSECAL).

378

Accepted Manuscript

ACCEPTED MANUSCRIPT

378 **References**

379 [1] Å. Bergman, J.J. Heindel, S. Jobling, K.A. Kidd, R.T. Zoeller, State of the science
380 of endocrine disrupting chemicals 2012. United Nations Environment Programme and
381 the World Health Organization (2013).
382 [2] R.J. Kavlock, G.P. Daston, C. DeRosa, P. Fenner-Crisp, L.E. Gray, S. Kaattari, G.
383 Lucier, M. Luster, M.J. Mac, C. Maczka, R. Miller, J. Moore, R. Rolland, G. Scott,
384 D.M. Sheehan, T. Sinks, H.A. Tilson, Research needs for the risk assessment of health
385 and environmental effects of endocrine disruptors: a report of the U.S. EPA-sponsored
386 workshop, Environ. Health Perspect. 104 (1996) 715–740.
387 [3] L.S. Birnbaum, S.E. Fenton, Cancer and developmental exposure to endocrine
388 disruptors, Environ. Health Perspect. 111 (2003) 389–394.
389 [4] A. Laganá, A. Bacaloni, I. De Leva, A. Feberi, G. Fago, A. Marino, Analytical
390 methodologies for determining the occurrence of endocrine disrupting chemicals in
391 sewage treatment plants and natural waters, Anal. Chim. Acta 501 (2004) 79-88.
392 [5] Council Directive 96/22 EC (1996) Off J Eur Comm L125-3.
393 [6] G. Kaklamanos, G. Theodoridis, Rapid multi-method for the determination of
394 growth promoters in bovine milk by liquid chromatography-tandem mass spectrometry,
395 J. Chromatogr. B 930 (2013) 22-29.
396 [7] E.M. Malone, C.T. Elliot, D.G. Kennedy, L. Regan, Rapid confirmatory method for
397 the determination of sixteen synthetic growth promoters and bisphenol A in bovine milk
398 using dispersive solid-phase extraction and liquid chromatography-tandem mass
399 spectrometry, J. Chromatogr. B 878 (2010) 1077-1084.
400 [8] T. Tso, D.D. Aga, A systematic investigation to optimize simultaneous extraction
401 and liquid chromatography tandem mass spectrometry analysis of estrogens and their
402 conjugated metabolites in milk, J. Chromatogr. A 1217 (2010) 4794-4795.

Accepted Manuscript

ACCEPTED MANUSCRIPT

403 [9] B. Shao, Z. Zhao, J. Meng, Y. Xue, G. Wu, J. Hu, X. Tu, Simultaneous
404 determination of residual hormonal chemical in meat, kidney, liver tissues and milk by
405 liquid chromatography-tandem mass spectrometry, Anal. Chim. Acta 548 (2005) 41-50.
406 [10] W. Yan, Y. Li, L. Zhao, J.M. Lin, Determination of estrogens and bisphenol A in
407 bovine milk by automated on-line C30 solid-phase extraction coupled with high-
408 performance liquid chromatography-mass spectrometry, J. Chromatogr. A 1216 (2009)
409 7539-7545.
410 [11] X. Xu, F. Liang, J. Shi, Z. Liu, L. Wu, Y. Song, H. Zhang, Z. Wang, Determination
411 of hormones in milk by hollow fiber-based stirring extraction bar liquid-liquid gas
412 chromatography mass spectrometry, Anal. Chim. Acta 790 (2013) 39-46.
413 [12] A. Azzouz, B. Jurado-Sánchez, B. Souhail, E. Ballesteros, Simultaneous
414 determination of 20 Pharmacologically active substances in cow´s milk, goat´s milk and
415 human breast milk by gas chromatography-mass spectrometry, J. Agr. Food Chem. 59
416 (2011) 5125-5132.
417 [13] F. Courant, J.P. Antignac, D. Maume, F. Monteau, F. Andre, B. Le Bizec,
418 Determination of naturally occurring oestrogens and androgens in retail samples of milk
419 and eggs, Food Addit. Contam. 24 (2007) 1358-1366.
420 [14] Y. Shi, D.D. Peng, C.H. Shi, X. Zhang, Y.T. Xie, B. Liu, Selective determination
421 of trace of 17β-estradiol in dairy and meat samples by molecular imprinted solid-phase
422 extraction and HPLC, Food Chem. 126 (2011) 1916-1925.
423 [15] B. Socas-Rodríguez, M. Asensio-Ramos, J. Hernández-Borges, M.A. Rodríguez-
424 Delgado, Hollow-fiber liquid-phase microextraction for determination of natural and
425 synthetic estrogens in milk samples, J. Chromatogr. A 1313 (2013) 175-184.
426 [16] V. Pérez-Fernandez, S. Morante-Zarcero, D. Pérez-Quintanilla, M.A. García, M.L.
427 Marina, I. Sierra, Evaluation of mesoporous silicas functionalized with C18 groups as

Accepted Manuscript

ACCEPTED MANUSCRIPT

428 stationary phases for the solid phase extraction of steroid hormones in milk.
429 Electrophoresis 35 (2014) 1666-1676.
430 [17] I. Sierra, D. Pérez-Quintanilla, Heavy metal complexation on hybrid mesoporous
431 silicas: an approach to analytical applications, Chem. Soc. Rev. 42 (2013) 3792-3807.
432 [18] L. Zhao, H. Qin, R. Wu, H. Zou, Recent advances of mesoporous materials in
433 sample preparation, J. Chromatogr. A 1228 (2012) 193-204.
434 [19] J. Gañán, D. Pérez-Quintanilla, S. Morante-Zarcero, I. Sierra, Comparison of
435 different mesoporous silicas for off-line solid phase extraction of 17β-estradiol from
436 waters and its determination by HPLC-DAD, J. Hazard. Mater. 260 (2013) 609-617.
437 [20] J. Gañán, S. Morante-Zarcero. D. Pérez-Quintanilla, I. Sierra, Evaluation of bi-
438 functionalized mesoporous silica for solid-phase extraction of twelve endocrine
439 disrupting compounds from water, Mater. Letters 132 (2014) 19-22.
440 [21] A. Carpio, D. Esquivel, L. Arce, F.J. Romero-Salguero, P. Van Der Voort, C.
441 Jiménez-Sanchidrián, M. Valcarcel, Evaluation of phenylene-bridged periodic
442 mesoporous organosilica as a stationary phase for solid-phase extraction, J. Chromatogr.
443 A 1370 (2014) 1666-1676.
444 [22] A.A. Al-Rashdi, A doubly functionalized mesoporous silica nonoscavenger for the
445 analytical extraction of triphenyltin from water, Sci. J. Anal. Chem. 1 (2013) 1-6.
446 [23] J. Gañán. S. Morante-Zarcero, D. Pérez-Quintanilla, I. Sierra, A novel hybrid
447 mesostructured silica for the solid phase extraction of estrogenic hormones from waters,
448 Anal. Methods 7 (2015) 4740-4749.
449

ACCEPTED MANUSCRIPT

449 **Fig. 1** Chromatographic separation obtained for twelve endocrine disrupting compounds
450 with the optimized gradient elution. Detection was recorded at a) 200 nm for E1, 17β-
451 E2, E3, EE2 and HEX and b) 242 nm for PRED, NAN, T, 17α-MT, AND, DES and P.
452 **Fig. 2.** XRD pattern of SBA-15-C_{18}-CO.
453 **Fig. 3**. Nitrogen adsorption-desorption isotherms and ore size distribution (inset) of
454 SBA-15-C_{18}-CO.
455 **Fig. 4.** Thermogravimetric curves and heat flow of SBA-15-C_{18}-CO.
456 **Fig. 5.** Effect of different elution solvents on the solid-phase extraction step of the
457 sample treatment procedure.
458 **Fig. 6**. Effect of different methanol elution volumes on the solid-phase extraction step
459 of the sample treatment procedure.
460 **Fig. 7.** Chromatograms corresponding to A) whole milk sample and B) skimmed milk
461 sample; a) 5 µg/mL spiked milk sample with twelve endocrine disrupting compounds
462 and b) blank milk sample after the optimized sample treatment method. Experimental
463 conditions as in Fig. 1.
464
465
466
467 ***Highlights***
468
469 ► SBA-15-C18-CO mesoporous silica was prepared by one-pot synthesis
470 ► Good recoveries were obtained for the determination of twelve EDCs in different milk samples by HPLC
471
472
473

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

Analyte	Calibration curve

Accepted Manuscript

ACCEPTED MANUSCRIPT

	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

476 [a] Linear range: IQL-100 µg/mL
477 [b] Linear range: MQL-15 µg/mL
478

479

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

Accepted Manuscript

ACCEPTED MANUSCRIPT

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

483

484 [a] MQL as low level

485 [b] 15 µg/mL as high level

486

487

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

Analyte	MQL	Low level[a]		High level[b]	
	(µg/mL)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)

Accepted Manuscript

ACCEPTED MANUSCRIPT

		Mean ± SD		Mean ± SD	
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

491

492 [a] MQL as low level

493 [b] 15 μg/mL as high level

494

495 **Table 4.** Comparison of SBA-15-C18-CO sorbent for SPE procedure with other sample
496 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]

Accepted Manuscript

ACCEPTED MANUSCRIPT

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

497 [a]Estimated time according to the works; * Total time (sample preparation + analysis) AND:
498 Androstenodione; DES: Diethylstilbestrol; DIE: Dienestrol; dSPE: dispersive solid phase extraction 17β-
499 E2: 17β-Estradiol; E1: Estrone; E3: Estriol; EE2: Ethinylestradiol; HEX: Hexestrol; HF-LPME: Hollow-
500 Fiber Liquid-phase microextraction; HLB: Hydrophilic Lipophilic balance; LLE: Liquid-liquid
501 extraction; 17α-MT: 17α-Methyltestorone; NAN: Nandrolone; P: Progesterone; PRED: Prednisolone;
502 SPE: solid phase extraction; T: Testosterone; TREM: Trembolone
503
504

Accepted Manuscript

ACCEPTED MANUSCRIPT

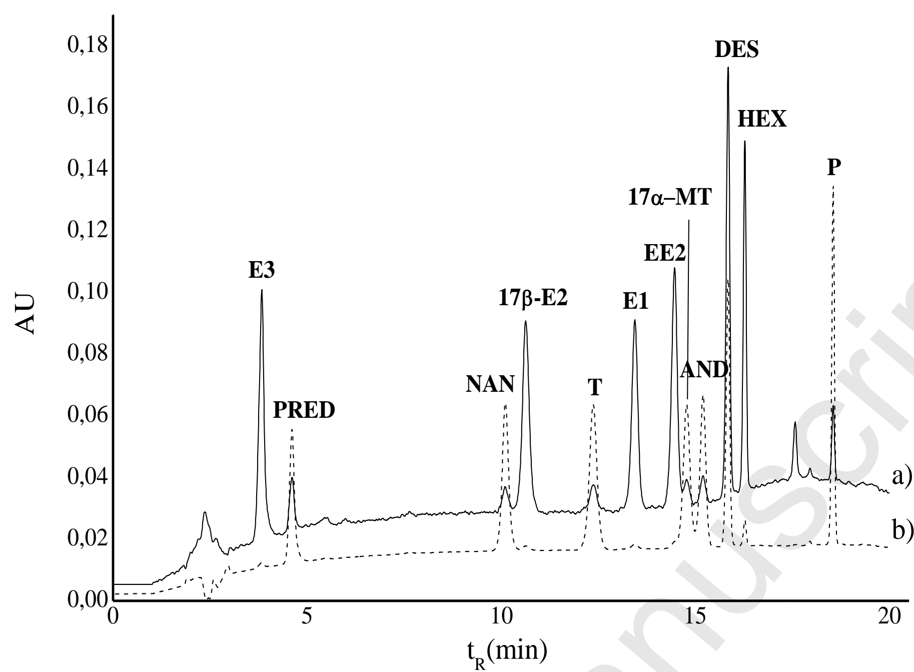
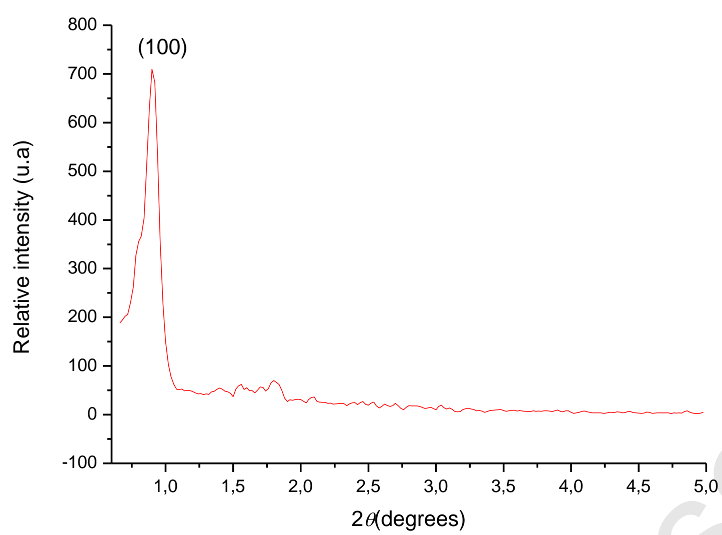


Fig. 1

Accepted Manuscript

ACCEPTED MANUSCRIPT



Accepted Manuscript

ACCEPTED MANUSCRIPT

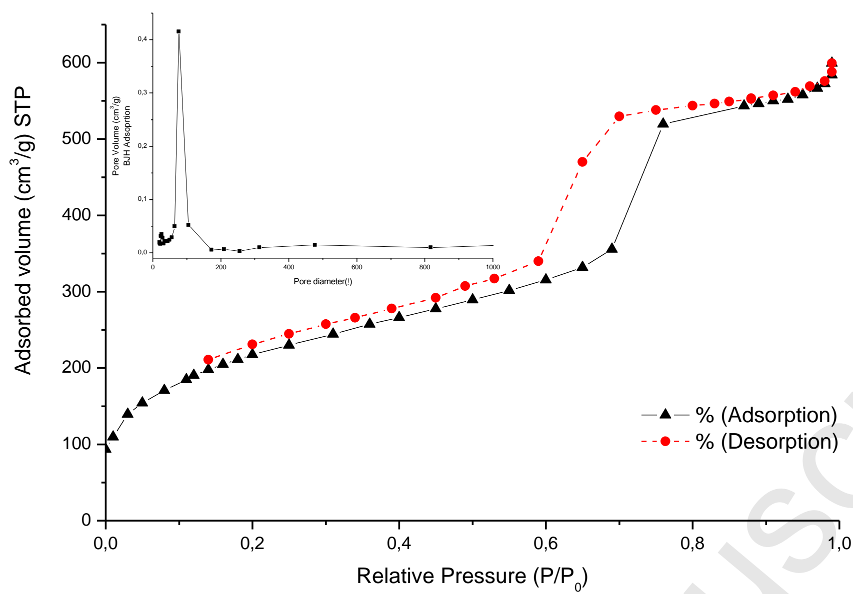


Fig. 3

Accepted Manuscript

ACCEPTED MANUSCRIPT

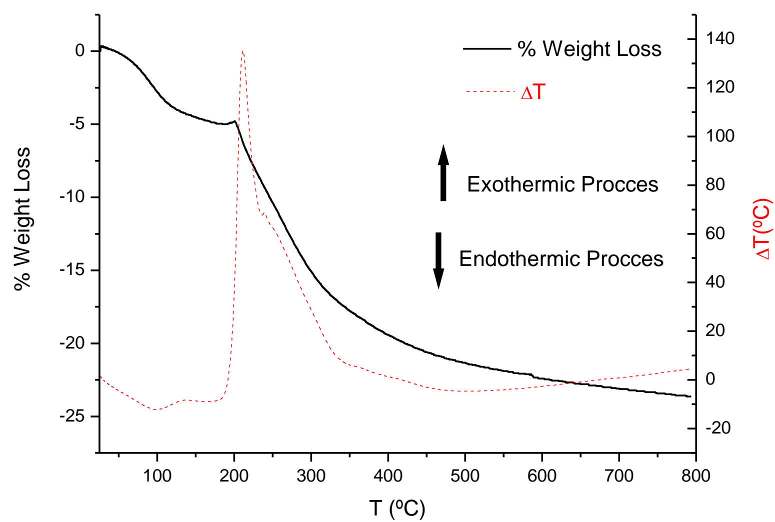


Fig. 4

Accepted Manuscript

ACCEPTED MANUSCRIPT

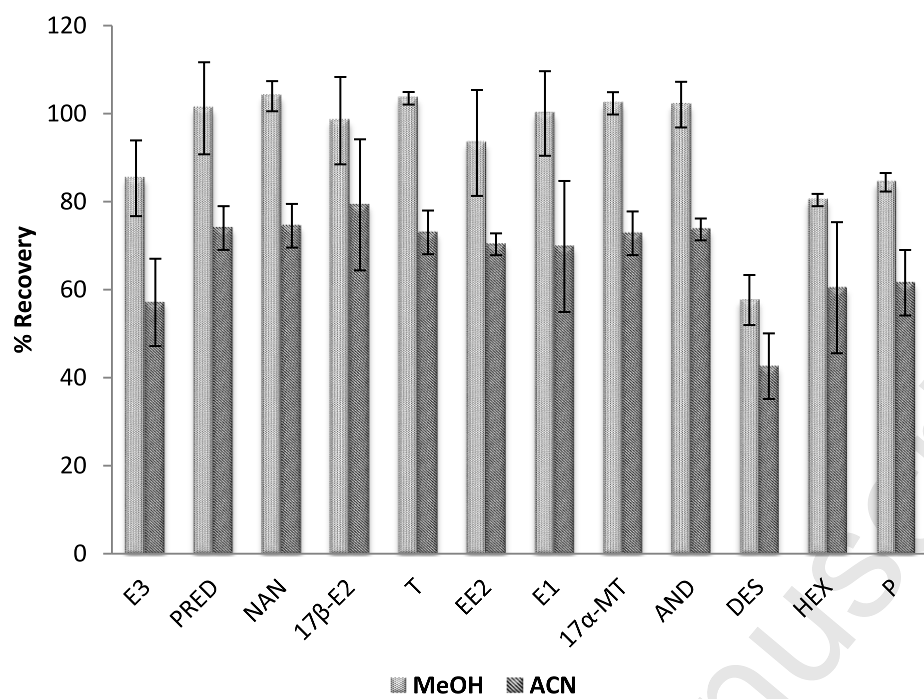


Fig. 5

Accepted Manuscript

ACCEPTED MANUSCRIPT

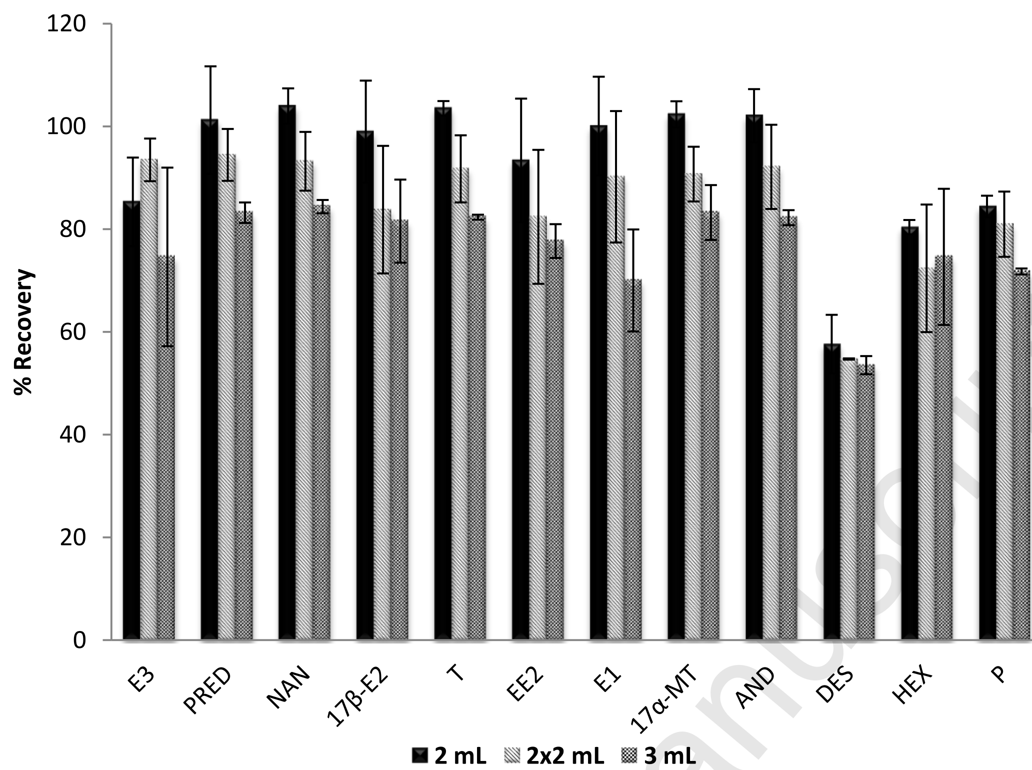


Fig. 6

Accepted Manuscript

ACCEPTED MANUSCRIPT

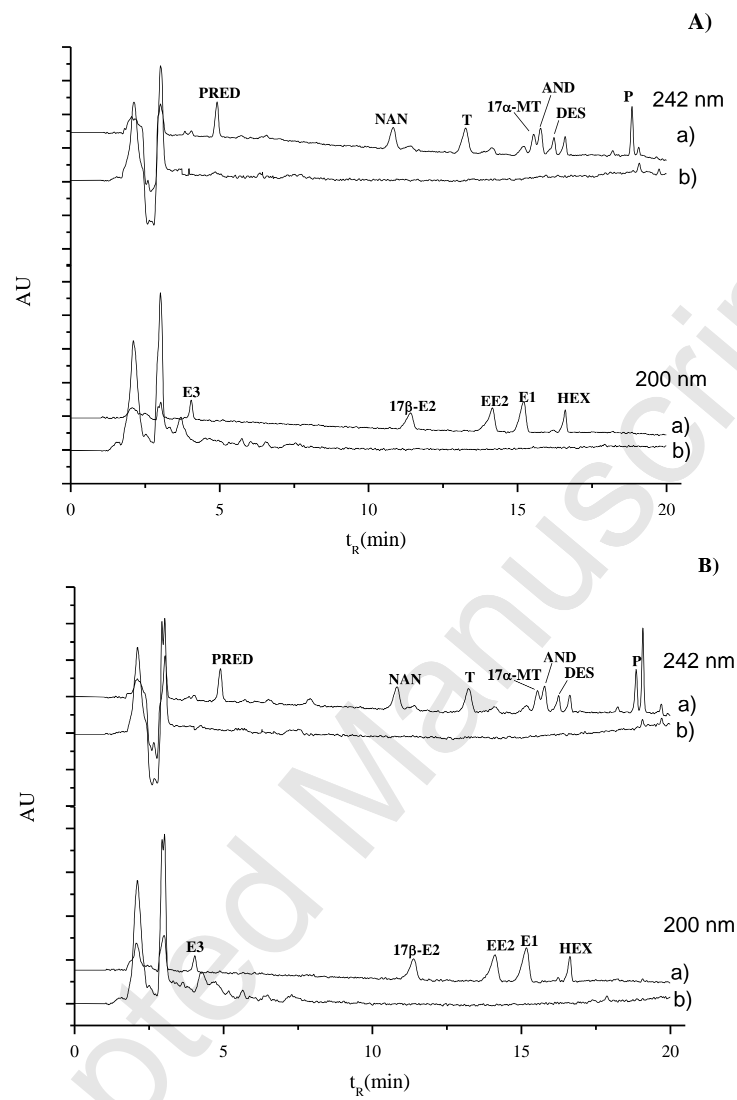


Fig. 7

Accepted Manuscript