Elsevier Editorial System(tm) for Journal of Chromatography A
Manuscript Draft

Manuscript Number: JCA-10-2200R1

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Article Type: ISC 2010

Keywords: Estrogens; molecularly imprinted polymers; ultra high performance liquid chromatography; mass spectrometry; water samples.

Corresponding Author: Dr Paolo Lucci,

Corresponding Author's Institution: University of Barcelona

First Author: Paolo Lucci

Order of Authors: Paolo Lucci; Oscar Núñez, Ph.D.; Maria Teresa Galceran, Professor

Solid-phase extraction using molecularly imprinted polymer for selective extraction of natural and synthetic estrogens from aqueous samples

Paolo Lucci*, Oscar Núñez, M.T. Galceran

Department of Analytical Chemistry, University of Barcelona, Av. Diagonal 647, 08028 Barcelona, Spain

Abstract

A method is proposed for the clean-up and preconcentration of natural and synthetic estrogens from aqueous samples employing molecularly imprinted polymer (MIP) as selective sorbent for solid-phase extraction (SPE). The selectivity of the MIP was checked toward several selected natural and synthetic estrogens such as estrone (E1), 17β-estradiol (β-E2), 17α-estradiol (α-E2), estriol (E3), 17α-ethinylestradiol (EE2), dienestrol (DIES) and diethylstilbestrol (DES). Ultrahigh pressure liquid chromatography (UHPLC) coupled to a TSQ triple quadrupole mass spectrometry (QqQ) was used for analysis of target analytes. The chromatographic separation of the selected compounds was performed in less than 2 min under isocratic conditions. The method was applied to the analysis of estrogens in spiked river and tap water samples. High recoveries (>82%) for estrone, 17β-estradiol, 17α-estradiol, estriol and 17α-ethinylestradiol were obtained. Lower but still satisfactory recoveries (>48%) were achieved for dienestrol and diethylstilbestrol. The method was validated and found to be linear in the range 50-500 ng L^{-1} with correlation coefficients (R^2) greater than 0.995 and repeatability relative standard deviation (RSD) below 8% in all cases. For analysis of 100-ml sample, the method detection limits (LOD) ranged from 4.5 to 9.8 ng L^{-1} and the limit of quantitation (LOQ) from 14.9 to 32.6 ng L^{-1}. To demonstrate the potential of the MIP obtained, a comparison with commercially available C_{18} SPE was performed. Molecularly imprinted SPE showed higher recoveries than commercially available C_{18} SPE for most of the compounds.

These results showed the suitability of the MIP-SPE method for the selective extraction of a class of structurally related compounds such as natural and synthetic estrogens.

KEYWORDS: Estrogens; molecularly imprinted polymers; ultrahigh pressure liquid chromatography; mass spectrometry; water samples.

* Corresponding author. Tel. 34-93-4021286:; Fax :34-93-4021233.
E-mail address : paololucci2001@yahoo.it (Paolo Lucci)

1. Introduction

Endocrine disrupting compounds (ECDs) are a heterogeneous group of substances that may interact with the endocrine system of organisms. Estrogens are important members of the ECDs group and they have been often recognized as the major contributors to the endocrine-disrupting activity observed in aquatic environments [1]. They are excreted into the aquatic environment through human and animal urine and the use of natural and synthetic estrogens in medicine or in veterinary have caused their presence in aquatic ecosystems. Although the environmental concentrations of estrogens are very low (up to 105 ng L^{-1}) [2,3,4], their adverse effect on the reproduction of wildlife and humans is not negligible [5]. To assess the ecological risk of these compounds, sensitive determination of estrogens in environment is needed.

Several analytical methods have been developed to identify and quantify ECDs in water samples [6], including high-performance liquid chromatography with several detection systems such as UV [7,8], fluorescence [9] and coupled to mass spectrometry [10,11,12,13], gas-chromatography after derivatization [14] and enzyme-linked immunosorbent assay [15].

Currently, liquid chromatography coupled with tandem mass spectrometry is the most common approach. However, as the concentrations of the estrogenic compounds in environmental matrices are very low, a clean-up and preconcentration step is usually required in order to minimize interferences and improve method accuracy and sensitivity. Solid-phase extraction (SPE) is a well-established method routinely used for clean-up and preconcentration step of this compounds [16]. The main drawback of conventional SPE sorbents is their lack of selectivity resulting in co-extraction of interfering matrix components, which can negatively affect quantitation. Selectivity can be obtained using sorbents based on molecularly imprinted polymers (MIP). These types of sorbents are synthetic materials possessing an artificially generated three-dimensional network that is able to specifically rebind a target analyte, or class of structurally related compounds. MIP has the advantages of being very selective, cost-effective, and not suffering from storage limitations and stability problems regarding organic solvents. MIPs have been proposed in recent years as sorbent for the extraction and/or removal of endocrine disrupting compounds [17,18,19]. In addition, the potential of MIP as SPE sorbent for extraction of diethylstilbestrol [20,21], 17β-estradiol [22] and 17α-ethinylestradiol [23] from aqueous samples has also been demonstrated. The aim of this work was to develop for the first time a group-selective extraction method based on molecularly imprinted polymer for the analysis of natural (estrone, 17β-estradiol, 17αestradiol, estriol) and synthetic estrogens (17α-ethinylestradiol, dienestrol and diethylstilbestrol) in aqueous samples. For analysis of the selected analytes ultrahigh pressure liquid chromatography (UHPLC) coupled to a TSQ triple quadrupole mass spectrometry (QqQ) with atmospheric pressure chemical ionization (APCI) was used. The applicability of the method was evaluated analyzing estrogens in river and tap water samples spiked at concentrations similar to those found in the aquatic environment.

2. Experimental

2.1 Materials and chemicals

HPLC-grade methanol, water and acetonitrile for the UHPLC analysis were purchased from Riedel-de Haën (Seelze, Germany). Acetonitrile, acetone, chloroform and methanol used for the synthesis and chromatographic evaluation of the polymers were supplied by Carlo Erba (Val de Reuil, France). Estrone (E1), 17β-estradiol (β-E2), 17α-estradiol (α-E2), estriol (E3), 17α-ethinylestradiol (EE2), dienestrol (DIES) and diethylstilbestrol (DES) (structures shown in **Fig.1**) were from Sigma-Aldrich (Steinheim, Germany). Nitrogen (99.8% pure) supplied by Claind Nitrogen Generator N2 FLO (Lenno, Italy) was used for the mass spectrometry ionization source. High-purity Argon (Ar_1) and helium, purchased from Air Liquide (Madrid, Spain), were used as a collision-induced gas (CID gas) in the triple quadrupole mass spectrometer.

Molecularly imprinted polymer (product code: AFFINIMIP) and non-imprinted polymer (NIP) were provided by POLYINTELL (Val de Reuil, France). MIPs are obtained by radical polymerization using initiatior 2,2´-azobis-isobutyronitrile from Sigma–Aldrich (Steinheim, Germany) and based on difunctional acrylic cross-linker monomers (Sigma–Aldrich, Steinheim, Germany). Isolute cartridges (3 mL) packed with 100 mg of C_{18} material were purchased from IST (Mid Glamorgan, UK).

2.2 Instrumentation

Chromatographic evaluation of the imprinted polymers was performed in an LC system from Gilson (Villiers le Bell, France) that consisted of a Pump 322 and a UV/VIS detector (UV/VIS-155). Stainless steel LC columns (250 mm x 2.1 mm) filled with molecularly imprinted and non-imprinted polymers were packed using 1666 HPLC column Slurry Packer (Alltech Associates Applied Science Ltd, Lancashire, UK). The UHPLC system used for the

MIP-SPE evaluation consisted of an Accela liquid chromatograph system (Thermo Fisher Scientific, San José, CA, USA) coupled to a triple quadrupole mass spectrometer TSQ Quantum Ultra AM (Thermo Fisher Scientific, San José, CA, USA) equipped with atmospheric pressure chemical ionization (APCI) source. The column used to analyze the various MIP-SPE fractions was an Ascentis Express Phenyl-Hexyl HPLC Column (150 mm × 2.1 mm i.d., 2.7 µm particle size) from Supelco (Bellefonte, PA, USA) ~~Sigma- Aldrich)~~. The Xcalibur software version 2.0 (Thermo Fisher Scientific, San José, CA, USA) was used to control the LC/MS system and to process data.

2.3 Procedure

2.3.1 Chromatographic evaluation of the imprinted polymers

Imprinted and non imprinted polymers (25-45 µm particles) were slurry-packed in chloroform/methanol (80:20, v/v) into LC columns using a slurry packer. The LC was carried out at 21 °C and the flow rate was kept constant at 1 mL min^{-1}. The analytical wavelength was set at 220 nm. Acetone was used as a void volume marker and the retention factor (k) for each analyte was calculated as $k = (t\text{-}t_0)\ t_0^{-1}$, where t and t_0 are the retention times of the analyte and the void marker (acetone), respectively. The imprinted factor (IF) was calculated as IF = $k_{\mathrm{MIP}}\ k_{\mathrm{NIP}}^{-1}$, *i.e.* the ratio of the retention factor of each analyte in the MIP column to that in the NIP column. The elution times of the void marker on MIP and NIP columns were 0.6 and 0.58 min, respectively.

2.3.2 Extraction and clean-up using MIP-SPE

Empty SPE cartridges of 4-mL capped with fritted polypropylene disks at the bottom and on the top were packed with 100 mg of each polymer particles (imprinted and non-imprinted). Before each use, sorbents were conditioned with acetonitrile (5 mL) followed by water (5 mL). For the MIP-SPE experiments, 100mL of Milli-Q, river and tap water samples free from analytes were filtered using 0.45µm pore size cellulose filters and spiked with different amounts of estrogens to reach a final concentration of 50, 100, 150 and 200 ng L^{-1}. The samples were percolated through the MIP-SPE cartridge at the flow rate of 2 ml min^{-1}. The sorbent was washed with 4 mL of water/acetonitrile (80:20, v/v) followed by 2 mL of water. Full vacuum was applied for 5 min to ensure the polymer was completely dry. Then, the sorbent was washed with acetonitrile (2 mL) followed by 2 mL of acetonitrile/methanol mixture (95:5, v/v). Estrogenic compounds were finally eluted from the cartridges with three aliquots (3 x 1 mL) of methanol.

Each fraction eluted from the MIP-SPE cartridge was evaporated to dryness under a stream of nitrogen and the residues were reconstituted in 500 µL of the UHPLC mobile phase. Extraction recovery was calculated by comparing the peak areas of the analytes from extracted samples with those of control samples corresponding to 100%. Recovery experiments were performed in triplicate.

2.3.3 Extraction using C_{18} SPE

C_{18} SPE columns were pre-treated with 4 mL of methanol followed by 10 mL of Milli-Q water. Then, spiked river water samples (100 ml) was loaded on the cartridge with a flow rate of 10 mL min^{-1} after which the column was dried under vacuum for 20 min. Acetone (3 mL) was used to elute the analytes from the extraction column [24]. The extract was evaporated under a gentle stream of nitrogen and redissolved in 500 µL of the ultrahigh pressure LC mobile phase.

2.3.4 ~~2.33~~ LC-MS conditions

The chromatographic separation of estrogens was performed at 35 °C using isocratic elution. A mobile phase consisting of a mixture of water/acetonitrile/methanol (51:44:5, v/v/v) at 450 µL min^{-1} flow rate was used. Injection volume was set to 10µL. Atmospheric pressure chemical ionization (APCI) interface in the positive (PI) ionization mode was used. Nitrogen (purity > 99.98%) was used as a sheath gas, ion sweep gas and auxiliary gas at flow rates of 50, 0 and 40 a.u. (arbitrary units), respectively. The vaporizer temperature was set at 350°C and corona discharge current at 10µA. Quantitative analysis was performed using selected reaction-monitoring mode (SRM). Argon was used as collision gas at 1.5mtorr and the optimum collision energy (CE) and the SRM transition with the best signal intensity was used for quantification (**Tab.1**).

Matrix-matched standard calibration curves, at seven concentration levels (5 to 1000 ng mL^{-1}) for each compound were obtained by spiking analytes into sample extracts. Good linearity of response by direct injection was obtained for all compounds. The resulting correlation coefficients (R^2) were higher than of 0.999 in all cases. The instrumental detection limits ranged from 8.3 to 25.1 pg injected, based on a signal to noise ratio of 3:1 (**Tab.1**).

3. Results and discussions

3.1 Evaluation of the MIP by LC

Chromatographic evaluation of the imprinted polymer was performed in order to assess the MIP activity. For this purpose, the chromatographic behaviour of β-E2 on the molecularly imprinted polymer packed column was compared with that of the column filled with non imprinted polymer. The choice of the mobile phase is crucial to identify the nature of the interactions involved in the retention process. Thus, different ACN/MeOH mixtures (MeOH content ranging from 0 to 10%) were used as mobile phases to characterize the MIP before SPE applications. β-E2 was totally retained on MIP when using acetonitrile as mobile phase (no elution of β-E2 after 75 min), whereas in NIP control, β-E2 has a retention time of 43 min (data not shown). These results reveal the successful imprinted process. Then, to obtain the optimal selectivity, a further set of experiments was performed using acetonitrile/methanol mixtures. In all polymers, the addition of methanol in the mobile phase resulted in a decrease in retention of β-E2. The highest imprinting factor (IF=3.9) was obtained using a mixture of ACN/MeOH (95:5, v/v), indicating that a moderate increase of the methanol content enhanced the selectivity of the MIP. As it is shown in **Fig.2**, a NIP retention time of 3.2 min for β-E2 whereas this compound was more strongly retained when the MIP polymer was used (t_{MIP} = 11.2 min). This behavior reveals the difference in the strength of the interactions between the analyte and the two sorbents. The strong retention of the MIP for β-E2 results from the presence of cavities with high affinity binding sites whereas β-E2 was adsorbed by the NIP through non-specific relative weak interactions which was easily eluted by a mobile phase containing low amounts of a polar protic solvent. This result was further supported by MIP-SPE procedure described below.

3.2 Study of the SPE retention mechanism

To develop the MIP-SPE method for the selective extraction of the selected estrogens in water, experiments for the optimization of conditioning, loading, washing and elution steps were performed. First, MIP performance was evaluated using Milli-Q water. After conditioning the imprinted polymer with 5mL of ACN followed by 5mL of water, a volume of 100 mL of Milli-Q water spiked with 200 ng L^{-1} of each estrogenic compound were percolated through the MIP. The same experiment was carried out on NIP. Under aqueous condition estrogens are principally retained on the polymer by non-specific interactions such as ionic and hydrophobic. In order to generate specific interactions between the target compounds and the MIP and to disrupt the non-specific interactions between the polymer and apolar matrix components that can be present in real samples, the sorbents were completely dried in vacuum during 5 min and, once the drying step was carried out, 2mL of acetonitrile were applied. A partial elution of the compounds (2-8%) was observed for NIP, while in MIP most of the compounds were completely retained (**Fig.3-w1**). The use of acetonitrile, a polar non protic solvent with a high dielectric constant, allowed the formation of specific interactions via hydrogen bonds between the molecules and the functional monomers. Each molecule displays at least one hydroxyl group able to interact specifically with imprinted cavities. In order to clearly demonstrate the real imprinting effect of the MIP, 2mL of a mixture of acetonitrile/methanol (95:5, v/v) were applied to the polymer in order to disrupt the residual non-specific interactions formed on the MIP and NIP by hydrogen bonds. Estrogens were completely desorbed in the non-imprinted polymer during the acetonitrile/methanol (95:5, v/v) washing step (**Fig.3-w2**) due to the presence of a protic polar solvent such as methanol and to the lack of MIP cavities. In contrast in the MIP most of the compounds were ~~manly~~ mainly retained and only DIES and DES were partially eluted. This can be explained because this analytes, besides the hydroxyl groups at para positions of the two benzene rings, have quite different chemical structure with a different number of aromatic rings (**Fig.1**). Finally, estrogens were eluted from MIP-SPE with 3x1mL of methanol. The results obtained from the analysis of the elution fractions showed a good recovery for all estrogenic compounds (**Fig.3-E**). High extraction recoveries (>95%) were obtained for E1, β-E2, α-E2, E3, and EE2 demonstrating the effectiveness of the newly prepared MIP. For DES and DIES, lower recoveries were found between 50% and 60%. Although these two compounds were more easily removed than the other estrogenic compounds during the acetonitrile/methanol (95:5, v/v) organic washing step, their MIP recoveries were relatively high. Thus, even if MIP exhibited a lower affinity for these compounds, it is clear that the synthesized polymer can recognize structurally related compounds.

3.3 Application of MIP-SPE procedure

To check the applicability of the developed MIP-SPE for the extraction of the selected estrogens in real matrices, river and tap water samples were collected and submitted to the MIP extraction procedure. In real samples an additional washing step was used in order to remove non-selectively bounded polar matrix components. Thus, after loading, 4mL of a mixture water/acetonitrile (80:20, v/v) followed by 2mL of water were applied to the polymers. As expected, there was no desorption from the MIP-SPE of estrogens during the additional aqueous washing steps (data not shown). Then, the same procedure as described above was applied. **Figure 4** shows the SRM chromatogram corresponding to the injection of the elution fraction after the purification of river water spiked at 100 ng L^{-1} on MIP. All compounds, including the two isomers of estradiol, were successfully separated in less then 2 min.

The linearity of the total analytical method, including the MIP-SPE step, was checked by analyzing water samples spiked at different concentrations ranging from 50 to 500 ng L^{-1}. Good linearity of the seven analytes was achieved in both river and tap water with correlation coefficients greater than 0.995 (**Tab.3**). The limits of detection (LODs), defined as the concentrations that yielded S/N ratios greater than or equal to 3, and the limits of quantification (LOQs), defined as the concentrations that yielded S/N ratios greater than or equal to 10, were determined through MIP-SPE extractions of spiked water samples. The LODs ranged from 4.5 to 9.8 ng L^{-1} whereas LOQs were in the range of 14.9-32.6 ng L^{-1} (**Tab.3**). The recovery, accuracy and precision of the developed MIP-SPE method were calculated in Milli-Q, river and tap water samples at four concentration levels. The recovery values obtained are presented in **Tab.2**. Comparable average recoveries at the different fortification levels were founded in Milli-Q and river water samples varying from 82 (E1) to 106% (EE2). Similar results were observed for tap water samples with a mean recovery in the elution fractions ranging from 82 (E1) to 95% (α-E2). For DES and DIES, recoveries between 48 and 63% were obtained. These results revealed the ability of MIP to extract estrogens in real water samples without suffering from matrix interferences during the rebinding process of the target compounds. The precision and linearity of the method were satisfactory with repeatability relative standard deviation (RSD) below 8% in all cases.

To demonstrate further the potential of the MIP obtained for the extractions of the selected estrogens in real matrices, a comparison between the MIP-SPE and commercially available C_{18} SPE was performed. The retention of the estrogenic compounds on both sorbents was evaluated under optimal conditions by percolating a river water samples spiked at 50 ng L^{-1}. Resulting elution profiles are described in **Fig.5**. The recoveries of MIP extraction were higher compared with C_{18} SPE and only DIES and DES were strongly retained on the C_{18} cartridges. However it should be pointed out that the MIP-SPE procedure included also a clean-up step.

The results obtained showed that the imprinted sorbent can be a good substitute of the traditional C_{18} sorbent, revealing the suitability of the method for the selective extraction of natural and synthetic estrogens from river and tap water samples.

4. Conclusions

In this work, we propose a MIP-SPE procedure for the group-selective extraction of natural and synthetic estrogens (estrone, 17β-estradiol, 17α-estradiol, estriol, 17α-ethinylestradiol, dienestrol and diethylstilbestrol) employing a new molecularly imprinted polymer (MIP) as selective sorbent. The new MIP has high specific recognition selectivity for estrogenic compounds with similar structure. Recovery, precision and accuracy found for the selective extraction of the target analytes from river and tap water samples spiked at concentrations similar to those observed in the aquatic environment allowed to propose this method for the determination of the selected estrogenic compounds at concentrations down to the ng L^{-1} level.

Acknowledgments:
This work has been supported by CARBOSORB project [(FP7 Marie Curie Industry-Academia Partnerships and Pathways (IAPP)]. Thanks to POLYINTELL team for their donation of MIP and NIP polymers and SPE cartridges.

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Figure captions

Fig.1. Selected estrogenic compounds

Fig. 2. Chromatograms of 17β-estradiol (2.2mM mM) on LC columns filled with non-imprinted (NIP) and imprinted polymer (MIP). Sample volume: 20 μL. Mobile phase: ACN/MeOH (95:5, v/v). Flow rate:1 mL min^{-1}. Column dimension: 250 mm × 2.1 mm. Detection at 220 nm. T: 21 °C.

Fig.3 Elution profiles of the estrogenic compounds obtained on MIP and NIP (100mg of sorbent) in MilliQ-water. W1: 2mL ACN, W2: 2mL ACN/MeOH (95/5, v/v), E: 3mL MeOH

Fig. 4. SRM Chromatogram of estrogens extracted from 100 mL river water spiked at 100 ng L^{-1}

Fig. 5. Comparison of extraction performance between the MIP and C_{18} in river water samples spiked at 50 ng L^{-1} of each compound. **Table 1.** LC/APCI-MS-MS parameters for the acquisition of the estrogenic compounds in positive ionization mode

Table 1. LC/APCI-MS-MS parameters for the acquisition of the estrogenic compounds in positive ionization mode

Compound	Precursor ion *(m/z)*	Quantitation ion *(m/z)*	CE (eV)	Tube lens (V)	Confirmation ion *(m/z)*	CE (eV)	Tube lens (V)	IDL (pg injected)	Linearity range (ng mL^{-1})
Estriol	271.2	253.0	12	54	157.0	21	54	24.0	5-1000
17β-estradiol	255.2	159.0	18	76	133.0	20	76	8.3	5-1000
17α-estradiol	255.2	159.0	18	76	133.0	20	76	8.5	5-1000
17α-ethynilestradiol	279.2	133.0	16	50	159.0	19	50	12.5	5-1000
Estrone	271.2	253.0	12	52	133.0	25	52	18.0	5-1000
Diethylstilbestrol	269.2	107.0	32	44	135.0	12	44	25.1	5-1000
Dienestrol	267.2	107.0	23	62	173.0	15	62	24.0	5-1000

Table 2. Recoveries of selected estrogens in MilliQ, river and tap water samples (n=3)

	Recovery (%)											
	MilliQ-water				*River water*				*Tap water*			
	Spike (ng L^{-1})				Spike (ng L^{-1})				Spike (ng L^{-1})			
Compound	50	100	150	200	50	100	150	200	50	100	150	200
Estriol	83	87	87	82	82	82	94	93	88	91	82	89
17β-estradiol	96	89	98	101	85	93	91	92	86	93	89	90
17α-estradiol	95	92	97	104	88	93	90	89	95	87	89	89
17α-ethynilestradiol	97	92	98	96	92	99	92	106	92	89	90	87
Estrone	98	94	103	96	94	89	88	95	94	92	85	94
Diethylstilbestrol	47	42	54	60	53	54	49	51	54	48	52	51
Dienestrol	56	53	69	71	50	54	61	63	63	57	61	63

Table 3. Linearity, detection and quantification limits of the MIP-SPE method in MilliQ, river and tap water samples (n=3)

	MilliQ-water			*River water*			*Tap water*			
Compound	LOD (ng L^{-1})	LOQ (ng L^{-1})	R^2	LOD (ng L^{-1})	LOQ (ng L^{-1})	R^2	LOD (ng L^{-1})	LOQ (ng L^{-1})	R^2	Linearity range (ng L^{-1})
Estriol	6.1	20.3	0.998	7.5	25.0	0.998	7.3	24.3	0.998	50-500
17β-estradiol	4.3	14.3	0.996	5.0	16.6	0.995	4.9	16.3	0.996	50-500
17α-estradiol	4.2	13.9	0.997	4.6	15.3	0.997	4.5	14.9	0.995	50-500
17α-ethynilestradiol	6.1	20.3	0.998	6.5	21.6	0.998	6.4	21.3	0.998	50-500
Estrone	5.7	18.9	0.996	6.0	19.9	0.996	5.8	19.3	0.996	50-500
Diethylstilbestrol	8.5	28.3	0.997	9.8	32.6	0.996	9.8	32.6	0.997	50-500
Dienestrol	8.3	27.6	0.996	9.5	31.6	0.995	9.4	31.2	0.995	50-500

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Figure 1

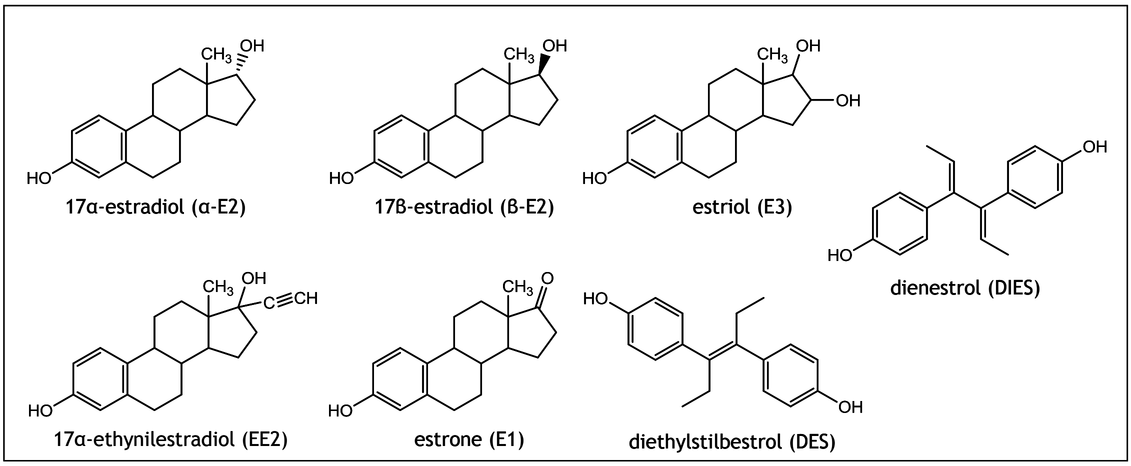
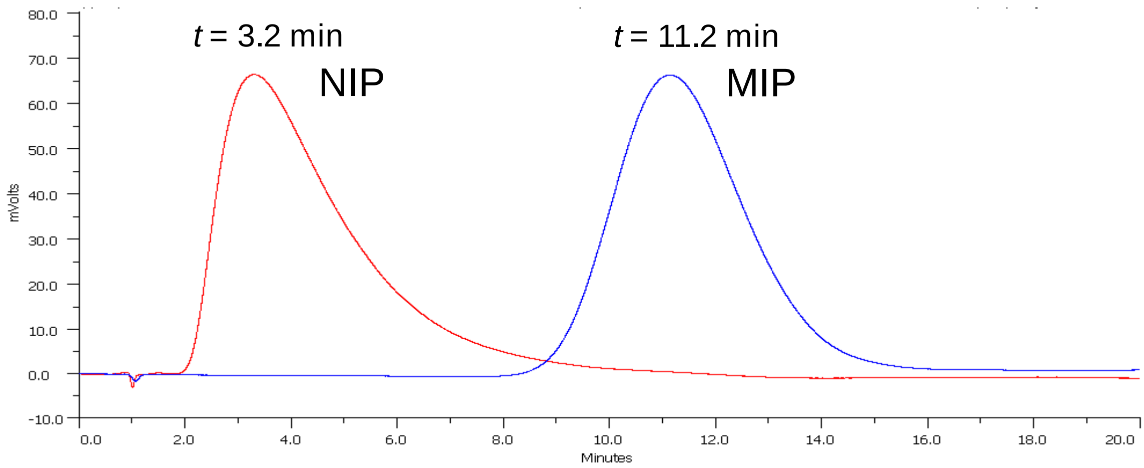

17α-estradiol (α-E2)
17β-estradiol (β-E2)
estriol (E3)
dienestrol (DIES)
17α-ethynilestradiol (EE2)
estrone (E1)
diethylstilbestrol (DES)

Figure 2


t = 3.2 min
NIP
t = 11.2 min
MIP
mVolts
80.0
70.0
60.0
50.0
40.0
30.0
20.0
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0.0
-10.0
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14.0
16.0
18.0
20.0
Minutes

Figure 3

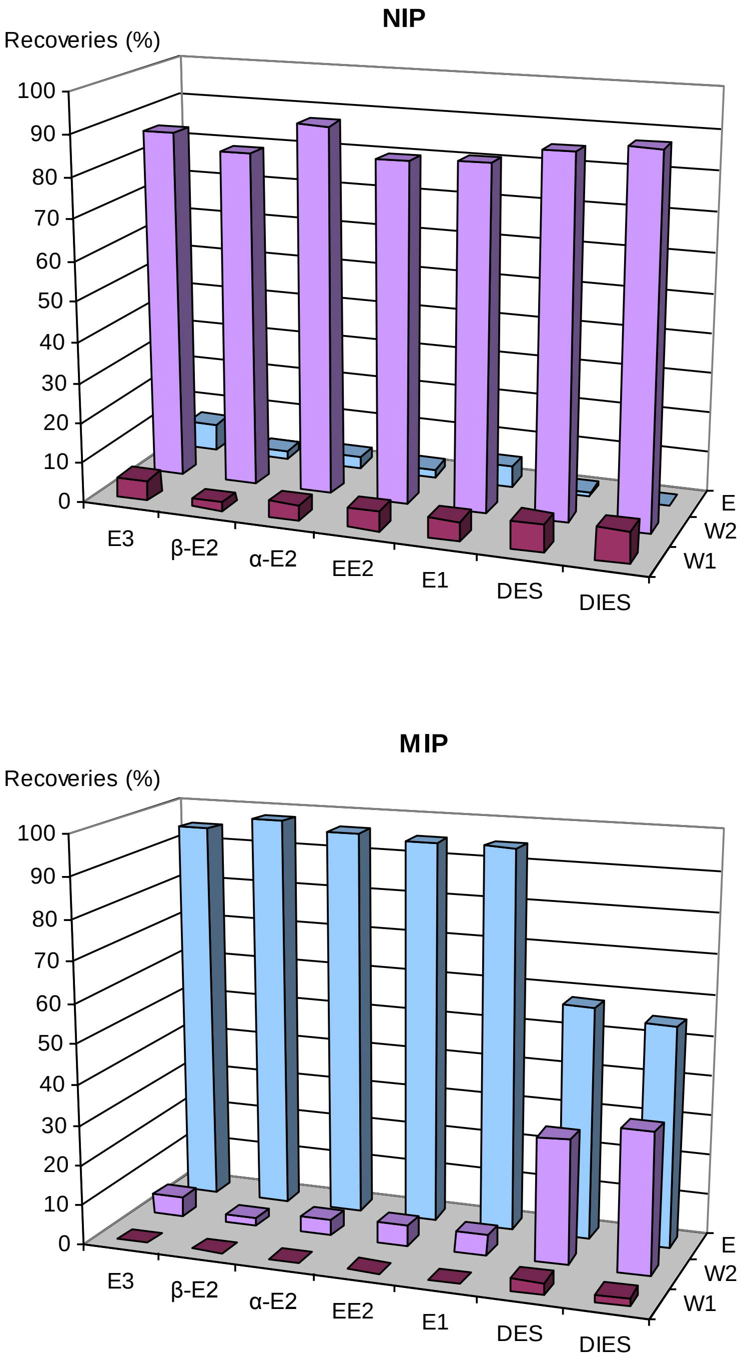
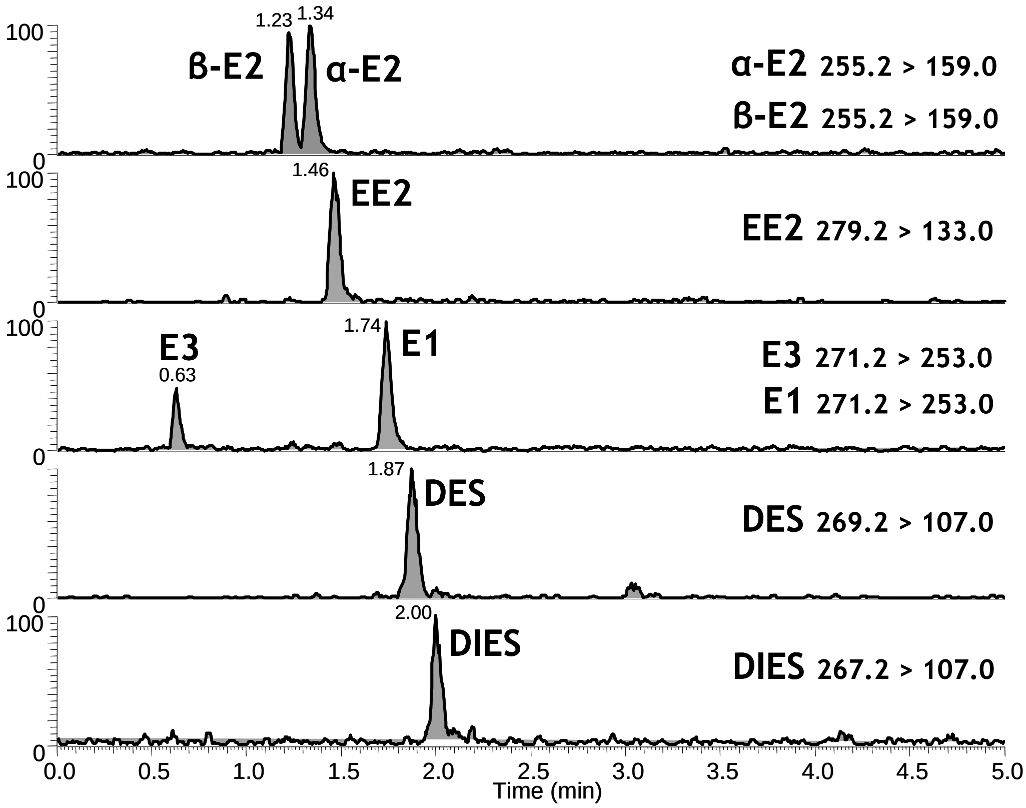
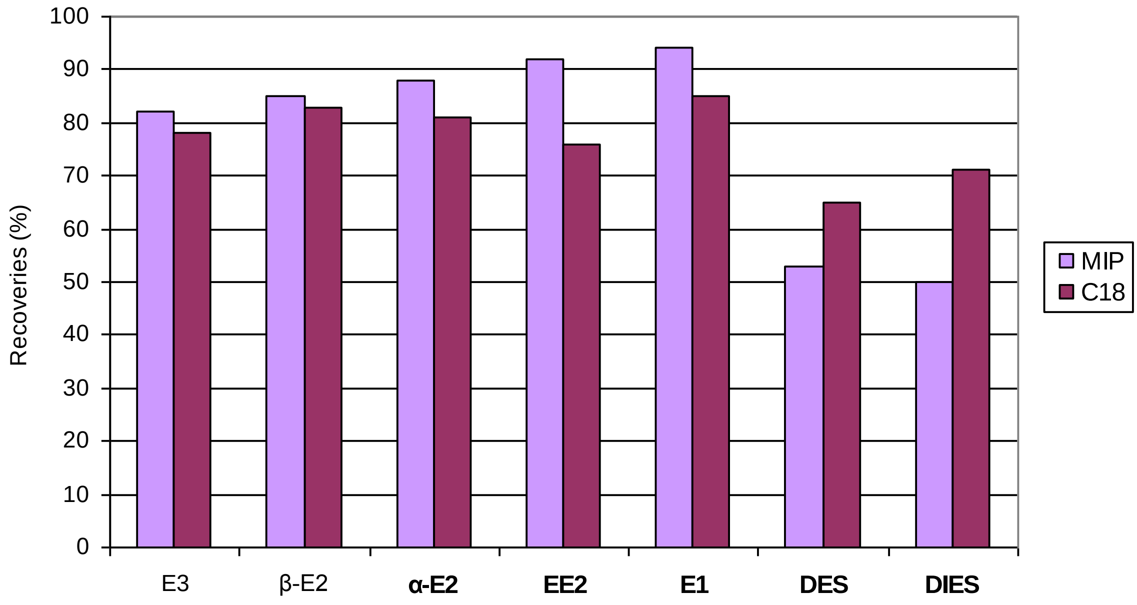

NIP
Recoveries (%)
100
90
80
70
60
50
40
30
20
10
0
E3
β-E2
α-E2
EE2
E1
DES
DIES
E
W2
W1
MIP
Recoveries (%)
100
90
80
70
60
50
40
30
20
10
0
E3
β-E2
α-E2
EE2
E1
DES
DIES
E
W2
W1

Figure 4



β-E2
α-E2
1.23
1.34
α-E2 255.2 > 159.0
β-E2 255.2 > 159.0
EE2
1.46
EE2 279.2 > 133.0
E3
0.63
E1
1.74
E3 271.2 > 253.0
E1 271.2 > 253.0
DES
1.87
DES 269.2 > 107.0
DIES
2.00
DIES 267.2 > 107.0
100
0
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0
Time (min)

Figure 5



Recoveries (%)
100
90
80
70
60
50
40
30
20
10
0
E3
β-E2
α-E2
EE2
E1
DES
DIES
MIP
C18