Progress in Sample Pretreatment for Analysis of Estrogens with Liquid Chromatography-Mass Spectrometry

YAN Wei¹, LIN Jin-Ming^{2,*}

¹ State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

² Department of Chemistry, Tsinghua University, Beijing 100084, China

Abstract: In this review, with 77 references cited, two sections are mainly discussed. One is about the various aspects of estrogens in environmental waters. The other is the introduction of current sample pretreatment methods for analyzing estrogens and their applications in recent years.

Key Words: Estrogens; Liquid chromatography-mass spectrometry; Sample pretreatment; Review

1 Introduction

Environmental Endocrine Disrupting Chemicals (EDCs) are classified under a large group of compounds, which can severely disrupt the normal physiological functions of hormones. Initially, people paid little attention to these compounds since they were usually present in extremely low concentrations in aquatic environment and had weak toxicity. In the 1980s, a malformed fish was found in a river of England and the teratogenic effect was proven to be caused by the estrogenic effect of polychlorinated biphenyls (PCBs), organotins, and pesticides present in the river. From then on, EDCs, both natural and synthetic have drawn considerable public attention due to their estrogenic effects.

The European Workshop for the first time, from December 2 to 4, 1996, discussed about the impact of endocrine disrupters on human health and wildlife in Weybridge, UK and classified the EDCs into 5 classes^[1]: (1) Halogenated Compounds such as dioxin, PCBs, and so on; (2) bisphenol A (BPA), phthalates, alkylphenols; (3) steroid estrogens; (4) pesticides; (5) phytoestrogen. Among them, steroid estrogens are of particular concern and have been investigated most

frequently because of their prominently high physiologic activity. In this paper, estrogens and their sample pretreatment methods are specifically reviewed.

2 Species, structures and origins of estrogens

Steroid estrogens are a large group of lipophilic, low-molecular weight, estrogen active compounds, which could be roughly classified as natural and synthetic estrogens. Natural estrogens (also called as endogenous estrogens) include Estrone (E₁), 17 β -estradiol (E₂), and Estriol (E₃). They have a similar tetracyclic molecular structure (Table 1): a phenol group, two hexamethylene groups, and a cyclopentane group namely a 6-6-6-5 structure. The only differences between them are the functional groups on C16 and C17 in the D-ring and their conformation^[2]. Usually, natural estrogens have relatively high hydrophobicity due to their ringed structures. Under the metabolic effect of organisms *in vivo*, free estrogens can be metabolized in conjugation with a glucuronide (GLU) or a sulfate (SUL) group linked to C3 and/or C17 positions. Besides, estrogens can also be hydroxylated or methoxylated with various enzymes *in vivo*.

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* Corresponding author. Email: jmlin@mail.tsinghua.edu.cn

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Table 1 Chemical structure of main estrogens and their conjugated forms

Estrogens	Abbreviation	Structure	Main conjugated formats
Natural estrogens			
Estrone	E ₁		Estrone-3-sulfate Estrone-3-glucuronide
17 α -estradiol	17 α -E ₂		Estradiol-3-sulfate Estrone-3-glucuronide Estradiol-17-sulfate Estradiol-17-glucuronide
17 β -estradiol	17 β -E ₂		Estradiol-3-sulfate-17-glucuronide Estradiol-3-glucuronide-17-sulfate Estradiol-3,17-disulfate Estradiol-3,17-diglucuronide
Estriol	E ₃		Estriol-3-glucuronide Estriol-16-glucuronide Estriol-17-glucuronide Estriol-3,17-disulphate Estriol-3-sulfate-17-glucuronide
Synthetic estrogens			
Ethinylestradiol	EE ₂		Ethinylestradiol-3-glucuronide
Mestranol	MES		
Dienestrol	DIE		
Diethylstilbestrol	DES		

The natural estrogens in an aquatic environment are mainly from the urine and feces of humans and livestock. It has been reported that the excretive amount of E_1 from pregnant women, menstrual women, menopausal women, and men are $550 \mu\text{g d}^{-1}$ ^[3], $11.7 \mu\text{g d}^{-1}$ ^[4], $1.4\text{--}8.5 \mu\text{g d}^{-1}$ ^[5], and $2.8\text{--}3.9 \mu\text{g d}^{-1}$ ^[6,7], respectively. The corresponding excretive amounts of E_2 are $340\text{--}445 \mu\text{g d}^{-1}$ ^[8], $1.7\text{--}4.6 \mu\text{g d}^{-1}$ ^[9], $0.0\text{--}3.5 \mu\text{g d}^{-1}$ ^[10], and $1.3\text{--}2.4 \mu\text{g d}^{-1}$ ^[11], respectively. What's more, for the purpose of behavior regulation and animal fattening or growth promotion, large amounts of steroid hormones and synthetic hormones are illegally used, which finally enter into the aquatic environment in the form of urine or feces. Maier *et al.*^[12] reported that 90% of estrogens in the environment came from animal excretion. Lange *et al.*^[13] also reported that the total estrogen excretive amount from cow, pig and chicken in USA per year is 45, 0.8 and 2.7 t, respectively. However, the report about estrogen excretive amount is limited in China.

In addition, synthetic estrogens such as dienestrol (DIE), diethylstilbestrol (DES), and ethinyl estradiol (EE_2) are used for the therapy of menstrual syndrome, hormone supplement, or as the main component of contraceptives. For this reason, the emergence of these synthetic estrogens in the environment owes to the human excretion and casual pills disposal.

Once estrogens enter into the aquatic environment, they induce vitellogenin (VTG) and *zona radiata* proteins (Zrp), which are peculiar to female fish, in the bodies of various fish (including male, female and infant fish) at very low concentration (ng L^{-1}). Both the proteins are developed in the liver of vertebrates under the stimulation of 17β -estradiol. Till date, though the influence of abnormally high content of VTG on fish reproduction and health is unclear, large numbers of experiments have proven that induction of VTG has affinity with a series of physiologic changes or lesions, such as rainbow trout's testicle heteroplasia, reduction in laying, damage of kidney and liver, and so forth.

3 Sample pretreatment and analysis by liquid chromatography-mass spectrometry

Currently, there are still some problems and challenges for the analysis of estrogens in real samples. Firstly, the matrices in which the estrogens exist are very complex such as river water and domestic sewage. If there is no effective cleanup pretreatment, the matrix effect would severely interfere with the analytical results. Secondly, the concentration of estrogens in environmental water, urine, or blood is extremely low, usually at the level of sub ng L^{-1} to ng L^{-1} . The third one is related to the physical properties of the estrogens themselves. As mentioned above, there are many forms of estrogen in water matrices that includes the free estrogens, estrogens conjugated with different functional groups (mainly glucuronide and sulfate groups) on different sites, and various metabolites. The different forms undoubtedly bring down the

concentration of estrogens, even though the total concentration is unchanged, which enhances the difficulty for their detection.

Currently, the main methods for estrogen detection are immunoassay method, LC, LC-MS, GC-MS and so on. Immunoassay method is extensively applied in the field of estrogen determination in biologic matrices, since it is of ultra-high sensitivity and high throughput, which can meet the demand of clinical analysis. Unfortunately, this method has failed to distinguish the estrogens from similar structures, which can easily lead to cross reaction. GC-MS has strong selectivity and sensitivity, by which each estrogen can be distinguished by the mass and charge of the molecule fragments. However, estrogens and their metabolites are compounds with low-molecular weight, weak volatility, and polarity. GC-MS is still not directly used for estrogen analysis, since estrogens cannot be easily isolated from the water samples, and complex hydrolysis and derivatizing reactions are requisite. Furthermore, estrogens and their metabolites lack highly ionizing functional groups, which cause weak-mass spectrometry signal response. For this reason, even the most advanced LC-MS methods cannot be used for the direct analysis of estrogens.

How to resolve the problem mentioned above? In our opinion, the crucial way to the successful detection of estrogens is to establish a rapid and effective sample pretreatment method.

3.1 Off-line sample pretreatment and analysis by LC-MS

3.1.1 Solid phase extraction (SPE)

SPE is probably the most frequently used pretreatment method, especially when employed for cleanup and enrichment of organic compounds in water samples. This method is compatible to subsequent LC-MS analysis because of its broad-spectrum applicability toward analytes and diversified choice of solid sorbents.

Typically, the process of SPE is described as follows: Prior to extraction, water sample is usually filtered through a glass fiber filter with a pore size of 0.45 or 0.22 μm to avoid the clogging of SPE cartridges. The water sample is loaded into SPE cartridges or disks^[14]. The most conventional extraction sorbents are silica gel bonded with C_{18} , NH_2 , CN group, or a polymer material such as HLB Oasis, which has been extensively used in recent years. Before use, these sorbents are usually washed and activated with water and organic solvent. After loading the sample, the dryness of the cartridge was controlled by vacuum to avoid incomplete fraction. Then, the extracts were eluted with organic solvents such as methanol or acetonitrile. Finally, the extracted solution was blown under nitrogen and reconstituted with methanol to a final fixed volume for subsequent HPLC-MS analysis. Part of application about the analysis of the estrogens is cited in Table 2.

Table 2 Solid-phase extraction (SPE) of estrogens in various matrices

Estrogens	Matrix	Sample treatment	Recovery (%)	LOD/LOQ (ng L ⁻¹)	Ref.
11 kinds of free and conjunct estrogens	River water, sewage	Oasis HLB extraction	46.0–87.0; E ₂ : 32.0	River water: 2–30; Sewage: 10–100	[15]
10 kinds of estrogens	River water, sewage	C ₁₈ extraction, Florisil clean up	70.4–106	15–70	[16]
15 kinds of estrogens	Urine	β -Cyclodextrin extraction, hexane elution	82.0–112.0	-	[17]
17 α -E ₂ , 17 β -E ₂	Calf serum	C ₁₈ and acetate buffer extraction	86.3–93.2	17 α -E ₂ : 60; 17 β -E ₂ : 30	[18]
E ₁ , E ₂ , E ₃ , EE ₂ , E ₁ -3S	Waste water	C ₁₈ extraction	83.0–100.0	0.1–0.2	[19]
E ₁ , E ₂	Human serum	PCH or PA derivation, BOND ELUT extraction	84.4–96.0	0.5–1	[20]
EE ₂	Rat serum	C ₁₈ extraction	89.0–94.0	30	[21]

3.1.2 Solid phase microextraction (SPME)

SPME, as a sample pretreatment technique, was first introduced by Pawliszyn research group^[22] in the 1990s and rapidly developed in the past decade. The principle of SPME is actually the same as that of SPE, and the only difference is the usage amount of sorbent material. The SPME device consists of a fiber holder and a fiber assembly^[23], and the latter contains an approximately 1-cm long retractable SPME fiber. The SPME fiber itself is a thin fused-silica optical fiber coated with different solid polymer films. The principle of SPME method is based on the extraction of solutes from a sample into the SPME absorptive polymer layer to achieve the preconcentration of organic compounds.

There are mainly three kinds of SPME extraction modes. The first one is the direct mode in which the SPME fiber is directly put into the liquid sample. The second one is called Headspace Solid Phase Microextraction (HS-SPME) in which the SPME fiber is put in the air up to the liquid or solid sample. The last one is SPME under the protection of dissepiments, which is not as usual as the two modes mentioned above. Direct SPME is adequate to relatively clean liquid samples. The merits include the ability to reach fast adsorption equilibrium and to have a low requirement for boiling point of target compounds. The shortcoming is that it is easily disturbed by matrix impurities. HS-SPME can overcome the defect of matrix interference, but it shows low adsorption equilibrium and enrichment effect for the compounds with high boiling point.

In the field of analytical research on EDCs, the successful application of SPME coupled with GC or GC-MSⁿ and LC or LC-MSⁿ has been gaining popularity. Practically, in recent years, a new mode of extraction called in-tube SPME^[24] has eminently developed the SPME technology. Compared to traditional fiber needle SPME, the in-tube SPME has a piece of open hollow tubular capillary column through which the sample flows. The capillary is packed with the extracting phase dispersed on an inert capillary column. Increasing the stationary phase film thickness and the length or interior diameter of capillary column helps to achieve a stationary phase volume 10 times higher than that of the traditional

mode, increasing the extraction efficiency highly.

Using the in-tube SPME technology, Feng group has made a series of studies on the analysis of estrogens^[25,26]. Fan *et al*^[25] introduced a poly(methacrylic acid-ethylene glycol dimethacrylate) monolithic capillary into in-tube SPME-HPLC, since it has higher surface specificity than normal, and this method has been successfully applied for the analysis of estrogens in various water matrices. Besides using the same method, this group^[26] also testified that poly(acrylamidevinylpyridine-*N,N'*-methylene bisacrylamide) (AA-VP-Bis) monolithic capillary could perform well as the extraction medium for in-tube SPME on-line coupled to HPLC. Based on in-tube SPME technology, Mitani *et al*^[27] achieved the on-line extraction and analysis of five estrogens in water samples and proved that the sensitivity is 34–90 times higher than that of direct sampling.

Till date, there have been only seven types of SPME coatings commercially available, and polyacrylate (PA) was found to be suitable for the analysis of estrogens. Pan *et al*^[28] selected PA fiber via head-space extraction mode to extract three alkylphenols from 2 mL of water sample. Carpinteiro *et al*^[29] directly exposed polyacrylate (PA) solid-phase microextraction (SPME) fiber to the water sample to extract five estrogens, which were then on-fiber silylated via the headspace of a vial containing 50 mL of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA). Five derivatized estrogens were determined via GC with MS/MS detection. The limit of detection was 0.2–3.0 ng/L, which was apparently superior to that of traditional SPE method.

It should be mentioned that sorbent coatings for estrogens are usually limited to PA coating. However, many commercial SPME coatings are not stable, and the coated phase is detached from silica core after two or three estrogen extractions. For this reason, many novel sorbents have been developed with good extraction results. Basher *et al*^[30] developed a new SPME sorbent, dihydroxylated polymethylmethacrylate (DHPMM), coated on hollow-fiber membrane. This novel polymer has a high number of functional groups (–OH) that makes it more amenable for the extraction of polar compounds.

In view of the defect that an extraction sorbent could only

extract compounds with similar polarity, Basher *et al.*^[31] also introduced another novel stationary phase that is amphiphilic and hydrophilic oligomers, which were synthesized and coated on fused-silica capillaries via a sol-gel technique. The new materials showed comparable selectivity and sensitivity toward both non-polar and polar analytes since they had both non-polar and polar functional groups. These materials have been successfully applied to the extraction and analysis of organochlorine pesticides, triazine herbicides, estrogens, alkylphenols, and bisphenol A.

3.1.3 Matrix solid phase dispersion (MSPD)

Matrix solid-phase dispersion (MSPD) was first introduced by Barker *et al.*^[32] in 1989 for the separation and detection of multiple residual pharmaceuticals from animal tissues. It has been proven to be an effective extraction method to separate pharmaceuticals from complicated plant and animal tissues.

The specific process of MSPD is described as follows^[33]. A sample (liver, fruit, and so on) is placed in a glass or an agate mortar containing an appropriate bonded-phase or other solid support material such as octadecylsilyl (ODS)-derivatized silica (C₁₈) or other suitable support. The solid support and the sample are manually blended together using a glass or agate pestle, which takes about 30 s. Internal standards or spikes may be added prior to this step. The blended material is then transferred and packed into a column suitable for conducting sequential elution with solvent.

Compared to traditional liquid-liquid extraction and solid-phase extraction, MSPD has evident superiority, especially when it is applied to solid or semi-solid samples, because it avoids troublesome pretreatment process. Reports have reviewed the mechanism of MSPD^[34,35].

According to the MSPD method for analysis of estrogens in solid samples, especially biologic tissue samples, Chinese scholars have carried out their endeavor. Ding *et al.*^[36] applied MSPD method combined with SPE to extract the residual DES in animal liver samples. The process involved 0.5 g of animal liver homogenate sample being spiked with 100 μ L of DES standard, which was manually blended together with 2 g of C₁₈ particle. The blended semi-solid sample was loaded into a 10 mL syringe with filter paper at its bottom. Then, using the syringe piston, the sample volume was made to 3.5 and 8 mL of ethyl acetate was added to elute the sample after 8 mL of *n*-hexane washing. Finally, the elute was determined by GC-MS. Liu *et al.*^[37] also developed a multi-residue determination method for the extraction of DES, hexestrol (HEX), and dienestrol (DE) in milk by matrix solid phase diverse technique (MSPD) with separation using HPLC. The results showed that the average recoveries of three estrogens were 84.1%–93.5% and the limit of detection for DES, HEX and DE were 0.004, 0.004 and 0.006 mg kg⁻¹, respectively.

3.1.4 Other methods

There still exist some off-line pretreatment methods for estrogen detection, such as Molecular Imprinting SPE (MISPE)^[38], magnetic particles extraction^[39], dispersive solid phase extraction (DSPE)^[40], Stir bar sorptive extraction (SBSE)^[41], Cloud point extraction (CPE)^[42], and so forth. These methods provide new ways for conventional sample pretreatment.

3.2 On-line sample pretreatment with analysis by LC-MS

Though it is believed that SPE is one of the most versatile and effective pretreatment methods, the main problems, which are cumbersome and time-consuming processes, limited the further development of this method. The advent of on-line SPE overcomes these defects perfectly and improves the efficiency of detection to a great extent. For this reason, on-line SPE is gaining widespread attention among current investigators.

Compared to off-line SPE, on-line SPE shows some apparent merits, which are described as follows^[43]: (1) Reduced analysis time and consequently high throughput achieved; (2) Total amount of extracted analytes could be analyzed; hence, the sensitivity is enhanced greatly; (3) Automatization makes minimal artificial error; (4) Minimal consumption of organic solvents and small amounts of sample needed, which result in low cost and only slight environmental pollution.

Though it has these merits, on-line SPE also exists with some inevitable problems such as long method setup time, expensive equipment, serious ion suppression, and so on. On the whole, it could be concluded that on-line SPE is suited for vast amount of samples or samples with strong toxicity, while off-line SPE is suited for small amount of samples.

Since on-line SPE cannot clean up different solvents as off-line SPE does, it is crucial to select an extraction column that can effectively retain analytes and has strong ability to resist matrix interference. This is particularly important to biologic sample on-line analysis.

3.2.1 Alkyl-bonded silica gel extraction column

As in the case of off-line method, alkyl-bonded silica gel extraction column (C₈, C₁₈), which is based on the effect of hydrophobic interaction, is one of the most frequently used columns. However, this kind of extraction column has serious problems in different matrices. For the analysis of environmental samples, the weak selectivity of alkyl-bonded silica gel makes it incapable of distinguishing polar analytes from matrix polar substances such as humic acid. The consequence is severe matrix effect. When it is applied for extraction of biologic samples, macromolecules in matrix,

such as proteins and lipids, easily block the column since its particles are relatively small and cause extremely high back pressure. For these reasons, many scholars are devoted to the exploitation of new extraction sorbents instead of using alkyl-bonded silica gel.

3.2.2 Restricted Access media (RAM)

The term restricted access media was first introduced by Desilets *et al*^[44]. This material has the property of limiting the accessibility of interaction sites within the pores to small molecules. Thus direct biologic sample injection could be achieved only because the small molecule analytes are permitted to enter the pore of the material and retained, and biologic macromolecules are excluded due to their size limit. Furthermore, the external surface is usually coated by a material with hydrophilic groups such as methylcellulose and diol. This design greatly reduces the adsorption of matrix proteins on the extraction material and thus alleviates the matrix effect.

According to the mechanism of protein exclusion, there are two kinds of RAM materials. One is physical protein exclusion material. It restricts macromolecules by its pore size. Its external surface is coated by a material with hydrophilic groups to decrease protein adsorption, while the internal surface is bonded with various alkyl chain (C_4 , C_8 and C_{18}) for retention of target compounds.

The other is chemical protein exclusion material. Its external surface is bonded with hydrophilic groups; the net structure forms outside the particle and excludes the matrix macromolecules. Usually, external and internal surfaces of material particles are synthesized respectively. The external surface is bonded with polyethenoxy ether to resist macromolecules, and the internal surface is bonded to a material with hydrophobic functional groups such as benzyl C_8 and C_{18} . This kind of RAM material has broad application in the analysis of biologic samples^[45,46].

3.2.3 Large particle support extraction column

Large particle support extraction column is also specially designed for biologic samples. In order to avoid the matrix proteins clogging the column, size of the packed particles is usually 30–50 μm . In this range, high flow rate of mobile phase is allowed without high back pressure. It is very important because the high flow rate of mobile phase helps proteins to rapidly percolate out of the column, while the analytes are retained on the column by hydrophobic effect. This concept was introduced in 1966^[47]. The packed column emerged in the 1980s^[48], and this extraction technique was patented in 1997 by Quinn and Takarewski under the name of turbulent flow chromatography (TFC).

Usually, the diameter of TFC column is approximately 1

mm i.d. and the systemic flow rate is 3–5 mL min^{-1} . Currently, there are various commercial TFC stationary phases such as micro- or capillary extraction columns packed with 50–60 μm of various sorbents. Among them, two kinds of TFC columns are most successfully used. One is silica particles coated by classical alkyl chains (C_2 , C_8 and C_{18}), phenyl groups, and mixed apolar/polar phase. Yan *et al*^[49] developed big particle triacontyl-bonded silica (C_{30}) as solid phase extraction material applied in the extraction of estrogens in water. By evaluating its extraction efficiency and comparing it with traditional C_{18} , the priority of C_{30} has been proven. The other is polymeric particles. The representative of this is Oasis HLB column, which is from Waters Company. It contains divinylbenzene-N-vinylpyrrolidone copolymer as sorbent and has the properties of both hydrophilicity and lipotropy.

3.2.4 Molecularly imprinted polymer (MIP)

MIP is a new kind of biomimetic material with strong specific molecular recognition ability. Its biggest advantage is that MIP can be synthetic for the purpose of selective recognition and enrichment of a target molecule. This specialty makes MIP easily adsorb target compounds with little matrix interference. Furthermore, the material itself resists heat, chemical erosion, and high pressure. It also needs no special demand for storage. So, MIP is gaining popularity in various fields.

Sellergren^[50] was the first to couple MIP technique with SPE, a new pretreatment technique (MIP-SPE, MISPE), and applied it for the selective adsorption and enrichment of some pharmaceuticals. After a great number of reports about its establishment and application, MISPE method was published^[51,52]. Owing to the high time consumption and bad repeatability of off-line MISPE, just like SPE, on-line MISPE has been rapidly developing.

Currently, there are two modes of on-line MISPE. The first mode is the most commonly used, in which a small amount of MIP particles is packed into a precolumn. After the process of sample loading and washing for cleanup, the analyte is eluted by mobile phase, separated by chromatography, and finally detected. This mode was first reported to be applied in the analysis of triazines in water with complicated matrices, apple juice, and urine samples^[53]. The second mode was also reported by Sellergren^[50]. The characteristic of this mode is that it does not involve separation of chromatographic column. So, the MIP column achieves both sample enrichment and separation. Normally, this kind of on-line MIP is used for only one analyte in real samples.

Attributable to its high selectivity, stability, reusability, and low cost, MIP particle as on-line enrichment material has been extensively applied to environmental, biologic, food, and pharmaceutical fields. Partial application of MISPE in recent years in different fields is cited in Table 3.

Table 3 On-line application of MISPE in various matrices

Analytes	Matrix	Template	MIP method	Ref.
Bisphenol A	River water	Bisphenol A	Non-covalent, bulk polymerization	[54]
4-chlorophenol	River water	4-chlorophenol, 4-nitrophenol	Non-covalent, suspension polymerization	[55]
Pirimicarb	Tap water, river water, spring water	Pirimicarb	Non-covalent, bulk polymerization	[56]
Pentachlorophenol	River water, lake water, waste water	Pentachlorophenol	Non-covalent, SiMIP	[57]
Terbuthylazine	River water	Triazines	Non-covalent, bulk polymerization	[58]
Cephalexin	Plasma, serum	Cephalexin	Non-covalent, bulk polymerization	[59]
Ropivacaine	Human serum	Ropivacaine	Non-covalent, bulk polymerization	[60]
Tramadol	Human urine	Tramadol	Non-covalent, bulk polymerization	[61]
Verapamil	Urine, plasma	Verapamil	Non-covalent, bulk polymerization	[62]
Ochratoxin A	Wheat extraction	Ochratoxin A	Non-covalent, bulk polymerization	[63]
Ceramide	Yeast	Ceramide	Non-covalent, in-situ polymerization	[64]

Though there is much successful application due to the merits mentioned above, some aspects can still be improved. Firstly, the preparation process of MIP particles seems too complex, time-consuming, and a large amount of organic solvent is needed to elute template molecule; secondly, the current common preparation method is non-covalent bulk polymerization. In the MIP synthesized using this method, the template molecules are hard to be eluted and the efficiency of special bonding sites is low, which results in low adsorption capacity and high nonspecific adsorption rate. Finally, template molecules often break away from the polymer if bulk polymerization method is used, and the interference with real determination is distinguished.

3.2.5 Other enrichment materials

In order to seek more effective, environmental friendly, and economic enrichment materials for the application of on-line SPE analysis, many scholars pay attention on the development of new materials, including amino column^[65], hydrosulfonyl column^[66], copper isonicotinate coordination polymer^[67], β -cyclodextrin silica gel^[68], polymer columns^[69,70], immunoaffinity column^[71,72], expanded graphite^[73], multi-walled carbon nanotube^[74], and some application even use cotton^[75] and cigarette filter^[76,77] for on-line adsorption of organic pollutants and perfect results could be obtained. The properties of these new adsorbents are low cost, easy producibility, some of which can be used repeatedly. The advent of these adsorbents has greatly expanded the applied range of the on-line SPE technique and also provided new thought for other researchers. Currently, the development of new on-line enrichment materials has become one of the most active research fields for the on-line SPE technique.

4 Prospects

The extremely high physiological activity of estrogens greatly threatens sensitive fish and other biotic population. With the increased usage of contraceptive and estrogen supplement, this threat has become much more significant.

Furthermore, it has been widely accepted that some estrogen metabolites play a key role in breast carcinogenesis. For these reasons, it is a very important to study the issue of concentration determination and toxic analysis of estrogens in environmental, ecological, and medical fields.

It is still a great challenge to identify and determine the types and contents of estrogens in complex matrices. SPE will continue to be the main method of estrogen pretreatment due to its broad applicability and low cost. However, cumbersome processes and large amount of organic solvent required modify the SPE method. There are maybe two directions to improve SPE method: One is developing new sorbents. The ideal sorbent should have a high adsorption capacity, selectivity, and elutibility. The other is to improve the extraction process. On-line SPE, MSPD, dispersive SPE, and so on are all modified SPE, which are developed according to the features of real samples and operation. It can be believed that there is an increased number of new extraction modes to appear in future.

In the aspect of detection, it has become state-of-the-art to use triple quadrupole instruments (or other tandem MS techniques) if extremely low concentrations in complex samples must be analyzed. Attributable to its powerful anti-interference and quantitative ability, LC-MS² will play an important role in the future analysis of estrogens. Meanwhile, some high resolution instruments, such as Q-TOF, IT-TOF, and so on will support the qualitative identification of estrogen types and provide more information on properties such as structure etc.

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