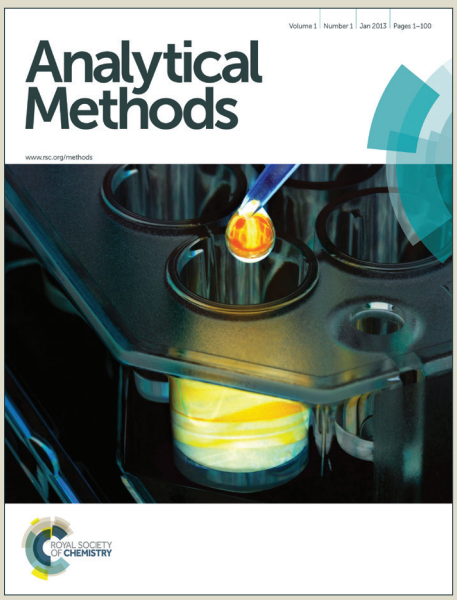
Analytical Methods

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/methods

Cite this: DOI: 10.1039/x0xx00000x

Received ooth January 2014,
Accepted ooth January 2014

DOI: 10.1039/x0xx00000x

www.rsc.org/

Simultaneous determination of progestogens, androgens, estrogens and phenols in water, sediment and biological samples by enolisation-silylation with ASE-GPC-SPE-GC/MS

B. Huang, W.W. Sun, X.M. Li, X.X. Yang, D. Ren, Y. Wang and X. J. Pan*

This research has developed an enolisation-silylation with accelerated solvent extraction (ASE) - automated gel permeation chromatography (GPC) - solid phase extraction (SPE) - gas chromatography/mass spectrometry (GC/MS) analytical method for the simultaneous determination of twelve steroidal and phenolic endocrine disrupting chemicals (EDCs) from water, sediment and biological samples. The parameters of ASE have been optimized as follows: ethyl acetate as extraction solvent, static extraction 5 min, and extraction 3 cycles at 80ºC and 60ºC for sediment and biological samples, respectively. The clean-up of extracts was carried out by GPC with Sep-Pak C18 cartridges. Target compounds were eluted in the fraction from 7-14 min retention time, then the extracts obtained by solid phase extraction with Sep-Pak C18 cartridges after the elution with 15 mL ethyl acetate. The final sample extracts were derivatized using *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) / trimethyliodosilane (TMIS) / dithioerythreitol (DTE) (1000:2:5; *v*/*v*/*w*) as derivatization reagent. Quantification was performed by gas chromatography-mass spectrometry (GC-MS) with electron ionisation (EI) and selected ion monitoring (SIM) mode. The method was validated by spiking experiments which showed low method detection limits, good recovery (60.28% to 95.46%) and reproducibility (RSD<10%). The proposed methods were successfully applied to the determination of the target EDCs in environmental samples taken from the Laoyu River and local market in Yunnan, China. This method had better practicability and feasibility for simultaneous determination of progestogens, androgens, estrogens and phenols in water, sediment and biological samples. The technique was more fast, simple and precise than some other methods, and has a wide range of application. It is beneficial to study the occurrence, fate and bioaccumulation of these compounds in environment.

Introduction

Endocrine disrupting chemicals (EDCs), particularly steroids and phenols, have attracted a great deal of scientific and public attention worldwide due to their potential adverse effects on the normal reproduction and growth of fish[1-5] and other wildlife or possibly even humans.[6,7] Up until now, most studies on the presence of natural and synthetic steroids in the environment focus on the estrogens, such as estrone (E1), 17β-estradiol (E2), estriol (E3) and 17α-ethynylestradiol (EE2).[8-10] However, the excretion masses by humans and livestock of other steroids, including androgens and progestogens, are several times or hundred times higher than estrogens. [9,10]

Recent studies have documented that androgens and progestogens also present a risk to exposed organisms and have been found to induce masculinization and impair immune function, reproduction and development of aquatic organisms.[10,11] In addition to phenolic EDCs, such as 4-nonylphenol (4-NP), bisphenol A (BPA), 4-tert-octylphenol (4-*t*-OP) and 4-cumylphenol (4-CP) were identified as the compounds responsible for the endocrine-disrupting activities in aquatic environments, which have been widely used in household, agriculture, and industrial processes in the past five decades.[8, 12]

The investigations on the occurrence, behavior, bioaccumulation and risk assessment of those trace pollutions in the environment require simultaneous and sensitive detection methods.[13] Several analytical methods have been developed for separation and determination of estrogens and phenols in marine and fresh waters, sewage treatment plants (STPs) and organisms around the world.[14-20] However, only limited studies have reported on the concentration levels of androgens and progestogens in surface water and sediment.[10, 21-25] So far, the understandings of their occurrence and removal at different stages of STPs, as well as bioaccumulation in various aquatic organisms were very scarce. [1] Therefore, there is a need for the development of a sensitive and reliable method to simultaneously

Faculty of Environmental Science and Engineering, Kunming University of Science and Technology, Kunming, Yunnan 650500, PR China

* Corresponding author. Tel. /fax: +86 871 65920510.

E-mail address: xjpan@kmust.edu.cn (X.J. Pan).

determination the target compounds in surface water, sediment, wastewater and biological samples to study the occurrence, fate and bioaccumulation of these steroids and phenols in environment. In the past decade, liquid-liquid extraction (LLE), pressurized liquid extraction (PLE), microwave assisted extraction (MAE), accelerated solvent extraction (ASE) and solid-phase extraction (SPE) techniques, combined with, gas chromatography (GC), high-performance liquid chromatography (HPLC), gas chromatography-(tandem) mass spectrometry (GC-MS(/MS)) and liquid chromatography-(tandem) mass spectrometry (LC-MS(/MS)) have been applied to determination of steroids and phenols.[26-38] In order to apply the superior resolution and identification power of GC-MS for the determination of steroids and phenols, derivatization is required to increase the volatility and thermal stability of the analytes and thus improve the chromatographic separation and sensitivity. The keto groups of androgens and progestogens are more difficult to derivatize because of free from active hydrogen atoms.[39] Among various derivatization, we have used a reliable silylation procedure with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) / trimethyliodosilane (TMIS) / Dithioerythreitol(DTE) (1000:2:5; *v*/*v*/*w*) reported in previous publications.[20,25,40] Before the detection step of GC-MS, the analytical procedures usually involve tedious and time-consuming steps for sample pretreatment, including extraction and clean-up. Among various methods of extraction and clean-up, MAE and ASE considered good alternative due to it streamlines sample preparation the entire procedure, reduces extraction time, uses small amounts of solvents and improves extraction yield.[41] The use of ASE for extraction of a variety of compounds has recently been reviewed and many environmental, food, polymer, and pharmaceutical applications have been reported in several recent studies.[42] For clean-up, many studies reported that the combination of automated gel permeation chromatography (GPC) with SPE could provide successful results and high purification efficiency. [43, 32]

The objective of this work is to develop an enolisation-silylation with ASE-GPC-SPE-GC/MS method for the simultaneous separation and determination of a wide range of EDCs including four phenols (4-*t*-OP, 4-CP, 4-NP and BPA), four estrogens (E1, E2, E3 and EE2), one progestogen (PROG) and three androgens including androstenedione (AND), dihydrotestosterone (DHT) and testosterone (TEST) in water, sediment and biological samples. The rapid and reliable sample extraction and clean-up procedure using ASE, automated GPC and SPE were examined to minimise matrix interferences. The optimized method was then verified by the investigation of linear range, limit of detection (LOD), limit of quantification (LOQ), recovery, and precision. Thereafter, the developed method were used to detemine the environmental levels of EDCs in water and sediment samples taken from the Laoyu River and biological samples from local market in Yunnan, China.

Experimental

Test chemicals and standard solution

All standards, four estrogens (E1, E2, E3 and EE2), one progestogen (PROG), phenols (4-*t*-OP, 4-CP, 4-NP and BPA) (purity>97%), internal standard (5*a*-androstane) and surrogates (estrone-d_4, test-d_3 and bisphenol A-d_{16}) were purchased from Sigma-Aldrich (USA). Androgenic hormones (AND, DIHYDRO and TEST) were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). MSTFA, DTE, TMIS were purchased from Fluka (Germany). Stock solutions of individual compounds were initially dissolved in 100% HPLC-grade methanol (Fisher Scientific) at 1 mg mL^{-1} and stored in a freezer at - 20ºC. The stock solutions were used to regularly prepare working standard solutions for spiking experiments.

Organic solvents (methanol, dichloromethane, ethyl acetate, hexane and cyclohexane) used for sample processing and analysis were HPLC grade, were all purchased from Merck (Germany). SPE cartridge of Sep-Pak C18 (6 mL, 500 mg) and Oasis HLB was obtained from Waters (Milford, MA, USA). All glassware was cleaned by SC 1160 automatic bottle washer (Salvis Lab, Switzerland) and then pyrolysed at 450ºC for 4 h prior to use.

Sample Collection and Preservation

The water and sediment samples were collected from Laoyu River and the fish samples from local market in Cheng gong, Yunnan, China. Laoyu River is the main inflow river of Dianchi Lake, and this area features a dense population.

Water samples were collected in 4 L amber glass bottles and 1% of methanol was added into the samples immediately to suppress potential biodegradation. Sediment samples were stored in 1 L glass bottles. All samples were placed into ice packed coolers and transported to the laboratory as soon as possible. Water samples were refrigerated at 4ºC and analyzed within 24 h to avoid degradation of target compounds, while sediment samples were frozen at - 40ºC until extracted.[26, 40]

Fish used in this study were high-back crucian carp (Carassius auratus) that purchased from a local market in Kunming, Yunnan Province, China. Fish were killed in a lethal dose of anaesthetic (MS222) and dissected with a clean scalpel blade to separate the tissues from the bones. Fish muscles were dissected, pooled, homogenised and freeze-dried (Eyela FDU-1200, Japan) for five days. [20] After lyophilisation, the samples were ground to powder and sieved to 51 mm and then stored in glass jars at room temperature until extraction. Fish muscle and sediment samples spiked with 100 ng g^{-1} dry mass of steroids and 25 ng g^{-1} dry mass of phenols were then analyzed to check recoveries and reproducibility of the improved method. The samples analysis procedure was shown in Fig. 1.

Acceleration Solvent Extraction

Sample extraction was conducted by an automated ASE 350 system (Dionex, USA). The extraction cell (stainless steel, 33 mL) was loaded from bottom to top: glass microfiber filters, diatomite, 2 g of fish sample or 5 g sediment and diatomite on the top. For sediment and biological samples, added 100 µL surrogates (estrone-d_4, test-d_3 and bisphenol A-d_{16}) before loading extraction cell. Moreover, the tested ASE parameters mainly included solvent (methanol, ethyl acetate, dichloromethane/ethyl acetate (1:1; *v*/*v*) and dichloromethane/methanol (1:1; *v*/*v*)), temperature (40-120ºC, 20ºC interval), holding time of static extraction (4, 5, 6, 7, 8 and 9 min)

and the number of static extraction cycles (1, 2 and 3), with a default pressure at 1500 psi, heated for 5 min, and purged into nitrogen for 120 s. All of the extracts (approximately 40 mL) were transferred to the round-bottomed flasks (250 mL), and then concentrated to near dryness leaving a small amount of residue in a rotary evaporator (Buchi Rotavapor RII, Switzerland). Finally, the concentrated extract was dissolved in 10 mL cyclohexane/ethyl acetate (1:1; *v*/*v*) and subjected to the GPC clean-up procedure.

Gel Permeation Chromatography

The extract after ASE contained a considerable amount of high-molecular weight lipids, and the extractable polar compounds in extraction solvent were co-extracted. It is difficult to pre-treat the extracts for the selective extraction of target EDCs, as well as remove the lipid interferences from the extracts. In this study, GPC was applied to remove the high-molecular weight lipids extracted from fish samples. Clean-up of EDCs in fish extracts was carried out in an AccuPrep MPS GPC (J2 Scientific, USA) using a GPC column of Bio-Beads S-X3 with cyclohexane/ethyl acetate (1:1; *v*/*v*) as the mobile phase. The recovery of compounds after GPC clean-up was assessed in triplicate by injection of 5 mL of a mixture of standard solutions containing the target analytes. Based on the results of the recovery tests, the fraction containing the target EDCs were chosen to be collected in round-bottomed flasks (250 mL), and then evaporated to near dryness leaving a small amount of residue. To completely remove all traces of the lipids, the residue was dissolved in 200 mL ultrapure Millipore-Q water and extracted by SPE.

Solid Phase Extraction

Water samples (1.0 L with 100 μL surrogates) were filtered through Millipore 0.45 μm GF/F glass fiber paper to remove suspended matter and then adjusted pH to 4.5. Oasis HLB cartridges and were preconditioned with 10 mL of ethyl acetate and 10 mL of methanol followed by 3 × 5 mL Milli-Q grade water. The filtered water samples passed through the cartridges for the extraction at a flow rate of less than 5 mL min^{-1}. Then, the cartridges were washed with 3 × 5 mL 5% MeOH in Milli-Q grade water (*v*/*v*) and dried under vacuum for 50 min. The target compounds were eluted from the cartridges using 3 × 5 mL ethyl acetate. The eluate was evaporated to nearly dry under a gentle stream of nitrogen. Finally, the dried residues were subjected to derivatization reaction.

Derivatization

The dried residues of SPE eluate or working standard solution were derivatized by adding 90 μL of MSTFA/TMIS/DTE (1000:2:5, *v*/*v*/*w*), then completely mixed using a vortex system and performed at 50ºC for 40 min. After the derivatization reaction, the derivatives were cooled to room temperature and 10 μL of internal standard (10 ng μL^{-1}) was added. Then, 1 μL of the mixtures was injected for GC-MS analysis.

GC–MS Analysis

Trace DSQ quadrupole mass spectrometer coupled to a Trace GC (Thermo Fisher Scientific, USA) fitted with with a 0.25 mm inner diameter, 30 m length and 0.25 mm film thicknesses DB-5 MS capillary column (J&W Scientific, USA) were used for all analyses. Helium carrier gas was maintained at a constant flow rate of 1 mL min^{-1}. The injector temperature was set at 280ºC temperature was 50ºC for 2 min, increased to 260ºC at 12ºC min^{-1} and maintained at this temperature for 8 min, then ramped at 3ºC min^{-1} to 280ºC and held for 5 min. The interface, MS transfer line, and ion source temperatures were set at 280, 300 and 250ºC, respectively. Mass spectra were scanned in full scan mode from m/z 50-600 mass range for qualitative analysis or selected ion monitoring (SIM) mode for quantitative analysis. Electron impact ionisation energy was 70 eV. Examples of chromatograms for the identification of target compounds are listed in Fig. 2A. The ions monitored for each compound are listed in table 1. Example of chromatograms for the identification of target compound in standard solutions and spiked fish tissue samples are shown in Fig. 2B.

Ethical Statement and Approval

All experiments about the use of live animals in the study were performed in compliance with the relevant laws and institutional guidelines, and approved by the local ethical review committee at the Fisheries Research Institute of Yunnan Province and the Kunming University of Science and Technology.

Results and Discussion

Optimization of derivatization parameters

In order to enhance detection sensitivity and separation resolution for the analysis of target compounds, derivatization is required to increase volatility and thermal stability, and reduce the polarity of target EDCs.[20] The effects of derivatization time were examined at 20, 30, 40, 50, 60 and 70 min, respectively; and the temperature at 30, 40, 50, 60, 70 and 80ºC. The results of the derivatization time and temperature optimization are shown in Fig. 3A and 3B.

The result showed that all the target compounds which include hydroxyl and ketone group could be derivatized to corresponding derivative products. The relative response factor (RRF) values of target compounds increased with the increasing temperature, and reached maximum at 50ºC. After 50ºC, with the increasing of temperature, the RRF values were beginning to stabilize. Thus, at 50ºC derivatization temperature was selected. The optimum response time could be chosen according to the target compounds. For multicomponent analysis, 40 min was used in the following experiment. Furthermore, in this experiment, hydroxyl and ketone groups of all the target compounds were completely derivatization at 50ºC for 40 min, the MSTFA/TMIS/DTE (1000:2:5; *v*/*v*/*w*) as derivatization reagent.[38-40] Yang et al. (2006) pointed out that the responses of OP, 4-*t*-NP, E1, E2, TEST and PROG reached almost 100% at 25ºC for 60 min.[29] In the derivatization procedure of MSTFA under the condition without heating (reaction at 20ºC for 30 min) and catalyst, E1, E2 and E3 were completely derivatized to their corresponding derivatives, but EE2 completely derivatized to di-TMS-EE2 needed to reacting at 70ºC for 30 min.[38] Liu et al. (2011) reported that four target phenols and steroids were completely derivatized by 40 mL BSTFA (1% TMCS) and 40 mL pyridine reacted at 70ºC for 30 min. For this study, twelve kinds of EDCs were completely derivatized and got the maximum RRF at

70ºC for 30 min when the MSTFA/TMIS/DTE (1000:2:5; *v*/*v*/*w*) as derivatization reagent.[20]

A 100 μL of standard mixture solution (10 ng μL^{-1}) was derivatized at 50ºC for 40 min. After reaction, this solution was repeatedly analyzed by GC-MS after being stored at - 20ºC for 0, 6, 12, 24, 36 and 48 h, and the stability of EDCs derivatives results are shown in Fig. 4. Had no observably changes were taken place, as the samples were deposited at - 20ºC for 48 h, respectively. The results indicated that the derivatives of target compounds were stable. The corresponding derivatives were stable for normalized peak areas with relative standard deviations (RSDs) less than 3.5%. The result suggested that the stability of target derivatives is sufficient for GC-MS analysis.

Optimization of accelerated solvent extraction

Accelerated solvent extraction had obvious advantages including higher yield, saving solvent amount, good selectivity, as well as extracted multiple samples simultaneously. Extraction solvent and temperature are the most important controlling factors of accelerated solvent extraction which can directly affect the extraction efficiency. In order to get higher recovery rate, static extraction time and extraction cycles should be consider.

Methanol, ethyl acetate, dichloromethane/ethyl acetate (1:1; *v*/*v*) and dichloromethane/methanol (1:1; *v*/*v*) were selected as the extraction solvents, due to their good solubility of the target EDCs and immiscibility with n-hexane, which was used to biological samples defatted. The extraction solvent is very critical for extraction efficiency.

As shown in Fig. 5, the recoveries of target compounds extraction by methanol, ethyl acetate obviously higher than dichloromethane/ ethyl acetate (1:1; *v*/*v*) and dichloromethane/methanol (1:1; *v*/*v*) for eight steroids, but four phenols shows no significant different. It could be due to the recoveries were dependent on the polarity of the target compounds and extraction solvent, the stronger of solvent polarity, the lower of weak polar compounds recoveries. The polarity of steroids stronger than phenols, their recoveries were better. Compared to methanol, ethyl acetate generally produces better extraction efficiency for majority target compounds due to its great extracting capacity for polar interferences. Therefore, ethyl acetate was chosen as the best extraction solvent for the ASE simultaneous extraction of the target compounds, with an extraction recovery of 65%-95.37%.

The extraction temperature was selected based on the optimized extraction solvent. The choice of extraction temperature primarily depends on the boiling point of the extraction solvent with higher temperatures favoring better extraction efficiency. However, the over-high temperature could lead to eventual degradation of the target compounds. As a result, a compromise must be made between high extraction efficiency and selectivity. To obtain higher recovery, the extraction temperature was changed from 40 to 120ºC. The recovery rates of target EDCs at different extraction temperatures are shown in Fig. 6. The recoveries of phenols and steroids increases first and then slightly decrease from 40 to 120ºC, especial E3, TEST and BPA. In the extraction process, heating was generally considered to increase the kinetics of the extraction, but it was shown that E3, TEST and BPA experience thermal degradation when the temperature is above 100ºC.[44] When the temperature reached 120ºC, the recoveries of most target compounds were significantly decreased. The recoveries decreased maybe caused by other impurities which co-extraction with target compounds when the temperature increasing. The best recoveries in excess of 74% for phenols and 84% for steroids were extracted from sediment at 80ºC, and the recoveries in excess of 68% for phenols and 83% for steroids were extracted from biological samples at 60ºC were also observed. The experiments reveal that the purity of samples has great effect on the results of the test. In order to achieve the purification sample, biological samples should be defatted by n-hexane.

The other important parameters affecting the extraction process are the static extraction time and extraction cycles. As shown in Fig. 7A, the recovery rates for 4-*t*-OP, 4-CP and 4-NP had no significant difference in various durations of static extraction. However, for another target compounds, static extraction 5 min shows the best recoveries. The extraction recoveries of the eight steroids and BPA were more than 80% in cycle 1, but 4-*t*-OP lower than 60%. The ranges of recoveries were from 65.21% to 94.35% in cycle 2, and 67.64% to 95.34% in cycle 3 (Fig. 7B). Compared with cycle 1, the ranges of increase recoveries were from 2.83% to 6.86% in cycle 2, cycle 3 had no significant increased compared with cycle 2 was also observed. Therefore, three cycles are sufficient for the complete extraction of target EDCs from sediment and biological samples. The most effective extraction of the five target EDCs from fish sample is achieved by using ASE with on-line purification and the parameters have been optimized as follows: extraction solvent used methanol-acetonitrile (1:1; *v*/*v*), extraction 3 cycles, static extraction 5 min at 60ºC reported by Ye et al. (2013).[45] Compared with the recoveries of target compounds extracted by ASE were better than MAE, and the extract was obvious cleaner.[20]

Optimization of gel permeation chromatograph

Biological, sediment and sewage water samples are highly complicated in terms of matrix components. The removal of co-extract interferences is critical and the GPC clean-up procedure was developed to minimize the negative effects. In order to minimize deterioration of chromatographic performance of the target compounds, it is essential to eliminate interferences from sample extracts. GPC represents a convenient alternative to conventional purification methods of sample preparation of Biological, sediment and sewage water samples. GPC allows for the handling of larger masses of lipids in each sample, and is significantly quicker and simpler than conventional purification methods.

The chromatogram of the target compounds in standard solution by GPC was shown in Fig. 8. On the one hand, the target compounds that appeared between 7 to 14 min could be successfully separated from most lipidic compounds. On the other hand, the recoveries of the target compounds in standard solution obtained after GPC were range from 90.2 to 98.5% when the fraction of 7-14 min was collected, and RSD < 10.2%. The analyte loss due to GPC clean-up could be attributed to higher losses in the evaporation accomplished by a rotary evaporator and transfer steps. Consequently, the fraction of 7-14 min was chosen to be collected for biological samples.

Optimization of Solid-phase extraction

In order to get the cleanest extracts from water, sediment and biological samples, SPE is usually necessary to remove the majority of co-extracted impurities. The optimization of the SPE procedure for extraction of the target compounds from water, sediment and biological samples included the type of cartridges and the organic solvent for the elution. In general, reversed solid-phase extraction column (SPE column) was suitable for extraction polarity and medium polar compounds from polar substrate. In this study, all the target steroidal and phenolic EDCs belong to polar compounds. Sep-Pak C18 and Oasis HLB cartridge are the most commonly used SPE cartridges [39] for the evaluation of extraction efficiency of target compounds. The results are shown in Fig. 9.

For all target compounds, their recoveries were nearly when used Sep-Pak C18 and Oasis HLB cartridges. For 4-*t*-OP, 4-NP, E1, AND, E2, EE2 and E3, improved recoveries were obtained with Sep-Pak C18 cartridges. The recoveries of all steroids were higher than 80% when Sep-Pak C18 and Oasis HLB cartridge as the SPE cartridges. But the recovery of 4-*t*-OP was lower than 50% when Oasis HLB cartridge as the SPE cartridges. In addition, water more easily adsorbed on the column filler due to Oasis HLB filler with hydrophilic functional, which lead to the drying time longer than Sep-Pak C18 cartridges. The Sep-Pak C18 cartridges resulted in best recoveries for the majority of the target compounds and taking into account their lower cost, Sep-Pak C18 cartridges were used for further experiments. For four phenols and steroids, best recoveries were obtained with Sep-Pak C18 cartridges reported by Liu et al. (2011). [20]

The effect of elution solvents on the recovery of steroids and phenols from spiked samples using Sep-Pak C18 were studied by testing methanol, ethyl acetate, dichloromethane/ ethyl acetate (1:1; *v*/*v*) and dichloromethane/methanol (1:1; *v*/*v*). The results (Fig. 10) showed that dichloromethane/ethyl acetate (1:1; *v*/*v*) and dichloromethane/methanol (1:1; *v*/*v*) produced poor recovery for 12 phenols and steroids. This is because the polarity of both the solvents was too weak to elute the analytes from cartridges completely. For the majority of the target compounds, the best recoveries (55.21%-97.02%) were achieved with elution by methanol or ethyl acetate. However, the recoveries of analytes will decrease if the highest polarity of methanol is used as elution solvent, as large amount of impurities eluted by methanol will disturb GC-MS analysis. Because ethyl acetate was more easily evaporated than methanol, ethyl acetate was chosen as the solvent for the elution. Huang et al. (2011) also pointed out that best recoveries were obtained when higher polarity of acetone and methanol were used, but large amount of impurities eluted by methanol will disturb GC-MS analysis.[26]

Linearity and Detection Limit

The linearity of calibration curves for the determination of 12 steroids and phenols were tested under the optimal conditions by increasing amounts of standards. The linear range of GC-MS for the determination of EDCs was tested by increasing amounts of standards at 1, 5, 10, 50, 100, 250, 500, 1000, 2500, 50000 and 10000 ng L^{-1} in ultrapure water, and the analytes were extracted and derivatized as described above. Linear equations, correlation coefficients (R^2) linear ranges, LOD and LOQ of the target compounds were obtained by linear regression analysis in which RRF of each compound was used as ordinate and corresponding concentration (ng L^{-1}) as abscissa.

Application

The developed method was applied to determination of target compounds in water and sediment samples from Laoyu River, and fish tissue samples from local market. Twelve peaks of target EDCs were observed in water, sediment and biological samples. The EDCs levels were shown in Table 4 and the chromatograms for the identification of target compounds in environment samples are listed in Fig. 11. The concentrations of target compounds in water ranged from n.d. (not detected)-62.1 ng L^{-1}, E3 was not detected. The concentrations of target compounds in sediment and fish muscle were ranged from n.d.-83.5 ng g^{-1} and 0-11.3 ng g^{-1}, respectively. Obviously, the concentrations of BPA were relatively higher than other EDCs in water, sediment and fish muscle samples analyzed in present study. All the results indicated that the improved method can be used to determination of 12 target EDCs in water, sediment and biological samples.

4. Conclusion

This study has developed a sensitive method based on the ASE-GPC-SPE-synchronous derivatization-GC-MS for simultaneous determination of four phenolic EDCs (4-*t*-OP, 4-CP, 4-NP and BPA) and eight steroid EDCs (E1, E2, EE2, E3, DIHYDRO, AND, TEST and PROG) in water, sediment and biological samples. During the method development, various key parameters were studied. The target compounds were first extracted by ASE using ethyl acetate as the solvent extraction 3 cycles at 80ºC for sediment sample (60ºC for biological sample) and static extraction 5 min. The clean-up of extracts was carried out by GPC, and the target compounds were eluted in the fraction from 7 to 14 min. Moreover, the cleanest extracts were extracted by SPE using Sep-Pak C18 cartridges, 15 mL ethyl acetate as the elution solvent, followed by derivatization with MSTFA/TMIS/DTE (1000:2:5; *v*/*v*/*w*) and analysis by GC-MS in SIM mode. This development method showed that the recovery ranges of target compounds from 60.28% to 95.46% and RSD lower than 10% in entire procedure. The LOD values ranged from 0.3 to 0.8 ng L^{-1} in water, 0.5 to 1 ng g^{-1} in sediments and 0.5 to 1 ng g^{-1} in biological samples. The proposed method was successfully used to detected water and sediment sample from Laoyu River, as well as detected the fish muscle from local market. All target EDCs (except EE2, E3, DIHYDRO and AND) were detected in all samples. The present method shows good recovery and reproducibility for the target compounds at ng L^{-1} level. Thus, the method had better practicability and feasibility for the simultaneous determination of 12 steroidal and phenolic EDCs (especially for progestogens and androgens) in complex environment medium with a wide range of application, higher extraction yield, lower cost and high target

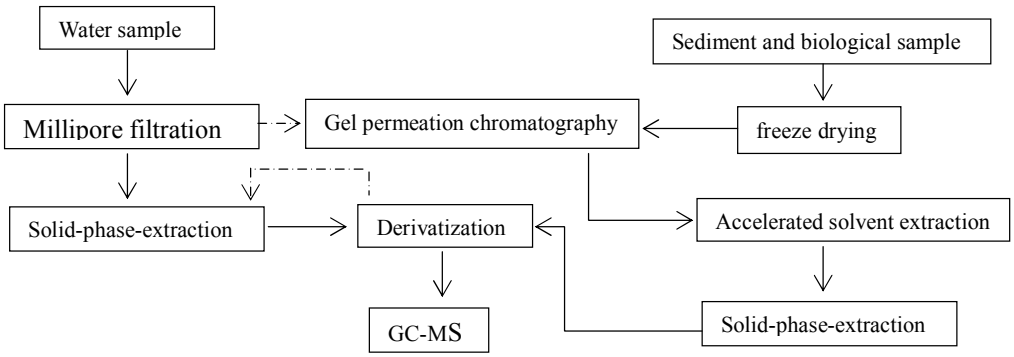
compound recovery. It is beneficial to study the occurrence, fate and bioaccumulation of these compounds in environment.

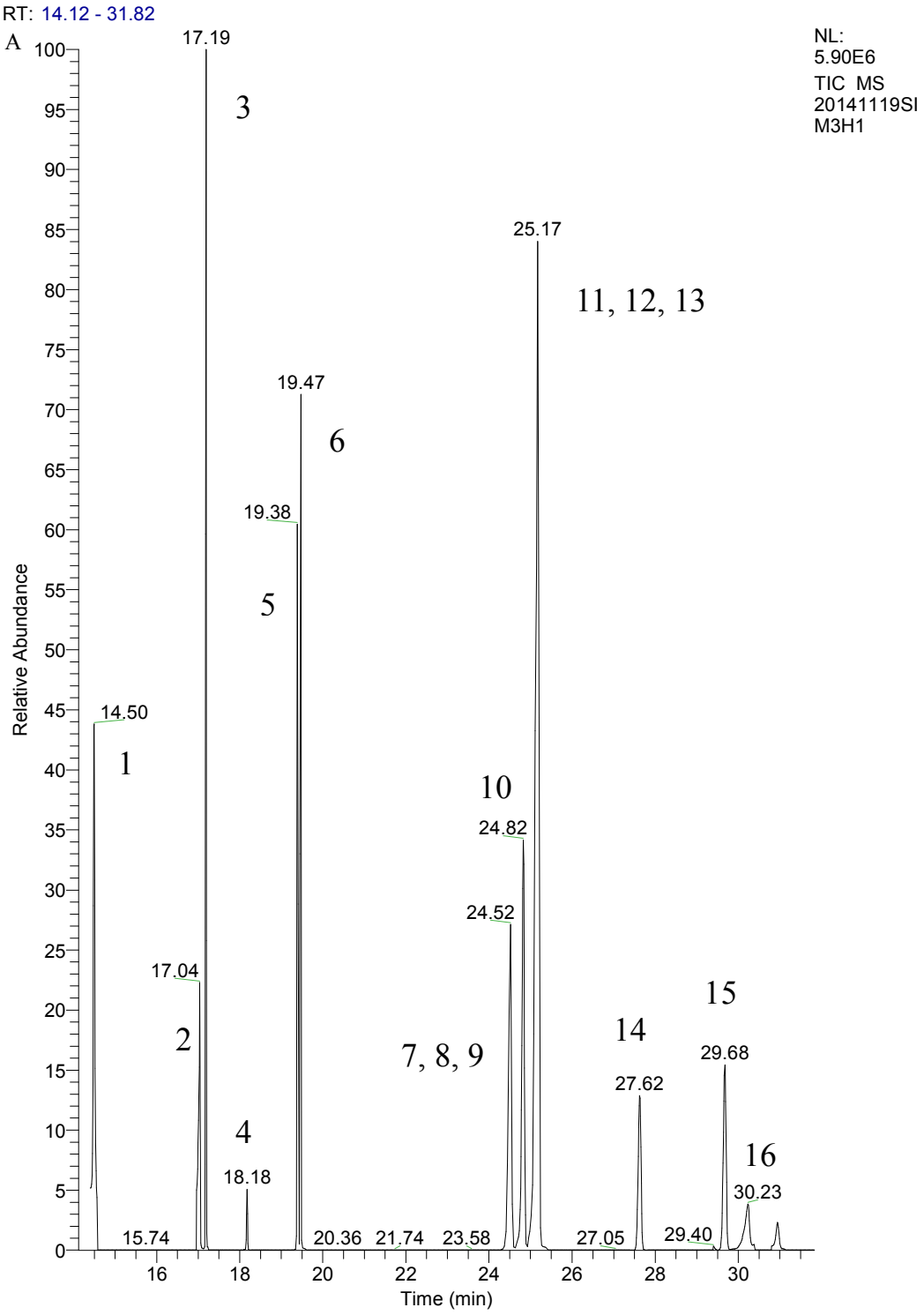
Acknowledgements

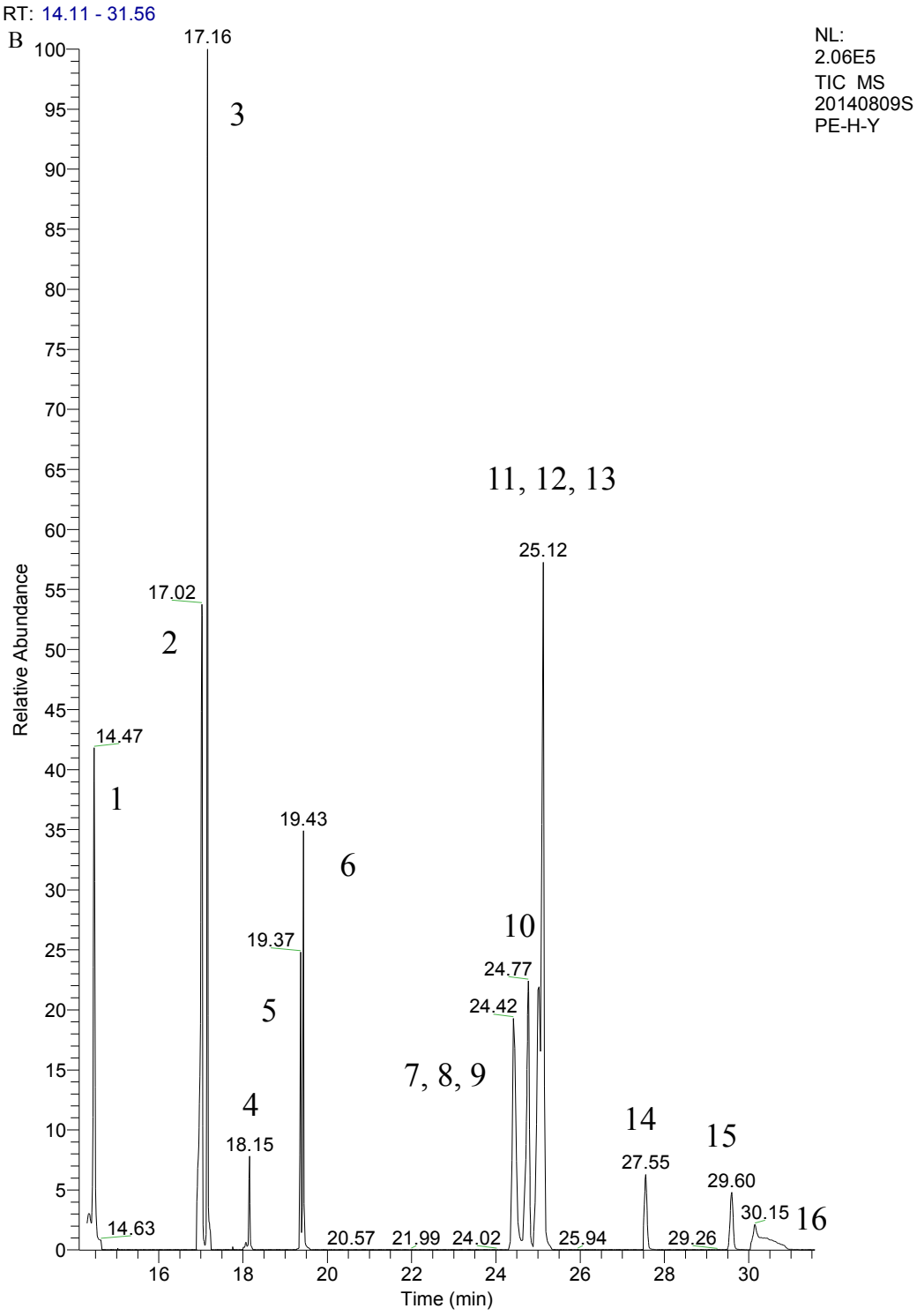
This project was sponsored by the National Natural Science Foundation of China (Grant No. 21267012, 41401558), China Postdoctoral Science Foundation (Grant No. 2014T70887), Application Fundamental Research Foundation of Yunnan Province, China (Grant No. 2012FB124), and State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences (Grant No. KF2013-04).

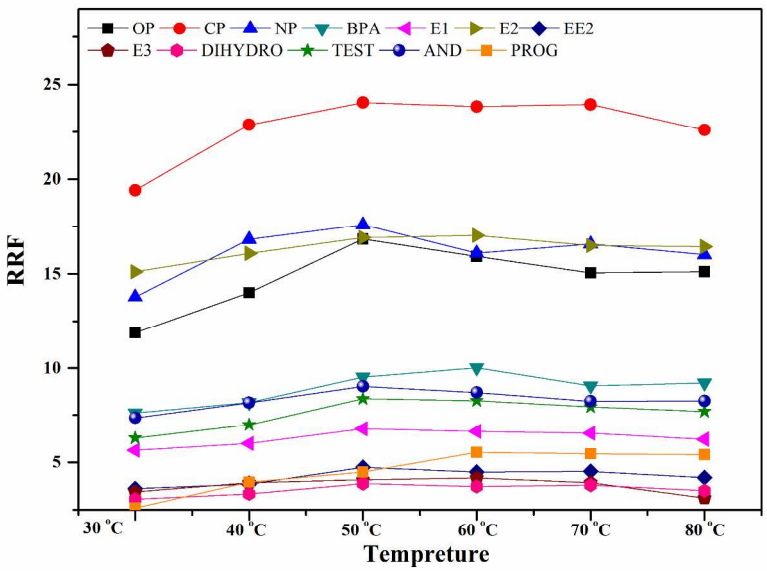
References

[1] S. Jobling, J. P. Sumpter, D. Sheahan, et al., Environ. Toxicol. Chem., 1996, 15, 194-202.
[2] L. B. Bjerregaard, B. Korsgaard and P. Bjerregaard, Ecotoxicol. Environ. Safety, 2006, 64, 321-328.
[3] L. A. Jones and R. A. Hajek, Environ. Health Perspect., 1995, 103, 63.
[4] O. Tsutsumi, J. Steroid Biochem. Mol. Biol., 2005, 93, 325-330.
[5] J. P. Sumpter, Acta Hydroch. Hydrob., 2005, 33, 9-16.
[6] D. L. Davis, M. B. Gottlieb and J. R. Stampnitzky, Jama, 1998, 279, 1018-1023.
[7] A. Casanova-Nakayama, M. Wenger, R. Burki, et al., Mar. Pollut. Bull., 2011, 63, 412-416.
[8] X. Peng, Y. Yu, C. Tang, et al., Sci. Total Environ., 2008, 397, 158-166.
[9] H. Chang, Y. Wan and S. Wu, et al., Water Res., 2011, 45, 732-740.
[10] S. Liu, G. G. Ying, J. L. Zhao, et al., Environ. Monit., 2012, 14, 482-491.
[11] Z. H. Liu, Y. Kanjo, and S. Mizutani, Sci. Total Environ., 2009, 407, 4975-4985.
[12] X. L. Jing, G. B. Jiang and G. Huang, Chemosphere, 2004, 56, 1113-1119.
[13] A. Soares, B. Guieysse, B. Jefferson, et al., Environ. Int., 2008, 34, 1033-1049.
[14] K. Inoue, Y. Yoshie, S. Kondo, et al., J. Chromatogr. A, 2002, 946, 291-294.
[15] M. Naassner, M. Mergler, K. Wolf, et al., J. Chromatogr. A, 2002, 945, 133-138.
[16] C. Y. Cheng and W. H. Ding, J. Chromatogr. A, 2002, 968, 143-150.
[17] Z. Zhu. and Y. G. Zuo. Adv. Environ. Res., 2013, 2, 179-202.
[18] Y. G. Zuo, K. Zhang and S. Zhou, Environ. Sci.-Proc. Imp. 2013, 15, 1529-1535.
[19] Y. Yu, and Wu, L, Talanta, 2012, 89, 258-263.
[20] J. L. Liu, X. J. Pan, B. Huang, et al., Int. J. Environ. Anal. Chem., 2012, 92, 1135-1149.
[21] R. Brix, C. Postigo, S. González, et al., Anal. Bioanal. Chem., 2010, 396, 1301-1309.
[22] W. J. Backe, C. Ort, A. J. Brewer, et al., Anal. Chem., 2011, 83, 2622-2630.
[23] S. Liu, G. G. Ying, J. L. Zhao, et al., J. Chromatogr. A, 1218, 1367-1378.
[24] Z. Fan, S. Wu, H. Chang, et al., Environ. Sci &Technol, 2011, 45, 2725-2733.
[25] H. Chang, S. Wu, J. Hu, et al, J. Chromatogr. A, 2008, 1195, 44-51.
[26] B. Huang, X. J. Pan, X. Wan, et al., J. Anal. Chem., 2011, 39, 449-454.
[27] R. Liu, J. L. Zhou and A. Wilding, J. Chromatogr. A, 2004, 1038, 19-26.
[28] R. Espejo, K. Valter, M. Simona, et al., J. Chromatogr. A, 2002, 976, 335-343.
[29] L. Yang, T. Luan and C. Lan, J. Chromatogr. A, 2006, 1104, 23-32.
[30] R. A. Trenholm, B. J. Vanderford, J. C. Holady, et al., Chemosphere, 2006, 65, 1990-1998.
[31] P. Navarro, J. Bustamante, A. Vallejo, et al., Arch Environ Contam Toxicol, 2011, 61, 93-100.
[33] R. Thibaut, A. Jumel, L. Debrauwer, et al., Analusis, 2000, 28 , 793.
[34] Y. G. Zuo and Z. Zhu, Adv. Environ. Res., 2015, 4, 39-48.
[35] Y. G. Zuo and Z. Zhu, Chemosphere, 2014, 107, 447-453.
[36] Y. G. Zuo, High-Performance Liquid Chromatography (HPLC): Principles, Procedure s and Practices. Nova Science Pub. Inc., New York, 2014.
[37] Y. G. Zuo, J. Chromatogr. A, 2007, 1148, 211-218,
[38] K. Fang, X. J. Pan and B. Huang, et al., Chromatogr., 2010a, 72, 949-956.
[39] K. Fang, X. J. Pan and B. Huang, et al., Chinese J. Anal. Chem., 2010b, 38, 743-751.
[40] W. Jin, B. Huang, B. Wang, et al., Chinese J. Anal. Chem., 2013, 41, 205-209.
[41] N. Fidalgo-Used, E. Blanco-González, and A. Sanz-Medel, Anal. Chim. Acta, 2007, 590, 1-16.
[42] LC-GC Mag. Sep. Sci. 17(6 Suppl. S) 1999, 22.
[43] P. Navarro, E. Cortazar and L. Bartolomé, et al., J. Chromatogr. A, 2006, 1128, 10-16.
[44] M. M. Schantz, Anal. Bioanaly. Chem., 2006, 386, 1043-1047.
[45] A. Ye, Y. Yang, J. Zhang, et al., J. Chromatogr. A, 2013, 1278, 126-132.

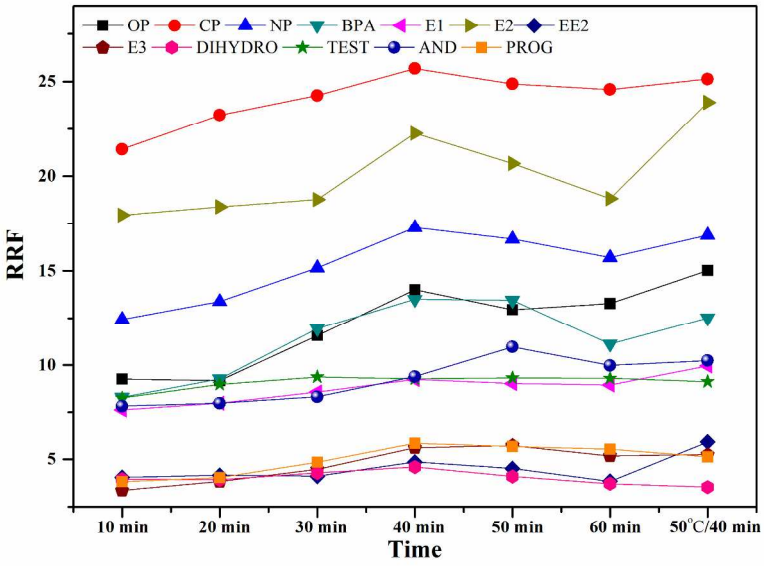




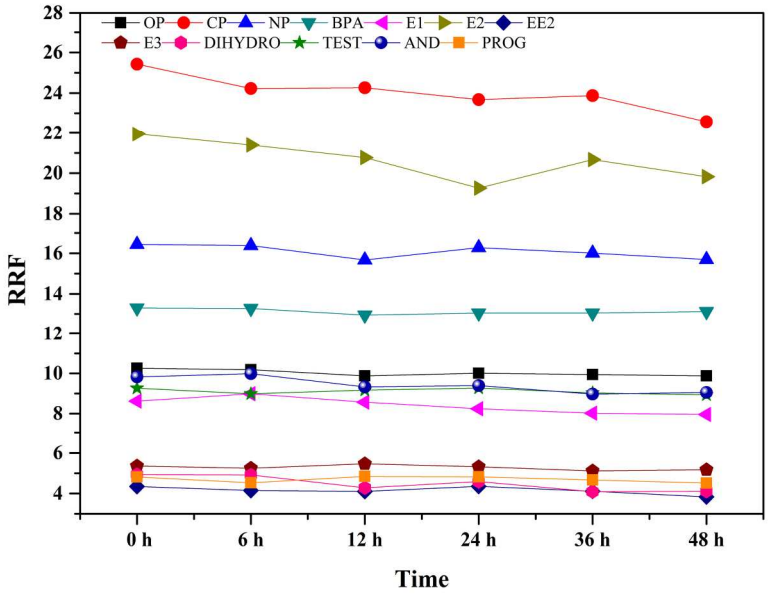




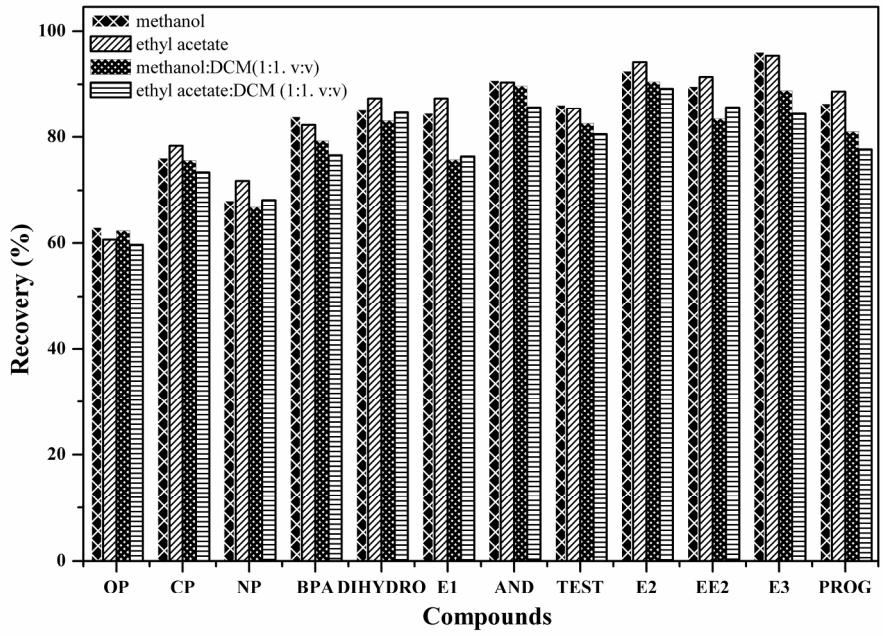
288x201mm (300 x 300 DPI)



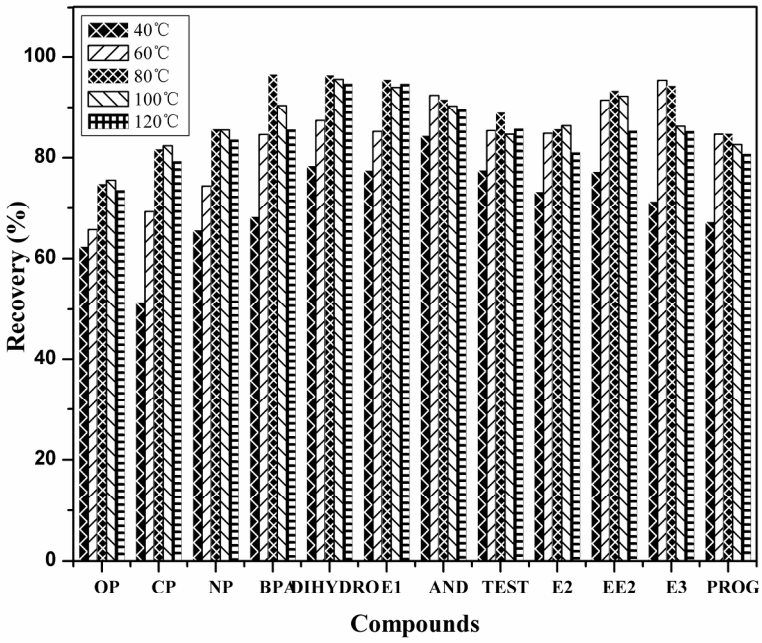
288x201mm (300 x 300 DPI)



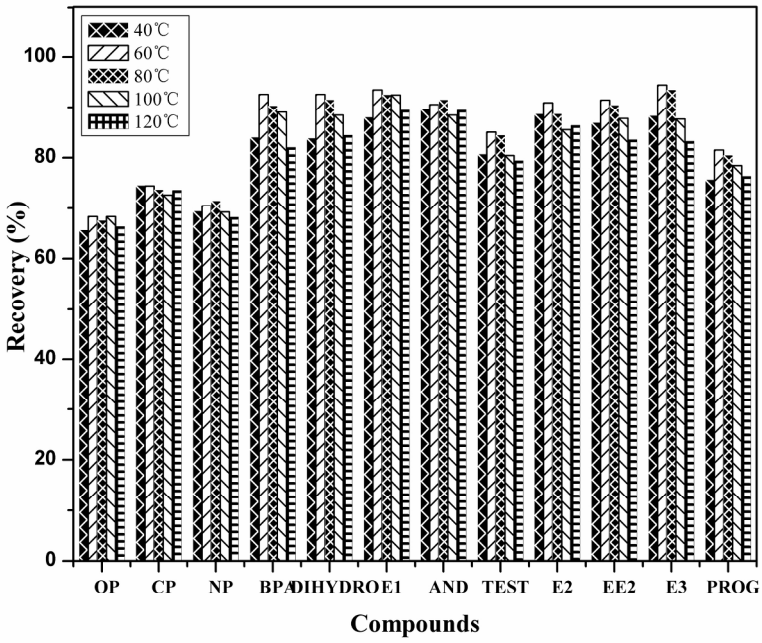
201x140mm (300 x 300 DPI)



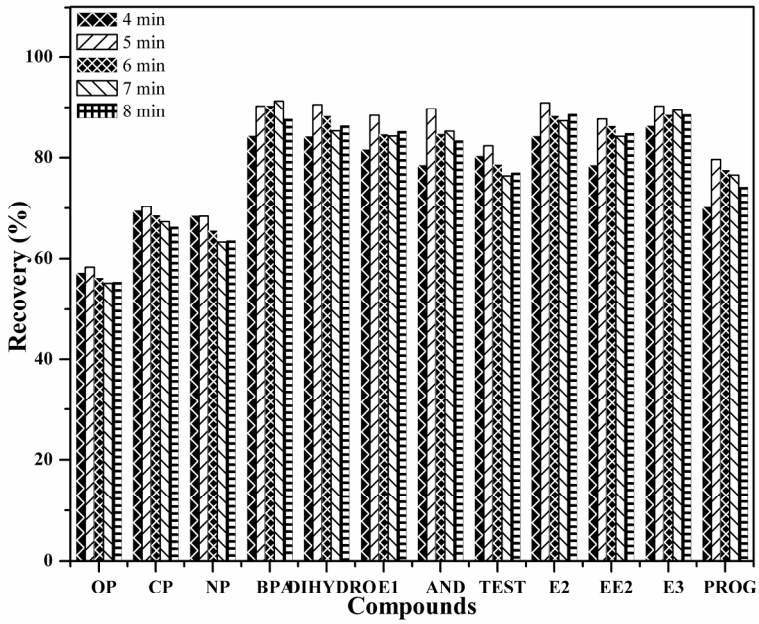
215x166mm (300 x 300 DPI)



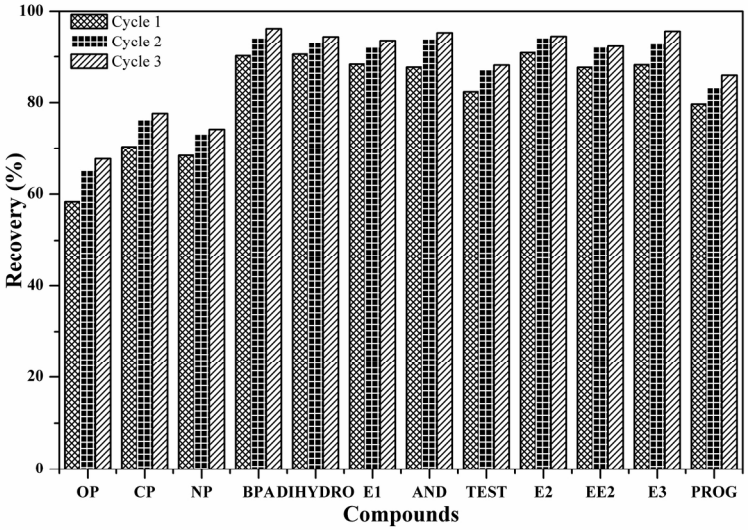
215x166mm (300 x 300 DPI)



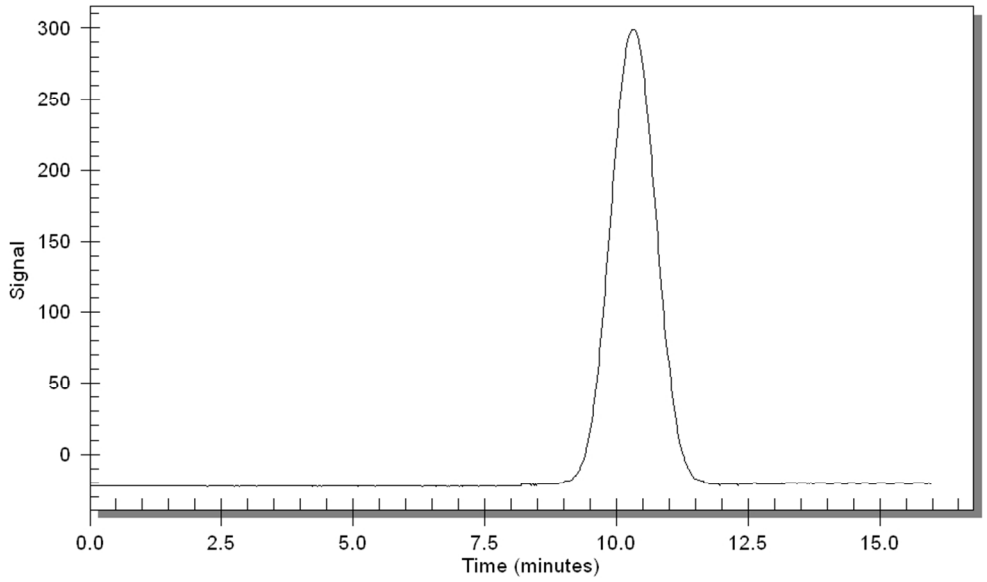
215x166mm (300 x 300 DPI)



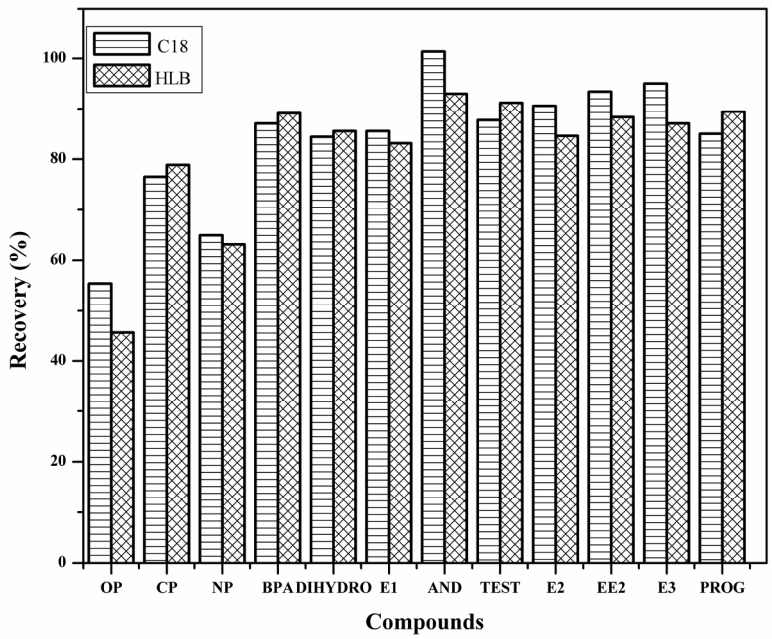
215x166mm (300 x 300 DPI)



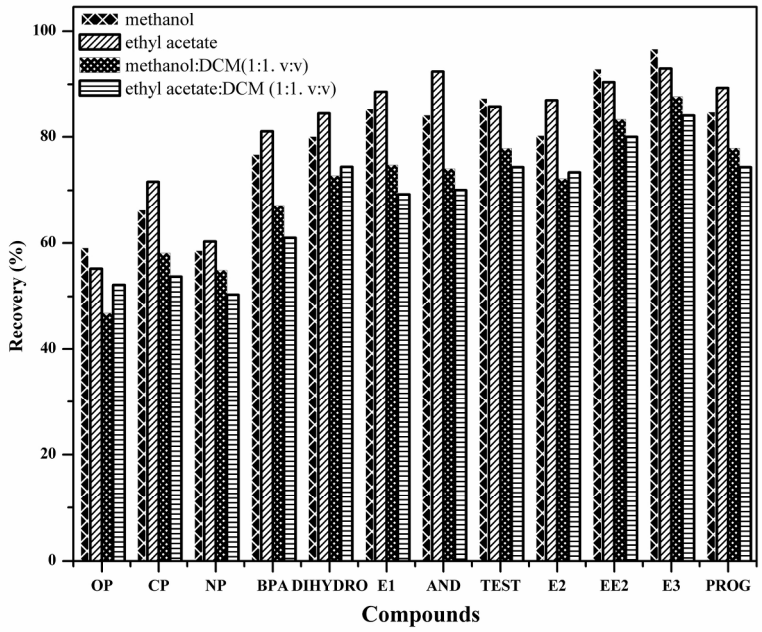
178x114mm (300 x 300 DPI)



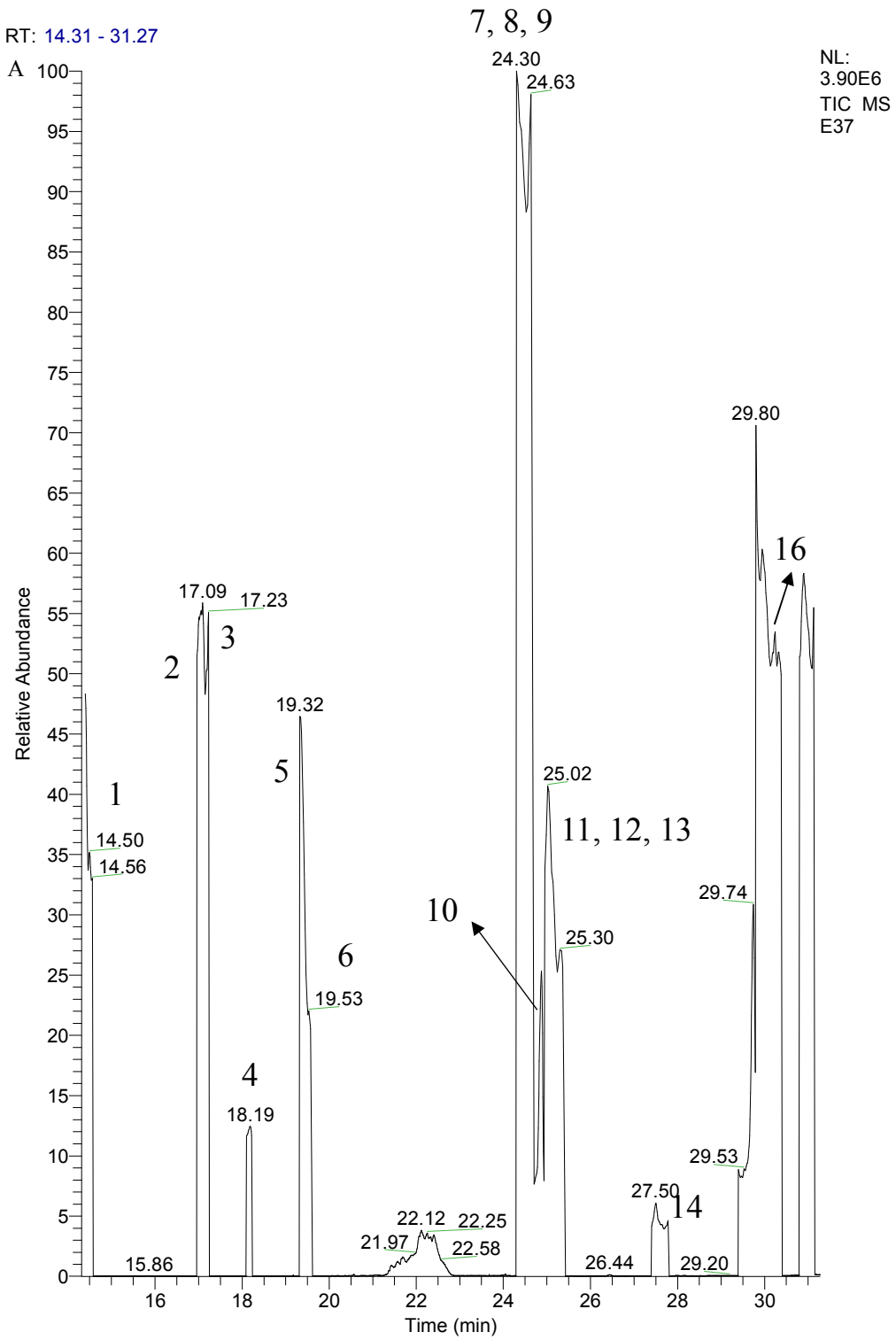
261x150mm (96 x 96 DPI)

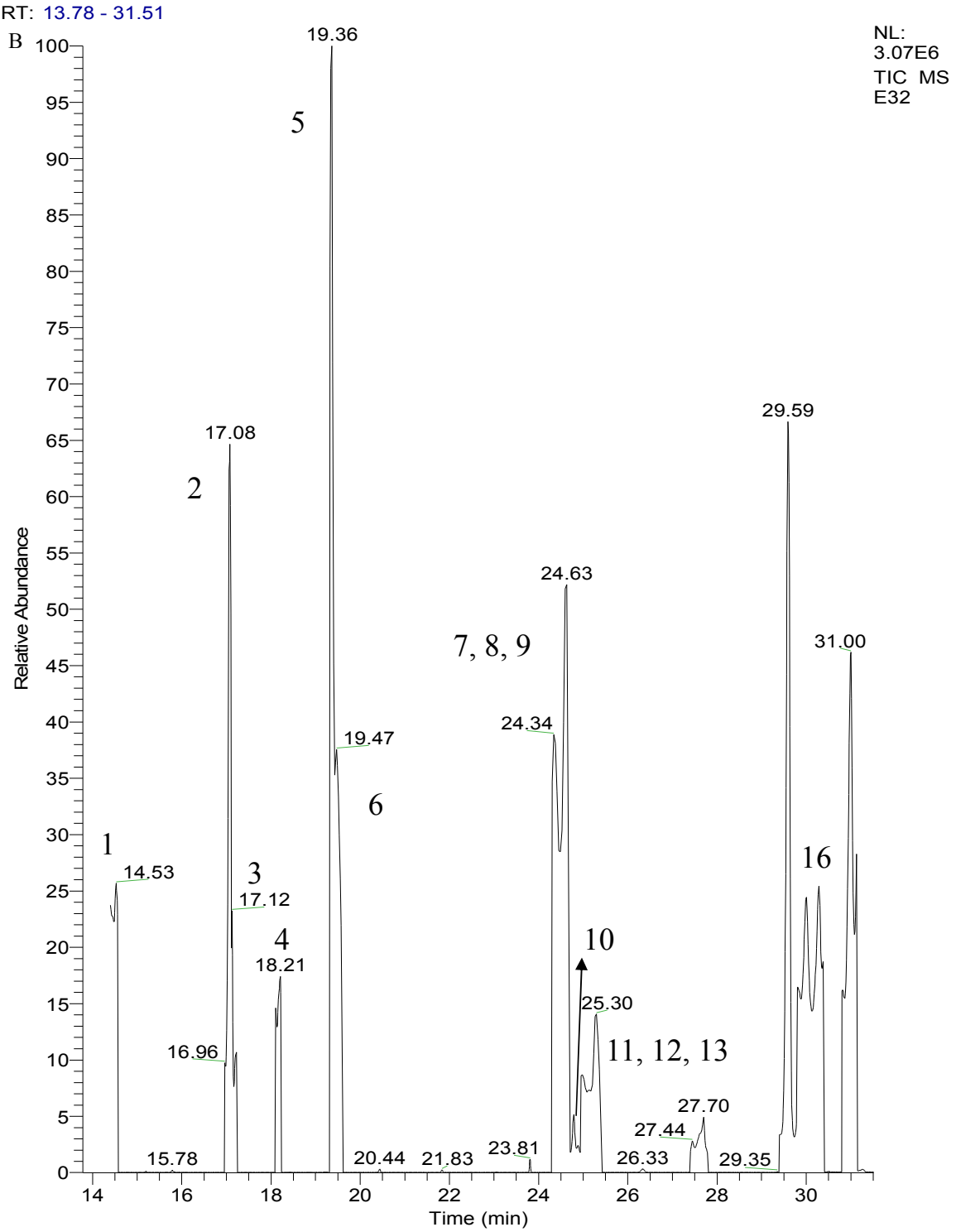


215x166mm (300 x 300 DPI)



215x166mm (300 x 300 DPI)





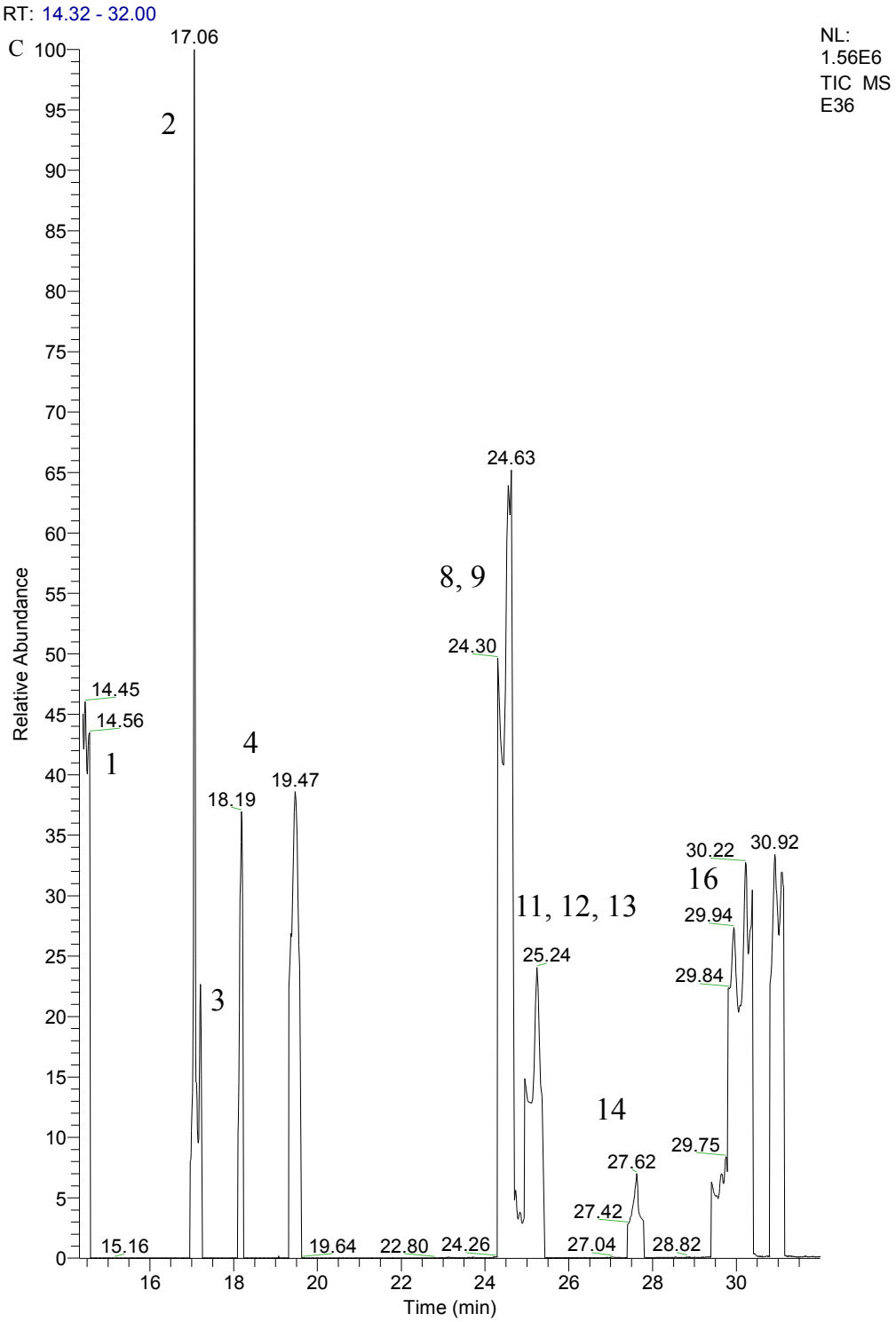


Table 1 Chromatogram information of derivatized target compounds, surrogates and internal standard

Compounds	Molecular mass	Retention times (min)	Confirmation ions (*m*/*z*)	Quantification ions (*m*/*z*)
4-*t*-OP	206	14.50	278	207
4-CP	212	17.04	284	269
4-NP	220	17.19	292	179
I.S.	260	18.18	217	260
BPA-d_{16}	244	19.38	386	368
BPA	228	19.47	372	357
E1-d_4	274	24.52	261	346
E1	270	24.52	342	257
E2	272	25.17	416	285
EE2	296	27.62	425	285
E3	288	29.68	504	345
DIHYDRO	260	24.52	434	405，419
TEST- d_3	293	25.17	435	432
TEST	290	25.17	420	417
AND	288	24.82	430	415
PROG	286	30.23 30.94	458	156，443

Table 2 Calibration curves, R^2, line range, LODs and LQDs for EDCs derivatives

Analyte	Calibration equation	R^2	Line range (ng L^{-1})	LOD (S/N=3)			LQD (S/N=10)		
				in water (ng L^{-1})	in sediment (ng g^{-1})	in biological (ng g^{-1})	in water (ng L^{-1})	in sediment (ng g^{-1})	in biological (ng g^{-1})
4-*t*-OP	Y=3.382X+0.016	0.995	5-1000	0.4	0.5	0.6	1.3	1.7	2.0
4-CP	Y=7.556X-0.089	0.998	5-1000	0.5	0.7	0.5	1.7	2.3	1.7
4-NP	Y=0.561X-0.033	0.994	5-1000	0.4	0.6	0.8	1.3	2.0	2.7
BPA	Y=3.817X+0.009	0.991	5-1000	0.6	0.8	0.7	2.0	2.7	2.3
E1	Y=0.358X+0.032	0.993	5-1000	0.3	0.5	0.6	1.0	1.7	2.0
E2	Y=1.749X+0.027	0.996	5-1000	0.4	0.5	0.5	1.3	1.7	1.7
EE2	Y=0.327X+0.005	0.995	5-1000	0.5	0.6	0.8	1.7	2.0	2.7
E3	Y=0.428X+0.04	0.99	5-1000	0.6	0.8	0.7	2.0	2.7	2.3
DIHYDRO	Y=0.437X-0.002	0.993	5-1000	0.7	0.8	0.8	2.3	2.7	2.7
TEST	Y=0.956X+0.05	0.992	5-1000	0.6	0.6	0.8	2.0	2.0	2.7
AND	Y=0.9182X+0.011	0.992	5-1000	0.8	1	0.9	2.7	3.3	3.0
PROG	Y=0.554X-0.01	0.997	5-1000	0.8	0.9	1	2.7	3.0	3.3

Table 3 The recovery for target EDCs in ultra-pure water, river water sediment and biological samples(n=3)

Matrix	Spiked level ($ng\ L^{-1}(g^{-1})$)	OP	CP	NP	BPA	E1	E2	EE2	E3	DIHYDRO	TEST	AND	PROG
Ultra-pure water	50.0	76.64±4.1	78.32±3.2	76.15±4.6	94.24±3.5	95.46±4.8	93.65±3.1	91.34±4.1	88.15±5.1	94.57±3.7	92.33±2.1	91.68±2.3	85.96±3.4
	100.0	72.65±6.5	75.26±4.3	77.35±2.6	92.35±2.4	90.34±5.1	88.21±4.7	85.21±2.7	85.64±4.6	87.35±4.2	88.65±3.4	85.26±3.4	82.35±4.7
	500.0	70.32±4.8	71.65±3.7	72.54±5.1	88.32±1.8	86.54±4.5	84.21±3.7	80.46±3.5	82.31±2.1	83.58±3.9	82.65±5.1	82.15±2.5	79.35±2.8
	1000.0	65.32±3.1	66.32±2.7	67.52±4.5	83.21±3.3	81.32±2.4	80.32±2.6	74.56±6.5	78.48±4.2	77.75±3.4	76.35±4.8	76.35±3.2	74.25±4.6
River water	50.0	74.34±2.8	75.72±2.4	75.45±3.2	90.35±2.8	92.78±5.1	89.25±4.6	90.88±3.4	86.37±4.9	92.87±4.5	90.54±3.3	90.34±3.4	82.88±4.1
	100.0	73.12±4.1	71.89±3.2	75.48±4.4	91.65±2.4	87.59±3.4	85.31±2.9	86.21±1.9	84.47±3.6	85.36±2.3	86.58±4.1	83.46±5.6	83.39±2.6
	500.0	71.35±2.1	72.88±2.9	70.34±3.4	85.76±3.2	83.12±4.3	82.36±5.4	82.15±3.8	79.32±2.3	81.76±4.3	81.19±2.8	80.87±3.5	76.38±4.3
	1000.0	63.54±4.3	68.53±3.6	66.87±3.1	80.13±4.1	77.65±5.1	76.58±5.7	73.54±3.9	75.58±3.9	73.65±2.7	72.38±2.5	75.32±2.6	73.45.±5.6
Sediment	50.0	72.41±3.7	71.44±2.3	74.46±1.8	90.13±2.9	91.44±5.3	89.42±3.6	87.41±3.8	84.54±4.5	90.78±4.4	88.45±3.4	87.12±3.6	82.54±4.3
	100.0	71.51±2.7	75.26±3.4	73.55±3.1	87.35±3.4	88.98±4.3	85.31±2.6	84.35±3.6	83.24±5.0	86.77±3.3	85.37±4.2	86.35±2.8	79.12±3.0
	500.0	67.89±2.9	66.67±5.3	67.14±3.3	84.72±2.4	85.58±3.7	80.61±4.5	78.33±2.9	76.72±3.5	79.65±4.2	76.32±4.8	80.77±3.8	76.49±5.3
	1000.0	63.79±4.5	59.34±3.7	62.28±4.8	80.27±4.5	78.32±3.0	74.66±3.9	70.13±4.9	70.25±5.1	72.34±5.7	70.65±5.5	76.35±3.2	69..34±5.8
Biological	50.0	74.44±3.8	77.64±2.8	74.14±3.7	91.18±2.5	91.16±4.1	92.21±2.8	89.97±4.0	85.17±3.7	90.17±2.8	89.43±3.3	87.87±5.6	82.91±4.3
	100.0	70.38±2.9	73.15±3.4	70.21±3.2	87.78±4.2	87.24±5.5	87.81±3.7	83.24±3.4	82.64±3.8	87.57±3.3	84.57±4.2	84.22±4.5	78.89±3.9
	500.0	64.35±5.1	64.35±2.7	66.65±4.3	81.252±3.5	83.33±4.2	82.57±4.4	76.99±5.3	77.67.±2.7	81.95±2.9	80.11±4.8	81.71±3.4	72.23±4.3
	1000.0	60.28±4.7	58.11±2.2	61.17±3.4	75.58±5.2	76.26±3.9	76.61±2.7	71.11±2.3	71.25±4.9	74.28±3.6	74.33±3.1	74.66±5.2	66.45.±5.3

Table 4 The concentrations of target compounds in water, sediment and biological samples

Compounds	Water samples (ng L^{-1})	Sediment samples (ng g^{-1})	Biological samples (ng g^{-1})
4-t-OP	9.5	4.6	2.2
4-CP	2.6	4.4	1.8
4-NP	8.3	18.9	2.4
BPA	62.1	83.5	11.3
E1	3.6	2.8	1.9
E2	3.1	3.1	2.1
EE2	2.3	-	2.3
E3	-	-	-
DIHYDRO	15.9	3.2	-
AND	2.8	2.2	-
TEST	1.2	2.4	2.4
PROG	3.9	8.1	5.1