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Review

Recent advances in the mass spectrometric analysis related to endocrine disrupting compounds in aquatic environmental samples

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Abstract

An overview of mass spectrometric methods used for the determination of endocrine disrupting compounds (EDCs) in environmental samples is presented. Among the EDCs we have selected five groups of compounds that are of priority within European Union and US research activities: alkylphenols, polychlorinated compounds (dioxins, furans and biphenyls), polybrominated diphenyl ethers, phthalates and steroid sex hormones. Various aspects of current LC–MS and GC–MS methodology, including sample preparation, are discussed.

Keywords: Reviews; Aquatic environmental samples; Mass spectrometry; Environmental analysis; Endocrine disrupting compounds; Alkylphenols; Polychlorinated compounds; Polybrominated diphenyl ethers; Phthalates; Steroids

Contents

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1. Introduction

There are two classes of substances which can cause endocrine disruption: natural substances (hormones found naturally in the body of humans and animals and phytoestrogens, substances contained in some plants) and man-made substances. The group of man-made substances comprises synthetically produced hormones designed intentionally to interfere with the endocrine system (e.g. oral contraceptives) and man-made chemicals designed for use in industry, agriculture and consumer goods that may have unforeseen adverse or synergistic effects. Man-made chemicals also include chemicals unintentionally formed or produced as a by-product of industrial processes or combustion. This review article discusses five composite groups: PCBs, dioxines/furans, PBDEs, PAEs, alkylphenols (see Table 1 for a list of compounds and acronyms) and bisphenol A, all considered as substances having high exposure concern and evidence of endocrine disruption [1]. Some of them, like nonylphenol, di(2-ethylhexylphthalate DEHP) and PBDEs, are now included in the priority list of 32 substances which are part of the Water Framework Directive (WFD). Additionally, a group of synthetic and natural hormones is included in this survey, since they stand out for their estrogenic potency, and some of them are already subject to a ban for growth promotion in stock-farming in the EU [2].

This globally increased concern toward endocrine disrupting compounds (EDCs) induced a necessity to develop highly sensitive and specific analytical tools for their determination in environmental samples. The diversity of chemical properties of EDCs, complexity of environmental matrices and low detection limits required make the analysis of EDCs very challenging and imply a need for a comprehensive approach for their quantitative determination. The analytical determination of the above mentioned groups of EDCs, has been dominated by chromatographic methods (GC and LC) coupled to sensitive and specific detection systems, such as MS, MS–MS or high-resolution MS (HRMS) and preceded by complicated, time- and labor-consuming sample preparation. Choice of the method generally depends on the compound's properties and a quite clear distinction could be made between the apolar (e.g. dioxins, PCBs) and moderately polar compounds (alkylphenols, phthalates) amenable to GC and the polar ones (alkylphenol carboxylates), suited for LC analysis, although some compounds (e.g. steroid sex hormones, alkylphenolic compounds) could be analyzed using both methods. Among the different groups of EDCs, some compounds are well studied and standardized methods are available for their determination, such as EPA methods for the determination of chlorinated dioxins, furans and biphenyls by isotopic dilution HRGC–HRMS [3,4], while other EDCs (e.g. steroid sex hormones, PBDE) are less well studied and appropriate methods still have to be developed or the proposed methods have to be validated.

Generally, GC–MS has been the technique most commonly employed for the environmental analysis of EDCs. However, due to the poor volatility of some compounds, derivatisation steps aimed to produce more volatile products are required to improve the sensitivity of subsequent GC analysis. Thus, the advantages of better sensitivity are sometimes largely offset by loss of sample during the additional manipulation [5]. Furthermore, each derivatisation step is generally focused on one group of target analytes, producing volatile derivatives of the

Table 1
List of target compounds and acronyms used

Compound	Acronym
Alkylphenolic compounds	
Alkylphenol ethoxylates	APE_nO, *n* is number of ethoxy groups
Alkylphenol	AP
Alkylphenoxy carboxylate	APEC
Nonylphenol ethoxylate	NPEO
Nonylphenol carboxylate	NPEC
Nonylphenol	NP
Octylphenol ethoxylate	OPEO
Octylphenol carboxylate	OPEC
Octylphenol	OP
Halogenated (chlorinated, brominated) alkylphenol ethoxylate and corresponding analogs	XAPEO (ClAPEO, BrAPEO)
Dicarboxylated alkylphenol ethoxylate	CAPEC
Bisphenol A	BPA
Phthalate esters	PAE
Dimethyl	DMP
Diethyl	DEP
Dibutyl	DBP
Butylbenzyl	BBP
Di(2-ethylhexyl)	DEHP
Di-*n*-octyl	DnOP
Polychlorinated and polybrominated compounds	
Polychlorinated biphenyls	PCB
Polychlorinated dibenzo-*p*-dioxins	PCDD
Polychlorinated dibenzofurans	PCDF
Tetrachlorodibenzo-*p*-dioxin	TCDD
Polybrominated diphenyl ethers	PBDE
Natural and synthetic steroids	
17β-Estradiol	E2
17α-Estradiol	17α-E2
Estriol	E3
Estrone	E1
Ethynyl estradiol	EE
Diethylstilbestrol	DES
Mestranol	MES
Progesterone	PRO
Levonorgestrel	LEVO
Norethindrone	NORE

expected compounds, and thus discriminating related compounds and metabolites, which are simultaneously present but differ in structure. This is the reason why, for some groups of EDCs, GC–MS methodology is partially substituted with LC–MS or LC–MS–MS. In the past, one of the obstacles to the routine analytical application of LC–MS had been the unavailability of rugged and reliable LC–MS interfaces. The development of Atmospheric Pressure Ionization (API) overcame limitations such as poor structural information or sensitivity seen with thermospray or particle beam, respectively. During the last 10 years, LC–MS has gained in popularity, due to the sensitivity, ruggedness and ease of use given by the newer API interfaces, such as electrospray (ESI) and atmospheric pressure chemical ionization

(APCI), [6]. Combined with a new generation of MS equipment (single quadrupole, triple quadrupole, ion-trap), LC–MS and LC–MS–MS have become not only the widespread, but also routine methods for several classes of EDCs, such as alkylphenolic compounds, synthetic and natural steroids and bisphenolic compounds.

This paper reviews GC–MS and LC–MS methods, published in the last 5–6 years, for the determination of alkylphenolic EDCs and their precursors, steroid sex hormones, phthalates, bisphenol A, polychlorinated (PCBs, PCDDs, PCDFs) and polybrominated compounds (PBDEs) in environmental samples. The current state-of-the-art in the MS and MS–MS analysis is surveyed and future perspectives outlined.

2. Sample preparation

2.1. Classical approaches

2.1.1. Aqueous samples (sea, river and drinking water)

Solvent sublation, steam distillation and liquid–liquid extraction methods, used in the past, have been replaced by more efficient and versatile solid-phase extraction (SPE) and solid-phase micro extraction (SPME) techniques. Today SPE, employing both disks, and most frequently disposable cartridges, and to a lesser extent SPME, are used to isolate and concentrate alkylphenolic compounds, steroid sex hormones and phthalates from aqueous environmental samples.

In analysis of specific groups of compounds different solid-phases have been applied. Octadecyl (C_{18}) bonded silica has been the SPE material most widely employed for extraction of both alkylphenolic compounds [7–12] and steroid sex hormones [5,13,14]. The use of graphitized carbon black (GCB) cartridges has also been reported for both groups of compounds [15,16]. Reported efficiency of extraction from wastewater and surface water, respectively, using C_{18} and GCB cartridges is generally higher than 80% for all compounds investigated.-However, for polymeric sorbents, e.g. styrene-divinylbenzene (SDB), a recent comparative study carried out for steroid sex hormones proved that this sorbent is unsuitable for quantitative extraction of the relatively polar compounds (estriol) due to the low breakthrough volume ($<$250 ml) [14].

This wide range of compounds is not easily extracted in a single step and sequential SPE procedures (two cartridges of different SPE material coupled in series) were developed to extract alkylphenol ethoxylates and their acidic and neutral degradation products [17,18]. Selective elution [17,19], applying solvents of different polarity and selective desorption potential, is also a prerequisite for successful analysis of target compounds and their degradation products by flow-injection analysis (FIA)-MS [20,21]. Otherwise, direct injection of extracts, by-passing the chromatographic column, can cause severe problems in quantification of target analytes due to the signal suppression effects. However, it is important to mention that fractionation and group separation depend on matrix and in the case of complex samples (untreated waste water, sewage sludge, polluted sediment and soil), it is not possible to achieve complete fractionation. But the extracts obtained by sequential SPE are cleaner and only minor interferences due to the matrix can be expected.

The main problem to be solved when analyzing phthalates is contamination during the extraction, clean-up and analysis through laboratory materials like tubing, pipet tips, septa for autosampler vials, solvents, chromatographic sorbents, drying agents and glassware. In order to minimize the risk of contamination, adequate precautionary measures should be undertaken [22,23]: (i) any contact with plastic material should be avoided, (ii) glassware should be properly cleaned and deactivated, (iii) blank samples should be run for each series of samples, (iv) just one GC or LC injection of a sample extract should be made from the same vial (if several injections need to be made, several separate aliquots of the sample extract should be prepared). Vitali et al. [24] determined contamination, coming from the use of sodium sulfate and glass-fiber filters in the water sample procedure, in a range from 10 ng/l (DEP, DBP, BBP and DOP) to 100 ng/l (DEHP). To solve the background contamination problem, simple and rapid analytical procedures, based on SPME, applying both direct immersion and headspace technique, and subsequent GC–MS analy-

sis, have been proposed for the analysis of phthalates in waters and landfill leachates [25–27].

2.1.2. Solid samples (sludge, sediment, soil)

The common approach that permits simultaneous extraction of non-polar alkylphenolic compounds (APEOs and APs) and moderately polar degradation products (APECs) from solid environmental samples includes either sonication [28,29] or pressurized liquid extraction (PLE) [30,31]. Recent studies report the use of efficient semi- or fully automated continuous-flow-high-temperature sonication [8,32, 33], subcritical hot-water extraction [34] and supercritical fluid extraction (SFE) [35,36]. However, only few methods permit simultaneous extraction and determination of parent compounds and metabolites, both lipophilic and acidic ones, while the others are applicable to the extraction of particular compounds (e.g. steam distillation is limited to the volatile, less polar compounds such as APs and APEOs with a few ethoxy units).

Estrogens and progestogens have been frequently investigated in environmental waters and in sludge, but only one study describing the determination of estrogens in sediments using LC–MS has been published. The main procedural steps of this recently developed method [37] applied to the determination of estrogens and progestogens in river sediments from the Catalonian area (NE Spain) include ultrasonic solvent extraction and subsequent clean-up using Sep Pak Plus C_{18} SPE cartridges. Potential improvements to this procedure include the use of newer extraction techniques such as PLE. By using PLE, the intermediate centrifugation step carried out after the ultrasonic extraction could be obviated, provided that the extracts are filtered at the time of extraction in the PLE.

For persistent polychlorinated (PCBs, PCDDs, PCDFs) and polybrominated compounds (PBDEs) Soxhlet extraction is still the most commonly used robust liquid–solid extraction technique. SFE [38], as well as microwave-assisted extraction (MAE) and PLE [39], have been tested by a number of laboratories for the extraction of toxic organics such as PAHs, chlorinated pesticides and other semivolatile contaminants, and PCDDs and PCDFs [40–42].

Because of the complexity of samples and low selectivity of exhaustive extraction techniques applied, a substantial amount of interfering substances is found in crude extracts and subsequent clean-up and fractionation are indispensable. The conventional approach for extract clean-up is based either on solid–liquid adsorption chromatography in open columns using different adsorbents (Florisil, Alumina, different types of carbon, etc.) or on off-line solid-phase extraction (SPE) using C_{18}, NH_2 or CN modified silica. However, the whole procedure is time- and labour-consuming, often constituting the bottleneck of the analytical method, and the final result, in terms of selectivity, sensitivity and reproducibility, is not always satisfactory.

2.2. Advanced sample preparation strategies

The growing number of samples to be analysed in laboratories carrying out monitoring studies requires development of high-throughput and fully automated analytical techniques. One of the well established and robust options is application of on-line coupling of SPE and LC, using special sample preparation units, e.g. PROSPEKT (Spark, Holland) or OSP-2 (Merck, Germany) and disposable extraction cartridges. An approach of these characteristics has been recently described for the analysis of the most relevant estrogens and progestogens, in terms of estrogenic potency and environmental occurrence, in water samples. The procedure, based on the on-line SPE of the water sample and subsequent analysis by LC/diode array detection (DAD) [43] or by LC–ESI-MS [37] allowed for the monitoring of the target compounds at the ng/l level in up to 16 samples, the maximum number that the PROSPEKT system can process, in a fully automated, unattended way.

Another still novel and not fully exploited approach is orientated toward application of more selective sorbents, like molecular imprinted polymers (MIPs), immunoaffinity sorbents and restricted-access materials (RAMs).

Current analysis for PCDDs, PCDFs and PCBs requires laborious clean-up procedures that involve multiple column procedures and consume large quantities of potentially hazardous solvents. The use of an antibody-based affinity column has been explored as a means to shorten the length of time needed for the dioxin analysis and decrease the amount of solvent consumption [44–46]. When

compared to classical clean-up and isolation methods, the immunochromatographic methods are >20 times faster and use 100 times less organic solvents, and their selectivity is enormously enhanced. Immunological methods, because of the specificity of antibody–antigen recognition are known to be highly selective. In immunoaffinity chromatography methods, antibodies are bound to the packing of a chromatographic column, into which the sample containing the antigen is injected. The antigen–antibody binding is a reversible reaction that allows the use of immunoaffinity chromatography as an isolation and concentration step of the analytes of interest that are present in the sample.

The use of immunoaffinity extraction coupled with LC–ESI-MS has recently been described for the analysis of the steroids β-estradiol and estrone in wastewater [47]. In this approach, the high selectivity of the immunosorbents has been shown to remove much of the isobaric noise and of the interfering sample matrix compounds that would otherwise cause severe ionisation suppression of the estrogens during the electrospray process, and to contribute to the achievement of very low detection limits (0.8 and 0.07 ng/l for β-estradiol and estrone, respectively).

Another advanced sample preparation strategy includes an integrated LC-sample preparation and analysis based on a dual column system (also called coupled column, multidimensional or column switching) [48,49]. Short LC columns, turbulent-flow chromatography (TFC) column and columns packed with MIPs or RAM, respectively, were successfully applied as precolumns in two-column LC systems [50–52]. RAMs have been successfully applied for direct extraction and enrichment of hydrophobic low molecular analytes from biological fluids carrying a high load of proteins (plasma, blood, urine, saliva, supernatants of cell cultures and tissue) and from food samples (milk, food homogenates) [53,54]. However, in environmental analysis RAMs have been seldom applied. The tailor-made RAM has been successfully applied for the separation of humic substances interfering in the analysis of pesticides [55–58] and just one application describes analysis of EDCs (alkylphenolic compounds and steroid sex hormones) [59]. This novel methodology based on column switching LC–MS, using LiChrospher ADS RAM precolumns (Merck, Darmstadt, Germany) is described for an integrated sample clean-up and analysis of EDCs (alkylphenolic compounds, BPA and steroid sex hormones) in sediment samples. The best results in terms of selectivity and sensitivity were obtained using a RAM column of low hydrophobicity (LiChrospher ADS C_4). It has been shown that a restricted access precolumn efficiently separates high-molecular matrix components (humic substances), polar impurities and inorganic salts, thus reducing significantly ion suppression effects in ESI-MS detection.

3. Alkylphenolic compounds

Table 2 reviews current methods applying MS detection used to quantify APEOs and their degradation products in various aqueous and solid matrices. Although not considered as EDCs, parental long-chain APEOs are often analysed simultaneously with their degradation products, listed in priority list of EDCs. Such multiresidue analysis permits the assessment of the degree of degradation of the parent surfactants and identification of the main sources of pollution (discharge of untreated, treated waste waters or sludges, respectively). This review also includes degradation products with insufficient data to determine their potential for endocrine activity, such as dicarboxylated ethoxylates and halogenated derivatives, respectively.

3.1. LC–MS

3.1.1. Alkylphenol ethoxylates (APEOs)

LC analysis of APEOs has been attempted using both normal-phase and reversed-phase systems. In normal-phase systems, the APEOs are separated according to the increasing number of ethylene oxide units, while corresponding oligomers with the same number of ethoxy units but different alkyl substituents (e.g. NPEO and OPEO) co-elute. Reversed-phase LC allows separation according to the character of the hydrophobic moiety and it is particularly well suited to separate surfactants containing various hydrophobic moieties (separation of alkyl-homologues). In this case, the length of the ethylene oxide chain does not influence the separation and the various oligomers containing the same hydrophobic

Table 2
Survey of MS methods used for quantitative determination of alkylphenolic compounds

Compounds	Matrix	Extraction	Clean-up	Derivatization	Separation and detection method	MS system	LOD	Ref.
NP, NPEO	Marine sediment	Soxhlet (hexane-2-propanole, 7:3)	SPE-CN	–	NP-LC–ESI-MS	VG Quattro tandem (Micromass)	2–10 ng/g (NPEO) 4 ng/g NP	[30]
NP, NPEO	Marine sediment	PLE (hexane–acetone, 1:1)	SPE-CN	–	NP-LC–ESI-MS	VG Quattro tandem (Micromass)	Low ng/g level	[61]
APEO (n_{EO}=1–3), APs, XAPs	Estuarine water and sediment	High-temperature continuous-flow sonication (methanol)	SPE-NH_2+ semipreparative RP-HPLC fractionation	–	RP-LC–ESI-MS	Platform LCZ (Micromass) a single quadrupole	0.2–0.92 ng/l	[8]
NP, NPEOs	Estuarine sediment	High-temperature continuous-flow sonication (methanol)	RP-HPLC fractionation (two columns in series)	–	Mixed mode LC–ESI-MS	Platform LCZ (Micromass) a single quadrupole	21.5 ng/g NP 0.78–37.3 ng/g NPEOs	[33]
APEOs, APs, APECs, XAPEOs, XAPs, XAPECs	Sludge, sediment, river water	Sonication (methanol–dichloromethane 7:3)	SPE-C_{18}	–	RP-LC–ESI-MS	HP 1100 (Hewlett-Packard) a single quadrupole	20–100 ng/l 2–25 ng/g	[28]
NPEO (n_{EO}>2) NPEC, CNPEC	STP samples	SPE-GCB	–	–	RP-LC–ESI-MS	Finnigan AQA (Thermoquest) a single quadrupole	Not reported	[66]
NPECs (n_{EO}=1–4)	Sludge, river water, STP effluents	Sub-critical (hot) water extraction (water–ethanol 7:3)	Anion exchange SAX disks	Methyl iodide	GC-(PCI)-MS NH_3 reagent gas	Finnigan 4023	0.2–2 μg/g (S/N=10)	[34,81]
NPEC, CNPEC	River water	SPE-GCB	–	Tetrabutyl ammonium hydrogen sulfate	GC–EI-MS	Saturn 2000 (Varian, USA) ion trap MS	100 ng/l (S/N=10)	[77]
NP, OP	STP effluent, river and drinking water	SPE-LiChrolut EN (polymeric)	–	Pentafluorobenzoyl chloride	GC-(NCI)-MS CH_4 reagent gas	HP 5973 MSD (Hewlett-Packard)	0.05 ng/l	[83]
NP, OP	Lake sediments	SFE (CO_2)	Silicagel	Acetic anhydride	GC-(EI)-MS	HP 5890 II (Hewlett-Packard)	46 ng/g NP 1 ng/g OP	[75]
OP, NP, NPE_1C, NPE_1O, NPE_2O	Groundwater, wastewater	LLE (dichloromethane)	–	N,O-bis(trimethylsilyl) trifluoro acetamide + trimethylchlorosilane	GC-(EI)-MS	VG Fisons MD800	5–64 ng/l	[90]

moiety elute in one peak. Eluting all the oligomers into one peak has the advantages of increasing the peak intensity and therefore, increasing the sensitivity of determination. Using MS detection, concentration of individual oligomers (NP_1EO, NP_2EO, NP_3EO, etc.) can be readily obtained by extracting total ion chromatograms for characteristic m/z values. However, quantitative results for individual APEOs should be interpreted with caution because of variations in the oligomer distribution attributed to the differences in the ionisation processes, different response factors of individual oligomers depending on the number of ethylene oxide groups, the interference from fragments of higher oligomers and ion suppression effects caused by complex matrices [60].

APEOs can be detected using both, ESI and APCI, under positive ionization (PI) conditions. The typical MS spectrum of polyethoxylates shows the characteristic pattern of equally spaced signals with mass differences of 44 Da (one ethylene oxide unit).

Using an APCI source [60], several series, corresponding to $[M+H]^+$, $[M-C_9H_{18}]^+$ and $[M+NH_4]^+$ (when an ammonium containing eluent is used) are observed. Additionally, a number of other adduct ions $[M+Na]^+$ and $[M+K]^+$ and clusters $[M+(H_2O)_n+H]^+$ are formed. Therefore, using APCI, the ionization of APEO molecules is dispersed among many molecular adduct ions and the abundance of each ion is highly variable, depending on operating parameters and concentrations of NH_4^+, Na^+, and K^+ ions, which are difficult to control due to their adventitious origin.

Using an ESI interface and aprotic solvent without any additive, APEOs show a great affinity for alkali

metal ions, and they give exclusively, evenly spaced sodium adducts $[M+Na]^+$, due to the ubiquity of sodium in the solvents and surfaces. Sodium adducts are relatively stable and generally no further structurally significant fragmentation is provided in the mass spectrum. Using a protic solvent (methanol) however, the formation of the intact protonated molecular ions $[M+H]^+$ and other adducts $[M+K]^+$ $[M+NH_4]^+$and cluster ions, such as $[M+(H_2O)_n+Na]^+$, $[M+(H_2O)_n+H]^+$ with $n=1–5$, are also reported [61], but sodium adducts still prevail. Formation of doubly charged ions $[M+2Na]^{2+}$ of highly ethoxylated APEOs that interfere with singly charged ions of less ethoxylates APEOs is reported by Shang et al. [61]. This interference, limited to odd numbered highly ethoxylated APEOs (e.g. pair $NPE_{15}O$/NPE_5O) can cause an error up to 40% in the quantification of less ethoxylated APEOs in reversed-phase LC–MS where all APEOs of a given alkyl chain (e.g. NPEOs) co-elute as a single peak. The problem could be eliminated by separating all ethoxymers with normal-phase LC [61], by removing highly ethoxylated APEOs applying appropriate sample preparation, e.g. normal-phase SPE [8] or by using additives, such as ammonium acetate, to enforce the formation of ammonium adducts over sodium or proton adducts and at the same time to improve the retention behaviour and peak shape [62,20].

Limits of detection reported were oligomer-dependent, since the relative response factor of individual-oligomers increases with increasing degree of ethoxylation. Shang et al. [61] reported detection limits for normal-phase LC–ESI-MS analysis of marine sediment in the range of 0.8–4 ng (injected) depending on the individual NPEO oligomer, while Crescenzi et al. [15] reported limits of quantification ($S/N=10$) for reversed-phase LC–ESI-MS analysis of STP influents, effluents, river water and drinking water of 2, 0.07, 0.007 and 0.0007 μg/l for preconcentration of 10, 100, 1000 and 4000 ml, respectively. Using SPE-ESI-MS, Petrovic et al. [28] reported detection limits ($S/N=3$), upon preconcentration of a 500-ml sample (river water), of 100, 40 and 25 ng/l for APE_1O, APE_2O and APEO with $n_{EO}=3–15$, respectively (SIM mode, 30 ions corresponding to OPEO and NPEO oligomers, respectively, $n_{EO}=1–15$). The lowest detection limits were reported by Ferguson et al. [8]. Using SPE (concentration factor 2000) and isocratic elution using narrow-bore C_8 column and ESI-MS detection, they reported method detection limits (MDLs) in the range of 0.2–1.0 ng/l for APEOs.

Methods using FIA-APCI-MS–MS applying precursor (parent) ion scanning of m/z 121 (characteristic for ethoxylates with 1–4 ethoxy unit), m/z 133 (characteristic for $n_{EO}=5$ to 16 homologues) and m/z 291 and multiple reaction monitoring (MRM) were reported for the identification of NPEOs in waste and surface water [63,64].

3.1.2. Alkylphenols (APs)

Alkylphenols (OP and NP) are detected under NI conditions, using both APCI and ESI interfaces. The sensitivity of detection, using an ESI source was approximately 40–50 times higher than that obtained with an APCI source [60]. Using an ESI, APs give exclusively $[M–H]^-$ ions, whereas using an APCI, at higher voltages, using so-called in-source CID, the spectra show fragmentation that closely resembles that obtained by the MS–MS technique. Alkylphenols give, in addition to the $[M–H]^-$ ion at m/z 205 (for OP) and m/z 219 (NP), respectively, fragment m/z 133, resulting from the loss of a C_5H_{12} (OP) and C_6H_{14} (NP) group [65]. A similar fragmentation pattern is obtained using a MS–MS (Fig. 1). Fragments m/z 147, m/z 133, m/z 119 and m/z 93 result from the progressive fragmentation of alkyl chain, whereas m/z 117, also observed by Pedersen and Lindholst [65], cannot straightforwardly be explained. Therefore, reaction channels m/z 205→m/z 133 (for OP) and m/z 219→m/z 133 (for NP) and precursor (parent) ion scan of m/z 133 can be used to monitor APs, but also APECs, since the dominant dissociative reaction for carboxylates is formation of deprotonated alkylphenols (Fig. 1, Inset A).

3.1.3. Alkylphenoxy carboxylates (APECs) and dicarboxylates (CAPECs)

Alkylphenoxy carboxylates (APE_nC) were detected in both the negative ionization (NI) mode [28,62,66] and PI mode [67]. In the NI mode, using ESI, APECs give two types of ions, one corresponding to the deprotonated molecule $[M–H]^-$ and the other to $[M–CH_2COOH]^-$ in the case of APE_1Cs and $[M–CH_2CH_2OCH_2COOH]^-$ for the

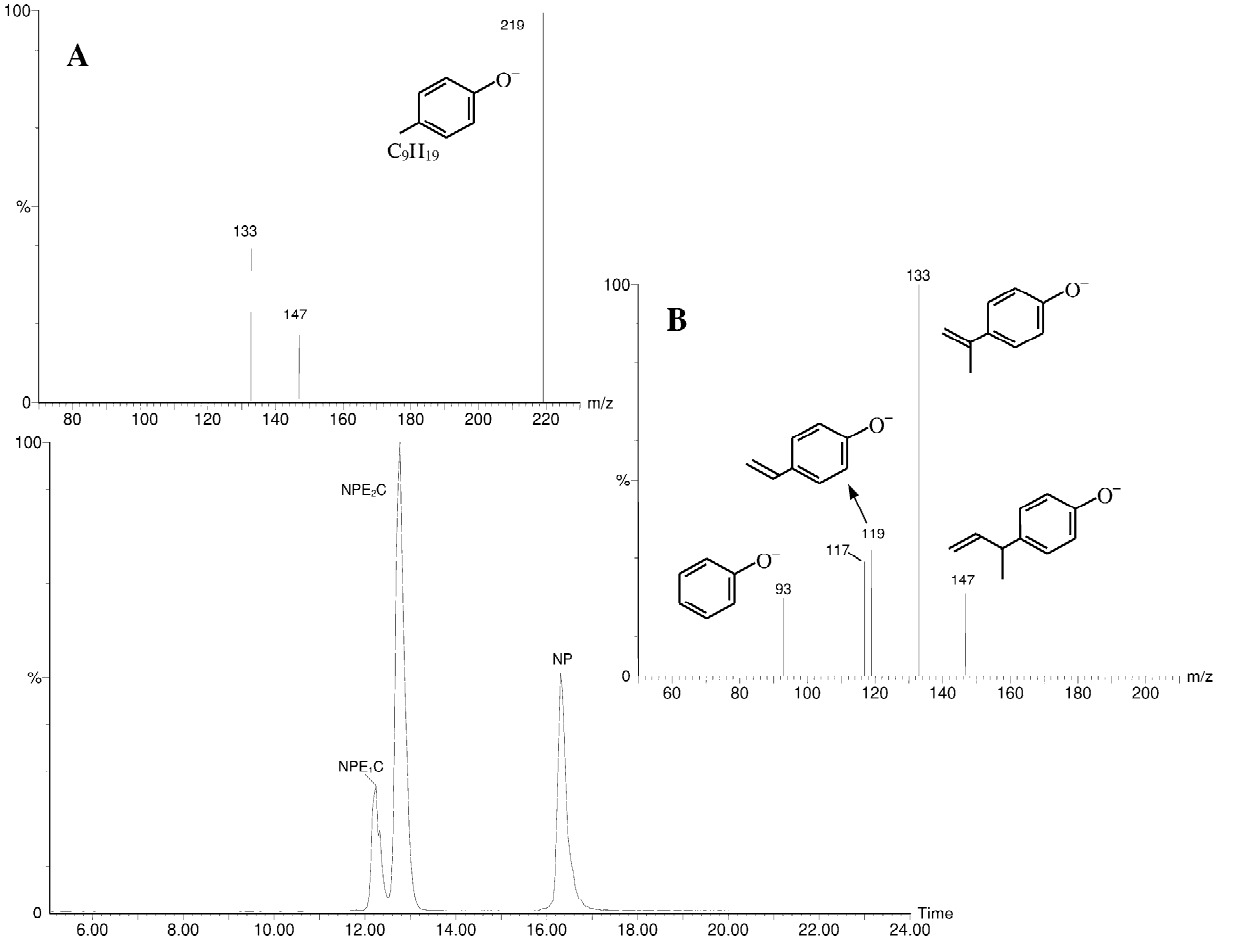

Fig. 1. MS–MS chromatogram (MRM channel m/z 219→133) of raw effluent (river water) treated in Barcelona drinking water treatment plant. Insets: product ion scan of NPE_2C (A) and NP (B), obtained using argon as collision gas at a collision energy of 40 eV.

APE_2Cs. The relative abundance of these two ions depends on the extraction voltage. Using a low voltage, the ESI source is capable of producing deprotonated molecular ions, and the spectra display only signals at m/z 277 and 263 corresponding to NPE_1C and OPE_1C and m/z 321 and 307 for NPE_2C and OPE_2C. At higher voltages, using so-called in-source CID, the spectra give fragmentation that closely resembles that obtained by the MS–MS technique [68]. Intense signals at m/z 219 and 205 are produced after the loss of the carboxylated (ethoxy) chain, while m/z 133 and 147 corresponded to the fragmentation of the alkyl chain, as described above for NP (Fig. 1, Inset A).

The identity of the dicarboxylated breakdown products was confirmed by LC–ESI-MS [66,67] and LC–ESI-MS–MS [62] Under NI conditions, at low cone voltage no CID process was possible and the spectrum displayed only signals tentatively assigned to the $[M-H]^-$ and $[MNa\text{-}2H]^-$. However, with the cone voltage of 55 V, structural confirmation of these species was achieved by observing different fragment ions (Fig. 2). Monocarboxylated and dicarboxylated metabolites, derivatized to yield the methyl esters, can also be detected by ESI in the PI mode [67]. At low extraction voltages, the in-source CID process is greatly inhibited and the spectra display intense signals for the protonated molecular ions. By raising the extraction voltage, in-source CID spectra were obtained. Neutral losses of the carboxylated

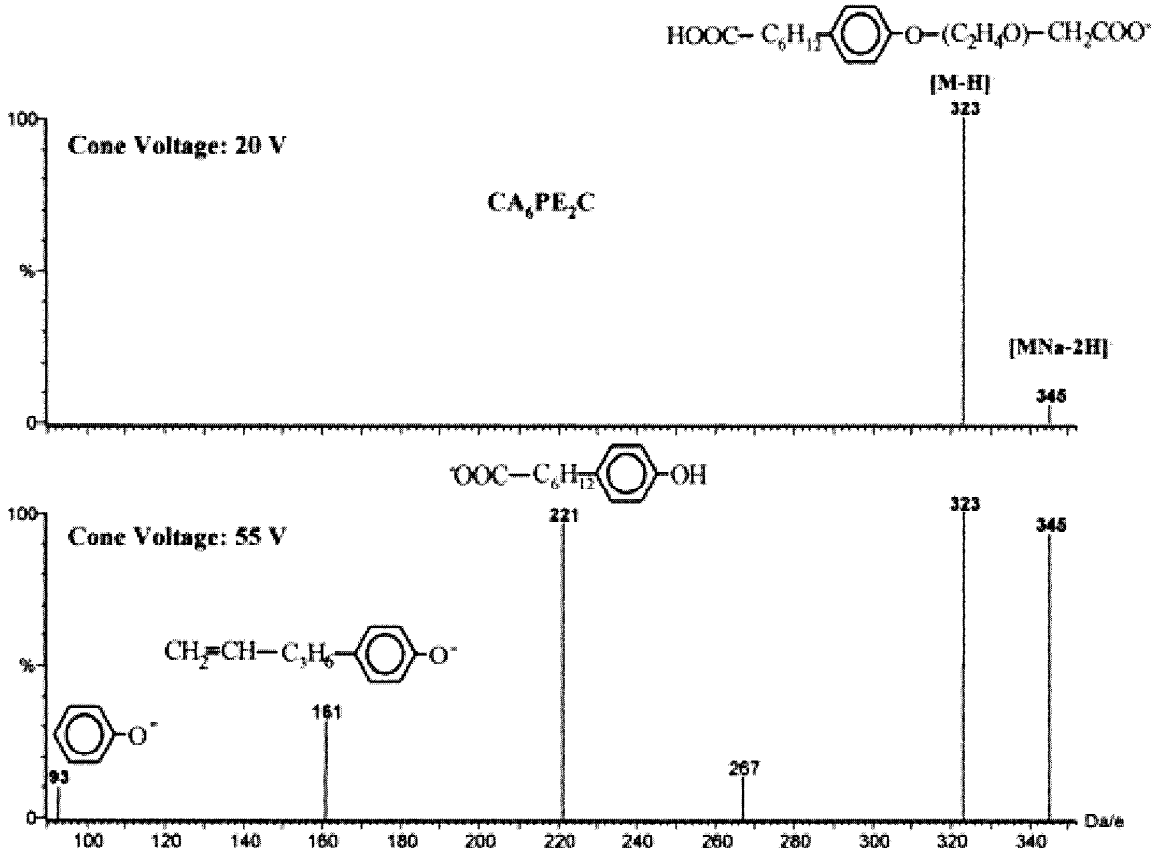

Fig. 2. NI mass spectra of the dicarboxylated (CA_6PE_2C) metabolite of NPEO taken at two different cone voltages. Reprinted with permission from [66] the American Chemical Society (Copyright 2000).

ethoxy chain and carboxylated alkyl chain, respectively, and methanol loss followed by formation of acylium ions, were found to be typical fragmentation patterns for methylated CAPECs.

3.1.4. Halogenated derivatives of alkylphenolic compounds

APEOs and their acidic and neutral metabolites can be halogenated to produce chlorinated and brominated products. The formation of these compounds has been reported during the chlorination processes at drinking water treatment plants [28,69,70] and chlorination after biological wastewater treatment [71].

Brominated and chlorinated APEOs, APs and APECs yield doublet signals characteristic for bromine and chlorine isotopes, respectively. Using an ESI interface, halogenated APEOs like their non-halogenated analogs show a great affinity for alkali metal ions, and they give exclusively evenly-spaced sodium adduct peaks $[M+Na]^+$ with no further structurally significant fragmentation. The problem arises from the fact that the chlorinated derivatives ($ClAPE_nO$ and $ClNPE_nC$) have the same molecular mass and they gave the same ions as brominated compounds with one ethoxy group less ($BrAPE_{n-1}O$ and $BrAPE_{n-1}C$, respectively). However, they can be distinguished by their different isotopic profiles. The doublet signal in the mass spectrum of brominated compounds shows the contribution of bromine isotopes of $^{79}Br/^{81}Br = 100:98$, while the contribution of chlorine isotopes is $^{35}Cl/^{37}Cl = 100:33$.

Therefore, chromatographic separation of these two groups of compounds is a prerequisite of their quantitative determination.

Halogenated APs and halogenated APECs were analysed in the NI mode using an ESI interface [8,28]. XNPs gave characteristic isotope doublet signal of the $[M-H]^-$ ions (m/z 297/299 for BrNP and m/z 253/255 for ClNP). XNPECs gave two signals, one corresponding to quasi-molecular ion and another to $[M-CH_2COOH]^-$ in the case of $XNPE_1Cs$ or $[M-CH_2CH_2OCH_2COOH]^-$ for $XNPE_2Cs$. The relative abundance and absolute intensity of these two ions, compared to quasimolecular ions, highly depends on the cone voltage. At higher values, the base peak, with high absolute intensity, for $ClNPE_1C$ and $ClNPE_2C$ is m/z 253/255 and for $BrNPE_1C$ and $BrNPE_2C$ m/z 297/299. Therefore, these compounds can be monitored using the same m/z channels as XNPs, increasing the relative instrument dwell time and enhancing sensitivity. Using MS–MS, further fragmentation can be obtained (Fig. 3). In addition to the neutral loss of the carboxylated (ethoxy) chain and sequential fragmentation of alkyl chain, the CID spectra of halogenated NPECs also display $[Cl]^-$ and $[Br]^-$ ions, respectively.

3.2. GC–MS

Although LC–MS has proved to be a more versatile technique applicable to the full range of APEOs oligomers and their degradation products, GC–MS is still a more readily available technique in many laboratories. However, the analysis of un-

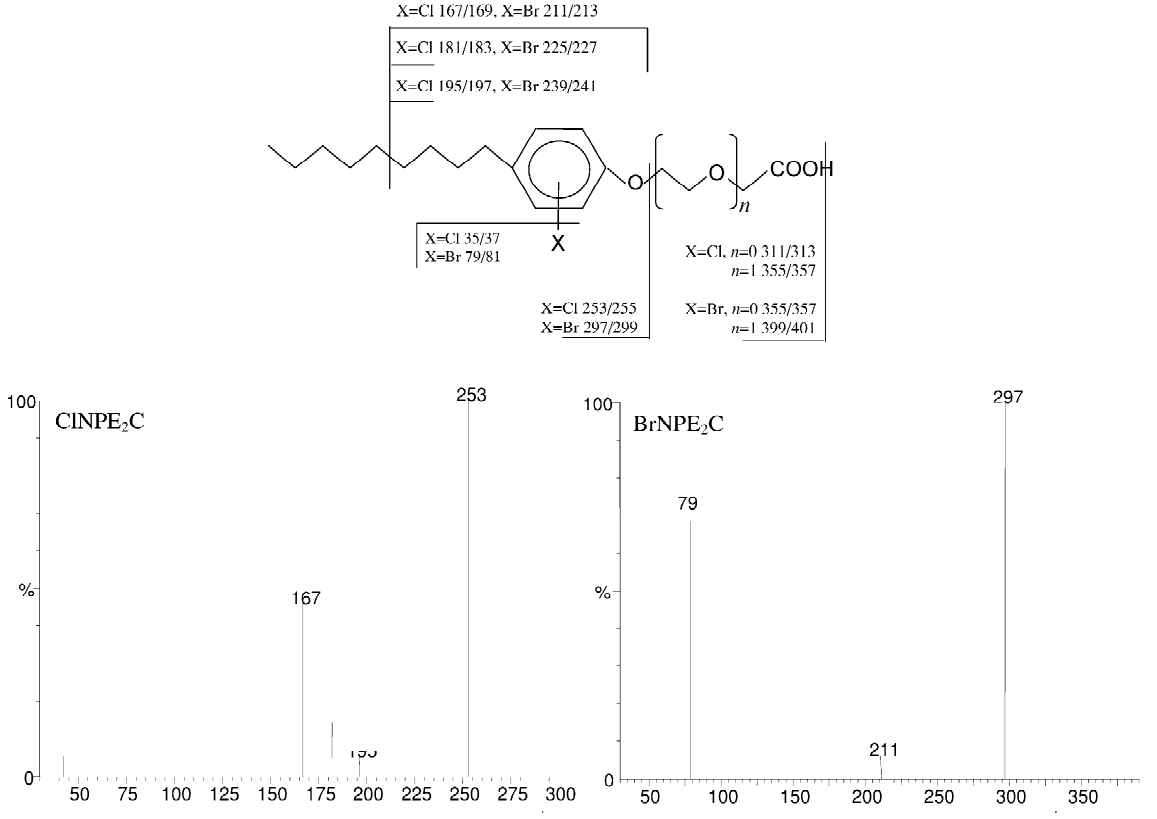

Fig. 3. CID spectra of (A) $ClNPE_2C$ (precursor ion m/z 355) and (B) $BrNPE_2C$ (precursor ion m/z 399) obtained at a collision energy of 30 eV and the proposed fragmentation pattern.

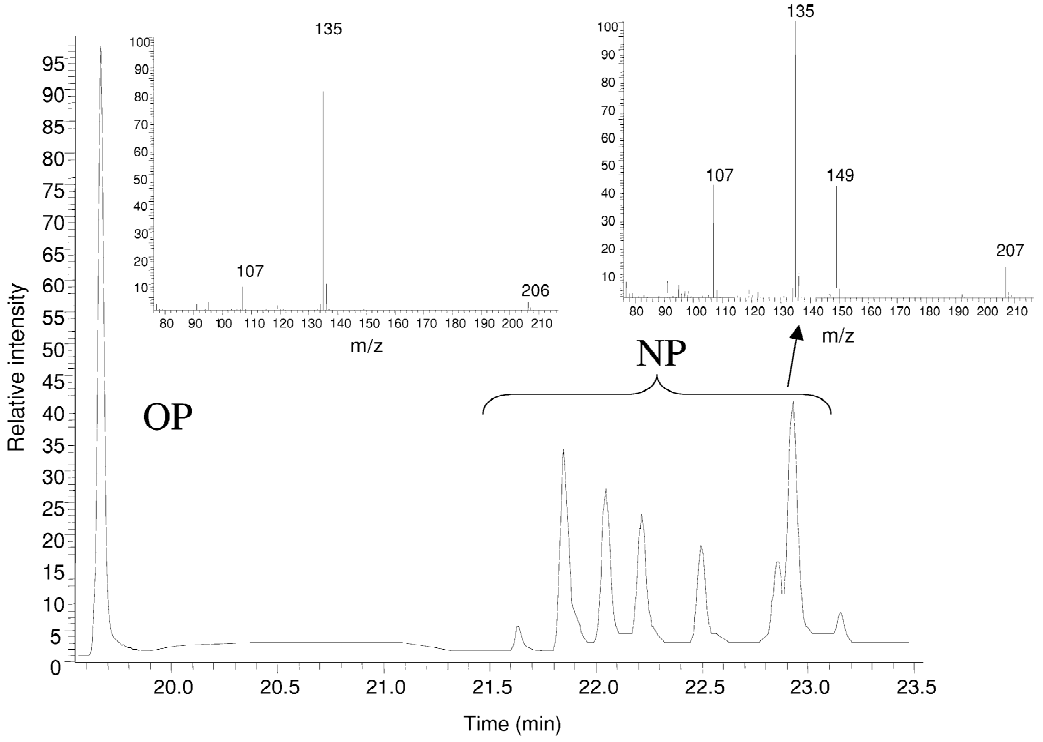

Fig. 4. GC–MS chromatogram of 4-*t*-OP and isomers of 4-NP. Insets: corresponding MS spectra.

derivatized alkylphenolic compounds by GC–MS is restricted to the most volatile degradation products, such as APs and APEOs with less than 4-ethoxy groups. Fig. 4 shows a GC–MS chromatogram, and corresponding EI-MS spectra, of underivatized 4-*t*-OP and 4-NP [72]. In contrast to the simple chromatographic profile, obtained for 4-NP by LC–MS, yielding a single broad peak, GC–MS analysis reveals the presence of different isomers in the alkyl chain. According to MS data, five distinct groups, comprising a total of 22 isomers, three of them having a tertiary alpha-carbon and two with a secondary alpha-carbon, can be distinguished [73].

To overcome the problem with the volatility, different derivatization off-line and on-line protocols, respectively, have been developed. Off-line derivatization to corresponding trimethylsilyl ethers, methyl ethers, acetyl esters, pentafluorobenzoyl or heptafluorobutyl esters, respectively was applied as a common approach in numerous studies, while an alternative derivatization method, applied to analyse NPEOs in solid and liquid environmental matrices, consists of in-situ derivatization and extraction [74–76]. An on-line method, using direct GC injection-port derivatization using ion-pair reagents (tetra-alkylammonium salts), has also been proposed [77].

Two complementary MS techniques, one, using EI and another, less commonly used, positive ion chemical ionization (PCI), have been evaluated for the analysis of APEOs, their acidic (APECs) and neutral metabolites (APs) and halogenated derivatives and reviewed by Lee [78].

The most significant ions in EI-MS of methylated NPECs were fragments produced by rupture of the benzylic bond in the branched nonyl side-chain [77,79,80]. The abundant fragment ions obtained using EI-MS were further analyzed by GC–MS–MS and product ions resulting from cleavage in the alkyl

moiety, cleavage in the carboxylated (derivatized to methyl esters) moiety and cleavage in both moieties, were detected [68].

GC–CI-MS spectra of the NPECs with isobutane as reagent gas showed characteristic hydride ion-abstracted fragment ions shifted by 1 Da from those in the corresponding EI mass spectra [68]. Using ammonia as reagent gas, intense ammonia-molecular ion adducts of the methyl esters, with little or no secondary fragmentation were reported for the detection of NPECs [81]. Ions selected in multiple ion detection mode were as follows: m/z 246, 310, 354 and 398 for NPE_1C, NPE_2C, NPE_3C and NPE_4C, respectively.

There are a few reports on NCI, used to analyse pentafluorobenzyl derivatives of NP and NPEOs employing methane as reagent gas [82,83].

An ion trap GC–MS system, with low-pressure CI and MS–MS capabilities, was developed for quick switching between EI and CI scans, as well as MS–MS modes. The method was successfully applied for the analysis of both NPEOs and their degradation products in river water and sewage effluents, employing a large-volume injection technique [77,84].

4. Bisphenol A (BPA)

Table 3 reviews LC–MS and GC–MS methods used to quantify BPA in environmental samples.

4.1. LC–MS

LC–MS is an alternative method for the analysis of BPA in environmental matrices (the first choice is GC-MC). Using an ESI interface, main fragment, in addition to the base ion m/z 227 $[M-H]^-$, is m/z 212 resulting from a cleavage of one of the CH_3 groups [85]. Using an APCI [65], additional fragments m/z 211 $[(C_6H_4)C_3H_6(C_6H_4OH)]^-$ and m/z 133, which is assigned to $[(C_6H_4)C(CH_3)CH_2]^-$, are observed. For quantitative purposes, a lower fragmentation voltage was used in order to maximize the signal from the molecular anion (m/z 227) and the limits of detection reported for water samples were in the range from 25 to 100 ng/l [59,65,85].

4.2. GC–MS

Bisphenol A is a polar compound and this characteristic ultimately affects the detection limit achieved

Table 3
Survey of MS methods used for quantitative determination of bisphenol A

Matrix	Extraction	Clean-up	Derivatization	Separation and detection method	MS system	LOD	Ref.
STP effluents	SPE-C_{18}	Silicagel cartridges	*N*-Methyl-*N*-trimethylsilyltrifluoro-acetamide	GC-(EI)-MS	HP 5972 A (Hewlett-Packard)	Not reported	[89]
Sea water, spring water	Liquid–liquid extraction (DCM)	–	–	GC-(EI)-MS	HP 5971 (Hewlett-Packard)	600 ng/l	[86]
River, sea, groundwater	Micro liquid–liquid extraction (DCM)		Trimethylchlorosilane + hexamethyldisilazane	GC–MS	HP 5971 (Hewlett-Packard)	0.4 ng/l	[88]
STP samples, sewage sludge	Sonication (methanol–DCM, 7:3)	SPE-C_{18}	–	LC–ESI-MS	HP 1100 (Hewlett-Packard)		[85,92]
Water	SPE-C_{18}	–	–	LC–APCI-MS	HP 1100 (Hewlett-Packard)	100 ng/l	[65]
River water	LLE (toluene)	–		GC-(EI)-MS (cool on-column injection)	HP 5989 (Hewlett-Packard)	1 μg/l	[87]
Wastewater, groundwater	LLE (dichloromethane)	–	*N,O*-Bis(trimethylsilyl) trifluoro acetamide + trimethylchlorosilane	GC-(EI)-MS	VG Fisons MD800	5.4 ng/l	[90]
Surface and drinking water	SPE-SDB	–	Pentafluorobenzoyl chloride	GC-(NCI)-MS (methane as reagent gas)	HP 5973 MSD (Hewlett-Packard)	0.02–0.04 ng/l	[83]

by GC–MS. Detection limits of 0.1–1 μg/l were reported for underivatized BPA [86,87]. Using EI-MS base ion at m/z 213 corresponded to $[M-CH_3]^{+\cdot}$. In addition, the spectrum showed a peak at m/z 228 corresponding to $[M]^{+\cdot}$. To improve the sensitivity, different GC–MS methodologies, based on high preconcentration using micro liquid–liquid extraction [88], derivatization to silyl BPA [88–90] or pentafluorobenzoylate ester [83] were proposed. Negative chemical ionization MS using methane as reagent gas enabled very sensitive determination of BPA-pentafluorobenzoylate at absolute femtogram amounts [83]. This ''soft ionization'' technique yielded a dominant molecular ion $[M]^-$ and the C_{13} isotope ion $[M+1]^-$ as the second intensive one. This highly sensitive and specific method gave a limit of detection of 20 pg/l.

5. Phthalate esters

Table 4 reviews LC–MS and GC–MS methods used to quantify phthalate esters in environmental samples.

5.1. LC–MS

Phthalate esters (DMP, DEP, DBP and DEHP) were detected in industrial effluents [91] and sewage sludge [92] using LC–APCI-MS under PI conditions. The LC separation of phthalic esters was performed on C_{18} stationary phases. At 20 V, base ion for DEP, DBP and DEHP was m/z 149 (protonated phthalic anhydride) and m/z 163 $[M+H-2CH_3]^+$ for DMP.

LC–ESI-MS based on the formation of sodium adducts can provide molecular ion information and was found to be a reliable tool for quantitative analysis of phthalate esters in various matrices [93]. The sodium adduct ions, characteristic for individual phthalate and each isomeric group, i.e. m/z 335, 385, 413, 441, 469 for C_6, C_7, C_8, C_9 and C_{10} isomers, were found to be formed with sodium ions from the spray mobile phase. LC–ESI-MS–MS study showed that the two major pathways of phthalate fragmentation in ESI(+)-MS–MS are similar to that in electron ionisation (EI)-GC–MS. One reaction is dominated by the loss of one of the substituents leading to the formation of monoester sodium adducts and another by the formation of sodiated phthalic anhydride ions $[(C_8H_4O_3Na]^+$ with m/z 171. Study with H^+ as mobile phase modifier showed that protonated molecular ions are more reliable for the identification of phthalates by MS–MS technique.

5.2. GC–MS

Different methods employing EI-MS, CI-MS with

Table 4
Survey of MS methods used for quantitative determination of phthalate esters

Compounds	Matrix	Extraction	Clean-up	Derivatization	Separation and detection method	MS system	LOD	Ref.
DEHP	Industrial effluents	SPE-SDB	–	–	LC–APCI-MS	VG Platform (Micromass)	100 ng/l	[91]
DEHP, DEP, DBP	Sewage sludge	Sonication	SPE-C_{18}	–	LC–APCI-MS	HP 1100 (Hewlett-Packard)	15–50 ng/g	[92]
9 PAEa	River water and sediment	LLE or sonication (dichloromethane)	–	–	CG-(EI)-MS	HP 5971 MSD (Hewlett-Packard)	6–12 ng/l 125–240 ng/kg	[24]
DBP, BBP	STP effluents	SPE-C_{18}	Silicagel cartridges	*N*-Methyl-*N*-trimethylsilyltrifluoroacetamide	GC-(EI)-MS	HP 5972 A (Hewlett-Packard)	Not reported	[89]
16 PAEs	Sewage sludge	Mechanical shaking (ethyl acetate)	–	–	GC-(EI)-MS GC-(CI)-MS (methane as reagent gas)	HP 5973 (Hewlett Packard)	10–632 ng/g	[23]
6 PAEs	River and sea water	SPME	–	–	GC-(EI)-MS	HP 5972 (Hewlett-Packard)	2–27 ng/l	[27]
DBP, DEHP	Surface and drinking water	On-line HPLC(C_{18})-GC-(EI) MS Large volume injection (10 ml)				QMD 100 (CE Instruments)	5–10 ng/l	[96]

methane as the reagent gas, either in the positive or negative mode, as well as tandem MS, under PCI conditions with isobutane as reagent gas, have been evaluated for the detection of phthalate esters in water, soil and sewage sludge samples [23,94]. PCI with methane as the reagent gas was found to be very useful to obtain molecular mass and information about the ester group. EI-MS was found to be the most sensitive detection technique and it is recommended for the quantification, although it gives little information on molecular mass and the nature of the alcohol moiety in the molecule. For all phthalates, the most abundant ion in the mass spectra was *m/z* 149, corresponding to the protonated phthalic anhydride ion $[C_8H_5O_3]^{+\cdot}$, except for dimethylphthalate, which gave a base ion at *m/z* 163 $[M\text{-}31]^{+\cdot}$. However, when analysing phthalate mixtures, better group profiling is needed with more characteristic ions. Beside these two ions, it is recommended to use the low abundance ions in the higher mass range for the assignment of structures in order to prevent any misidentification [95].

Hyötyläinen et al. [96] proposed on-line coupling of reversed-phase HPLC to GC–MS by the vaporizer/precolumn solvent split/gas discharge interface for the analysis of PAEs in drinking and surface water. Applying large volume injection (10 ml), without any sample pretreatment, limits of detection were 5–10 ng/l.

6. Synthetic and natural steroids

Table 5 reviews MS methods for the quantitative determination of estrogens and progestogens in environmental samples.

6.1. LC–MS

LC coupled with different detection systems, such as diode array detection (DAD) [43], fluorescence (FL) [97] and MS [14,16,37,47,98–101] has been used for the separation and final analysis of estrogens and progestogens in environmental matrices.

The LC separation of estrogens and progestogens is usually performed on octadecyl silica stationary phases (25 cm×4.6 mm I.D., 5-μm particle size) using water–acetonitrile mixtures and gradient elution from 20–50 to 100% organic solvent as mobile phases.

Both the ESI and the APCI interfaces, operating in the PI mode of ionisation, have been used for the LC–MS determination of progestogens. In general, MS conditions provoking light fragmentation and single predominant ions have been selected as optimum in order to obtain maximum sensitivity under SIM conditions. These predominant ions correspond to adducts of the analyte molecule with one sodium atom ($[M+Na]^+$), when the interface employed is ESI, and to the protonated molecular ion ($[M+H]^+$), when the interface employed is APCI. In terms of sensitivity, the ESI interface has been shown to provide limits of detection about one order of magnitude better than those achieved with the APCI interface [14].

For the MS determination of estrogens, ESI, operating in the NI mode of ionisation, has been the interface most widely used because of its observed better sensitivity compared to the APCI interface [8,14,16,102]. A recent study, by Lagana et al. [100], of the trace analysis of estrogens in sewage effluent by use of HPLC-tandem mass spectrometry has, however, shown that an APCI interface operating in the positive-ion mode of ionisation can furnish sensitivity (LOQ between 0.5 and 1 ng/l) almost as good as that achieved with an ESI interface under similar analytical conditions (LOQ between 0.08 and 0.6 ng/l) [16].

In the LC–ESI-MS analysis of estrogens, selected ion monitoring of the $[M-H]^-$ ions is usually carried out for maximum sensitivity [8,14]. In the LC–ESI-MS–MS analysis of the most environmentally relevant estrogens, the following precursor ion-product ion transitions have been recorded in the MRM mode: *m/z* 287→171 and *m/z* 287→145 for estriol, *m/z* 271→183 and *m/z* 271→145 for estradiol, *m/z* 295→159 and *m/z* 295→145 for ethynyl estradiol, and *m/z* 269→145 and *m/z* 269→143 for estrone [16,99,103]. Fig. 5 shows the CID spectra obtained for these four estrogens under full scan product-ion conditions. The confirmation criteria considered for analyte identification in these approaches have been:

1. LC retention times of the analytes should be within 2% of the retention times of the standards;
2. the absolute relative abundances of at least two selected precursor ion–product ion transitions

Table 5
Survey of MS methods used for quantitative determination of natural and synthetic steroids

Compounds	Matrix	Extraction	Clean-up	Derivatization	Separation and detection method	MS system	LOD (ng/l)	Ref.
E2, E3, E1, EE, DES, PROG, LEV, NOR	Drinking and surface water, STP effluent	SPE (C_{18} col.)	–	–	LC–ESI-MS	HP 1100 (Hewlett-Packard)	2–500	[14]
E2, E3, E1, EE	River water, STP influent and effluent	SPE (Carbograph-4 col.)	–	–	LC-(NI)ESI-MS–MS	Sciex API 2000 triple-quadrupole (Perkin-Elmer)	0.08–0.6[a]	[16]
E2, E3, E1, EE, DES, PROG, LEV, NOR	Water	On-line SPE (HySphere-Resin-GP col.)	–	–	LC–ESI-MS	HP 1100 (Hewlett-Packard)	<1	[37]
E2, E1	STP effluent	SPE (LiChrolut EN+C_{18} col.)	Immunoaffinity extraction	–	LC-(NI)ESI-MS	Platform LCZ (Micromass)	0.07–0.18	[47]
E2, E3, E1	River water	SPE (SDB-SC disk)	–	–	LC-(NI)ESI-MS	HP 1100 (Hewlett-Packard)	1–50	[98]
E2, E3, E1, EE	STP influent and effluent	SPE (Carbograph-4 col.)	–	–	LC-(NI)ESI-MS–MS	Sciex API 2000 triple-quadrupole (Perkin-Elmer)	0.2–0.5	[99]
E2, E3, E1, EE	STP influent and effluent	SPE (ENVI-CARB col.)	–	–	LC-(PI)APCI-MS–MS	Sciex API 365 triple-quadrupole (Perkin-Elmer)	0.5–1[a]	[100]
E2, E3, E1, EE, MES, equilin, testosterone, dihydrotestosterone, cyproterone	River water	SPE (C_{18} col.)	–	–	LC-(PI)APCI-MS–MS	Sciex API 365 triple-quadrupole (Perkin-Elmer)	1–10	[101]
E2, E1, EE	STP effluents	SPE (C_{18} col.)	-SPE (C_{18} col) -HPLC fraction. -LLE	–	GC-(EI)MS	Finnigan MAT Magnum ion trap	0.2	[5]
E2, E1, EE	STP effluents	SPE (C_{18} col.)	HPLC fraction.	–	GC–MS	Not reported	0.5–1	[13]
E2, 17α-E2, E1, EE	Surface and drinking water, STP effluent	SPE (LiChrolut EN)		PFBCl	GC-(NCI)-MS	HP 5973 MSD (Hewlett-Packard)	0.05–0.15	[83]
E2, E1, EE	STP effluent	SPE (ENV+ col.)	-LLE, GPC (BioBeads SX-3) -hydrolysis	Acetic anhydride	GC-(EI)MS	Not reported	Not reported	[105]

E2, E1, EE, 17α-E2 (SS)	River water and STP effluent	Continuous LLE	–	Bis-(trimethylsilyl) trifluoroacetamide with 10% trimethylchlorosilane	GC-(EI)MS	HP 5970 MSD (Hewlett-Packard)	58 (only E2)	[106]
E2, E3, E1, EE, MES, LEV, NOR-acetate	STP effluent	SPE (LiChrolut EN/ Bondesil C_{18} col.)	Silicagel 60	MSTFA/TMSI/DTE (1000:4:2)	GC-(EI)MS	HP 5970B MSD (Hewlett-Packard)	1*	[107]
EE	STP effluent	SPE (Empore C_{18} disk)	–	–	GC-(EI)MS	Finnigan Voyager	74	[108]
E2, EE	Surface water	SPE (C_{18} col. or disks or PS–DVB col.) or LLE	–	MTBSTFA	GC-(EI)MS	Voyager (Interscience)	50–300	[109]
E2, E3, E1	STP effluent	SPE (C_{18} col)	–	Pentafluoropropionic acid anhydride	GC-(EI)MS	HP 5890 MSD (Hewlett-Packard)	5–10	[110]
EE	Tap and river water	In-sample acetylation on-line SPE (PLRP-s)	–	Acetic anhydride	GC-(EI)MS	Finnigan MAT 44 S	15	[111]
E2, E1, EE	STP influent and effluent	SPE (SBD-XC disk)	-SPE (C_{18}/NH_2 col.) -HPLC fraction.	–	GC-(EI)MS–MS	Not reported	0.1–1.8	[99]
E2, 17α-E2, E1, MES, E2-17-valer., 16α-OH-E1, E2-17-acet.	River water, STP influent and effluent	SPE (C_{18}/EN col.)	Silicagel	MSTFA/TMSI/DTE (1000:2:2)	GC-(EI)MS–MS	Varian Saturn 4	0.5–1	[112]
E2, EE, E2gluc, E2sulf	Surface water, STP effluent	SPE (C_{18} disk)	-Hydrolysis -HPLC fraction.	Heptafluorobutyric anhydride	GC-(EI)MS–MS (only E2)	Finnigan GCQ ion trap (ThermoQuest)	0.2–0.4	[113]
E2, 17α-E2, E1, EE, glucuronides	Surface and waste water	SPE (SDB-XC disk)	-hydrolysis -SPE (C_{18}/NH_2) -HPLC fraction.	SIL A reagent	GC-(EI)MS–MS	Saturn IV ion trap (Varian)	0.1–2.4	[114]
E2, E1, EE	Reservoir and river water, STP effluent	SPE (C_{18} disk)	–	MTBSTFA with 1% TBDMCS	GC-(EI)MS–MS	ThermoQuest GCQ ion-trap	1	[115]

Abbreviations (not included in the text): STP, sewage treatment plant; col., column or cartridge; fraction., fractionation; PFBCl, pentafluorobenzylbenzene; GPC, gel permeation chromatography; SS, surrogate standard; NOR-acetate, norethindrone acetate; MSTFA, *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide; TMSI trimethylsilyl-imidazole; DTE, dithioerytrol; MTBSTFA, *N*-methyl-*N*-(*tert.*)-butyldimethylsilyltrifluoroacetamide; E2-17-valer., estradiol-17-valerate; 16α-OH-E1, 16α-hydroxy-estrone; E2-17-acet., estradiol-17-acetate; E2gluc, estradiol glucuronide; E2sulf, estradiol sulfate; TBDMCS, *tert.*-butyldimethylchlorosilane.

[a] Limit of quantification.

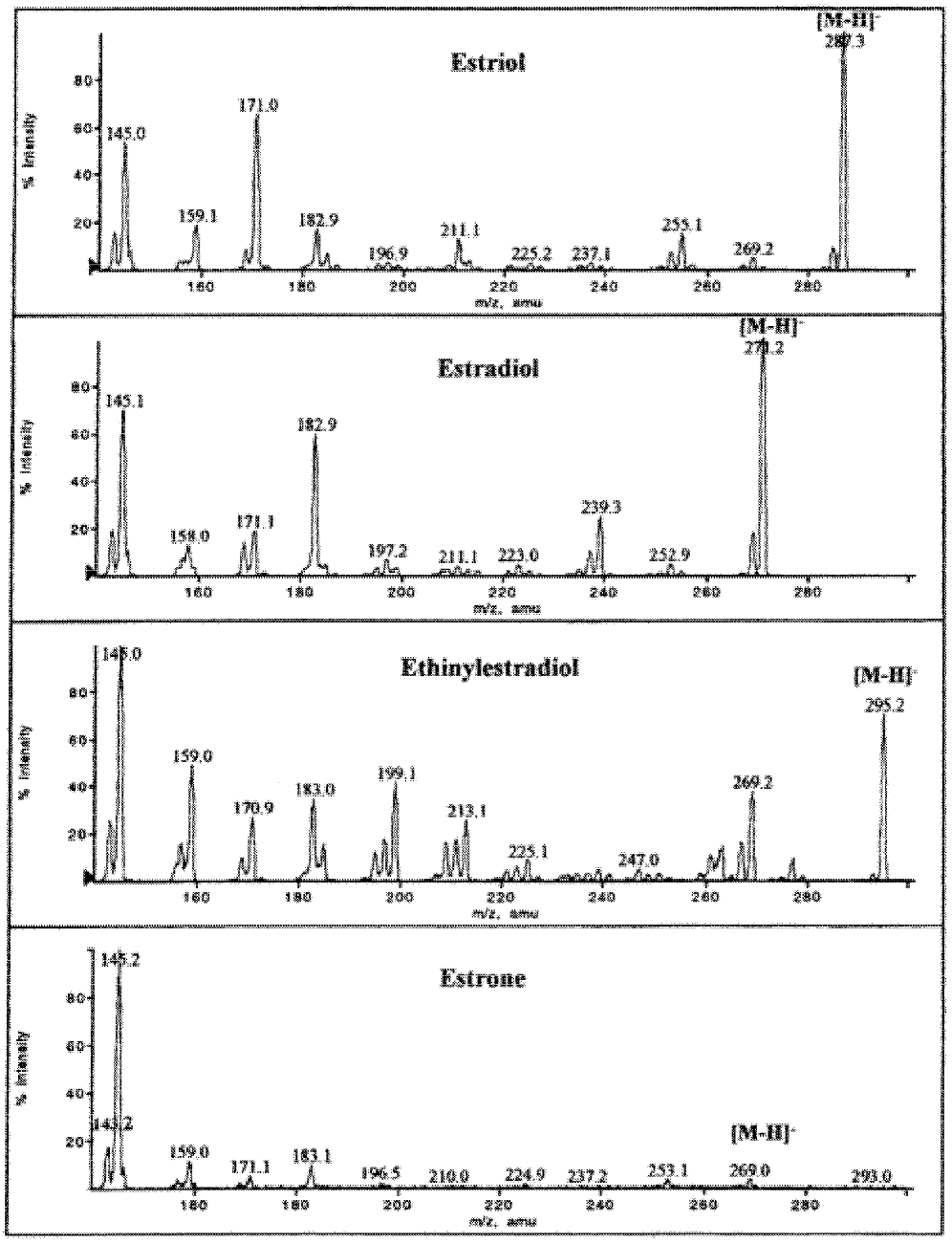

Fig. 5. CID spectra of four estrogens with their respective deprotonated molecules as precursor ions. Reprinted with permission from [16] the American Chemical Society (Copyright 2000).

should be within 20% of the ion ratios obtained for the standards.

Modification of the mobile phase, when carried out in an attempt to improve the sensitivity of MS detection, has always been postcolumn. Methanolic ammonia [16] and triethylamine [99] have been used as modifiers to promote deprotonation of the weakly acidic estrogens and to increase the response of the

mass spectrometer operated in the negative-ion mode of ionisation with the electrospray interface.

The use of triple quadrupole mass spectrometers in HPLC–MS–MS systems has substantially increased the selectivity and sensitivity of the determination, resulting in limits of detection, in the analysis of estrogens and progestogens in wastewaters (0.08–1 ng/l), far better than those achieved with single quadrupole HPLC–MS (2–500 ng/l) and GC–MS (0.2–74 ng/l), and comparable or slightly lower than those achieved with GC–MS–MS (ion trap) (0.1–2.4 ng/l) [104].

An advantage of LC–MS, compared with GC–MS, is that it enables the determination of both conjugated and unconjugated forms of estrogens and progestogens without the need for derivatization.

6.2. GC–MS

The analytical determination of estrogens and progestogens in environmental matrices has been dominated by the use of GC–MS [5,13,83,105–111] and GC–MS–MS [99,103,112–115].

GC separation is performed with a variety of capillary columns using helium as carrier gas, with temperature programs from approximately 45 to 300 °C. Both conventional MS and MS–MS (ion trap) detection are accomplished in the EI mode. The use of NCI has been reported only once [83]. To improve the stability of the compounds and the sensitivity and precision of the GC–MS or GC–MS–MS analysis, the analytes are usually derivatized in the –OH groups of the steroid ring. Several derivatization agents, such as bis-(trimethylsilyl)-trifluoroacetamide [106], *N*-methyl-*N*-(*tert.*)-butyl-dimethylsilyltrifluoroacetamide (MTBSTFA) [115,116], and heptafluoro-butyric anhydride [113], have been used for this purpose. The ion masses selected for quantitation in each case vary depending on the derivatizating reaction performed. As an example, Fig. 6 illustrates the GC–MS–MS spectra of the most relevant estrogens after derivatization with pentafluoropropionic acid anhydride and the purported fragmentation scheme [103].

The sensitivity and general performance of the techniques GC–MS–MS, LC–MS, and LC–MS–MS for the determination and quantitation of steroid hormones in complex environmental matrices has been compared in a recent study published by Croley et al. [103]. According to these authors, the sensitivity of these techniques is improved in the order LC–MS (LOD 200 pg/μl)<GC–MS–MS (LOQ 20 pg/μl)<LC–MS–MS (LOQ 5 pg/μl).

In terms of accuracy and repeatability, all three techniques are in general satisfactory, although the derivatization step, usually carried out prior to the GC–MS or GC–MS–MS analysis, in addition to being time-consuming, can constitute a source of inaccuracy [100,103,104]. An advantage of GC–MS, compared with LC–MS, is the availability of extensive libraries of mass spectra useful for identification of unknown peaks in estrogenically active fractions.

A recent study has also discussed the possibilities of high-resolution mass spectrometry (HRMS) combined with two analytical tools developed by the U.S. EPA's Environmental Sciences Division, termed Mass Peak Profiling from Selected Ion Recording Data (MPPSIRD) and Profile Generation Model (PGM), for characterizing or identifying environmental contaminants [117]. This study has shown that, with 20 000 resolution, 10 pg of ethynyl estradiol can be detected. This amount corresponds to a detection limit of 1 ppt if the ethynyl estradiol in 1 liter of water were concentrated into a 0.1-ml extract followed by injection of 1 μl of the extract into the probe tip capillary for analysis.

7. Polychlorinated and polybrominated compounds

The method of choice for the determination of many halogenated contaminants is GC (an overview is given in Table 6). The volatility of these compounds allows a GC determination and the use of sensitive detection methods such as electron-capture detection (ECD) or MS. The development of capillary columns in GC enabled a congener-specific determination of a number of these mixtures such as PCBs, PCDDs and PCDFs. However, more and more scientists have become aware of the limitations of single-column capillary GC for this type of determination. Injection on two different columns for an unambiguous determination was recommended [118].

In recent years, comprehensive two-dimensional

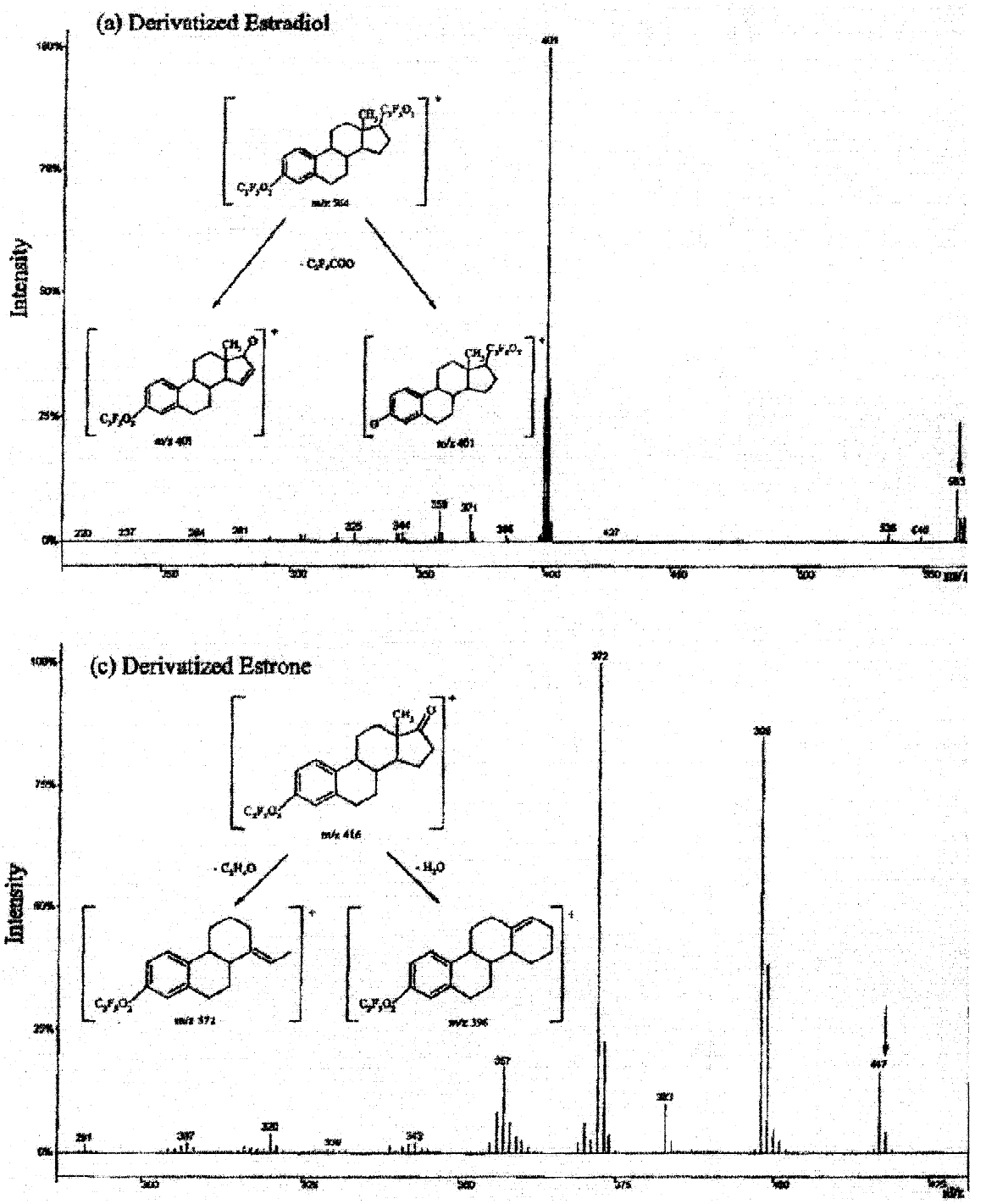

Fig. 6. GC–MS–MS spectra of the derivatized estrogens and the proposed fragmentation scheme. Insets: (a) estradiol, (b) estrone. Reprinted with permission from [103] John Wiley & Sons Limited (Copyright 2000).

Table 6
Survey of MS methods used for quantitative determination of polychlorinated and polybrominated compounds

Compounds	Matrix	Extraction	Clean-up	Separation and detection method	MS system	LOD	Ref.
PCBs	Liquid samples	Immunoaffinity chromatography		GC–LRMS	Varian 2000	n.r.	[45]
PCDDs/Fs				GC–HRMS	AutoSpec Ultima—Micromass		
PCBs	Standards	–	–	GC×GC-μECD	HP6890	1 pg/μl	[120]
PCDDs/Fs	Environmental samples	Soxhlet (toluene)	Silica+Florisil+alumina	GC–LRMS	Fisons MD-800	3–17 pg	[127]
				GC–HRMS	VG AutoSpec Q	350 fg–2 pg	
PCDDs/Fs	Environmental samples	Soxhlet	Silica+carbon	GC–MS–MS	Finnigan MAT TSQ 70	200–600 fg	[128]
PCDDs/Fs	Food samples	Soxhlet (CH_2Cl_2/pentane 1:1])	Automated system (silica+alumina+carbon)	PTVLV-GC-ITD-MS	ThermoQuest Trace GC PolarisQ	200 fg (TCDD)	[132]
PCDDs/Fs	Waste water Soil	n.r.	n.r.	GC-ITD-MS	ThermoQuest GCQ	n.r.	[133]
PCBs	Biological samples	SFE–LC (supercritical CO_2, 40 °C, 300 atm.)		GC-ToF-MS	Thermoquest Trace 2000 GC–HD Technology Sprint	3.5 pg/μl	[139]
PCBs	Biological samples	n.r.	n.r.	GC-ToF-MS	Pegasus II–LECO Corp.	iLOD=5 pg/μl (PCB≠149) mLOD=30 ppt	[142]
PBDEs	Standards	–	–	GC–EI-MS	Agilent 6890—Agilent 5973 Network	0.53–32.09 pg	[146]
				GC–NCI-MS		30 fg–1.72 pg	
PBDEs	Human milk	SPE (Oasis™ HLB)	Silica	GC–NCI-MS	n.r.	0.3–1.0 pg/g	[148]
PBDEs	Fish	SPE	GPC	GC–HRMS	VG AutoSpec Q	0.1–4.8 pg	[149]

n.r., not reported; PTVLV, Programmable Temperature Vaporisation Large Volume; iLOD, instrumental LOD; mLOD, method LOD; GPC, gel permeation chromatography.

chromatography (GC×GC) has been shown to be very useful for the separation of various complex samples. In GC×GC, two independent GC separations are applied to the sample. The most obvious advantage of the GC×GC is the large peak capacity. Because retentions in the two dimensions are almost independent, the peak capacity that can be achieved is close to the product of the peak capacities of the two individual columns [119]. Another advantage of the GC×GC system is the increase in the signal-to-noise ratios, which leads to an improvement of the detection limit. Finally, two time co-ordinates that make the identification more reliable describe all peaks in the chromatogram. Recent studies [120,121] examined the potential of GC×GC for the qualitative analysis and characterisation of complex mixtures of halogenated contaminants, such as PCBs, with emphasis on the non- and mono-*ortho* PCB congeners.

7.1. PCDDs and PCDFs

MS has been the leading technique used in dioxin analysis ever since Banghman and Meselson first reported its use for the characterisation and quantification of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2378-TCDD) in samples from South Vietnam that were contaminated after using a herbicide called Agent Orange [122]. The analytical method based on high resolution gas chromatography (HRGC) coupled to low resolution mass spectrometry (LRMS) provides an assay for the determination of 2378-TCDD in environmental samples at ppb and potentially at low ppt levels, when it is combined with state-of-the-art sample preparation techniques. However, is not applicable to samples that contain a high level of interfering compounds because of the low specificity of LRMS. In addition, the absolute instrumental sensitivity of LRMS (quadrupole MS) is generally 2–3 orders of magnitude poorer than that achievable by HRMS (magnetic sector MS). As a result, the HRMS method (EPA method 1613 [3]) has emerged as the leading and routine analytical method for the determination of PCDDs and PCDFs at ppt or ppq levels in complex matrices. Until the 1990s, ultra-trace determinations of PCDDs/PCDFs are routinely performed using HRGC–HRMS, operating in the electron impact ionisation mode (electron energy

~38 eV) at 10 000 resolving power [3]. Quantification was carried out by an isotopic dilution technique, based on the addition of known amounts of 2378-substituted labelled standards (surrogates) prior to the extraction process.

Other MS techniques such as triple quadrupole MS–MS and hybrid MS–MS have also been investigated for the analysis of dioxins. It is generally believed that the MS–MS technique surpasses others in analytical specificity, but it is not widely used owing to its relatively poor sensitivity and reproducibility compared to the HRMS method. Despite the drawbacks reported for MS–MS, monitoring the loss of COCl from PCDDs and PCDFs by MS–MS gives selectivity greater than HRMS at 10 000 resolution in the presence of some interferences, especially PCBs [123]. Instrumental parameters affecting the formation of daughter ions, and thus sensitivity of tandem MS–MS are collision gas, collision energy and collision gas pressure. Charles et al. [124] have shown that the sensitivity of MS–MS for the analysis of PCDDs and PCDFs is enhanced at increased collision gas pressures and at lower collision energies.

Different studies have been published on the comparison between LRMS, HRMS and tandem MS–MS in their application to PCDD/PCDF analyses [125–129]. PCDDs and PCDFs can also be analysed using negative ion chemical ionisation (NCI)-MS. Characteristics of the NCI mass spectra of PCDDs/PCDFs have been extensively studied [130]. The fragmentation and sensitivity depend on the degree of chlorination and the substitution pattern. For instance, 2378-TCDD was found to be less sensitive than other TCDDs and PCDFs were more sensitive than PCDDs.

The application of HRMS has proved to provide the required sensitivity and specificity. The required specificity could be provided by tandem MS as well. While MS–MS with sector or quadrupole instruments needs a series of mass analysers in space, ion trap uses one mass analyser to perform MS–MS in time. Recently, the ion trap detection (ITD)-MS was developed to analyse PCDDs and PCDFs. The advantage of ITD-MS systems is the much lower price, which could reduce analyses costs. However, sensitivity of ITD-MS instruments is considerably lower than of HRMS instruments. The limit of determination for ITD-MS systems for TCDD (S/N 3:1) can be assumed to be in the range of about 100–300 fg, whereas modern HRMS instruments have a LOD of about 3 fg [131]. The lack of sensitivity can be compensated up to a certain degree by much higher sample amounts for extraction and clean-up. However, the need to use about 10-fold or more higher sample amounts causes many problems for the availability of sample material and the analytical procedure. Another possibility is to increase the amount of analyte injected. Eppe et al. [132] tested the large volume injection in order to increase the method sensitivity and to reduce the evaporation time before analysis. The final volume (30 μl) is a good compromise between the amount of liquid injected in the liner and the time saved during the evaporation step before analysis.

Another disadvantage of the ITD-MS is the reproducibility of the quantification. Kemmochi et al. [133] optimised the ionisation conditions in order to perform the reproducible PCDD and PCDF quantitative analysis using ITD-MS. The voltage, current and temperature of the chamber are the parameters for the ionisation condition optimisation. As shown from their results, reproducibility can be obtained working at an emission current of 150 μA or at electron energy of 90 eV. Quantification at these optimised conditions is equivalent to those obtained by the conventional method.

Kemmochi and Tsutusmi [134] presented a review on the advantages of the ITD-MS for the determination of PCDDs and PCDFs in fly ash and soil samples. The advantages included rapid determinations and economic, simplicity in operation and maintenance, and high selectivity for dioxin isomers.

Different studies of the comparison of ITD-MS versus HRMS and MS–MS were carried out. The dioxin and furan content of extracts from sludges, fly ash, soil, compost, sediment, and rabbit liver was analysed by HRMS as well as ITD-MS [135]. Moreover, March et al. [136] compared HRMS, ITD-MS and MS–MS methods for the determination of dioxins and furans. The factors considered in this comparison were the tuning of each instrument, the preparation and comparison of calibration curves, the 2378-TCDD detection limit for each instrument, ion signals due to HxCDDs obtained with each instrument from two real samples (air and pyrolyzed

polychlorinated phenols), relative response factors, and ionisation cross-sections.

7.2. PCBs

Different methods for the determination of PCBs have been developed. A number of countries have chosen to monitor PCBs as a set of seven indicator PCBs (IUPAC Nos. 28, 52, 101, 118, 138, 153 and 180). These PCBs were analysed by HRGC with electron-capture detection system (ECD) or by HRGC–LRMS. However, when dioxin-like PCBs (non-*ortho* and mono-*ortho* PCBs) were studied, comprehensive analytical procedures are necessary because they occur at concentrations lower than the indicator PCBs mentioned above, and are therefore very elaborate and complicated to analyse. In these cases, a HRGC–HRMS with quantification by isotopic dilution method was required [4].

Recently, the ITD-MS was also developed to analyse PCBs. In order to increase the qualitative information on PCB congeners, Guidugli [137] investigated a procedure based on the use of a wide band waveform to isolate the whole isotopic cluster. The isotopic cluster, related to the product ions, is detected, this giving immediate information on the number of chlorine atom still present in the fragment ion. The detection limit for PCB in water samples is at the pg/ml level.

The powerful HRMS instrumentation that is used for the analysis of these contaminants suffers some limitations that are related to the limited accelerating voltage working range for a given group of ions in the SIM mode. For example, co-eluting compounds with wide differences in their masses cannot be effectively monitored in the same window [138] and the chromatographic run thus needs to be prolonged for adequate component speciation. Due to their non-scanning character, time of flight mass spectrometers (ToF-MS) are valuable tools for fast GC because they are able to monitor the entire mass range in very short times. Different recent studies showed the capabilities of ToF for the analysis of PCBs in different types of samples.

Van Bavel et al. [139] quantified 30 different PCB congeners in biological samples above the detection limit in less than 7 min. Routinely, around 40 congeners are detected above the detection limit using SIM on a quadrupole GC–MS in 45 min [140]. The detection limit using the GC-ToF seemed to be somewhat higher. The GC-ToF showed good linearity until a concentration of 17.5 pg PCB injected on the column, while the LRMS showed good linearity down to 3.5 pg injected splitless running in SIM mode. Dimandja et al. [141] compared the HRMS with the ToF techniques for assessing human exposure to PCBs. A ToF analysis was carried out for 38 PCBs in <5 min. The sample throughput gained by the ToF method was significant. The same co-eluting pairs in the 40-min HRMS run were co-eluting in the ToF run. An isotope dilution calibration curve for PCB 206 showed good linearity over the selected range, with limits of detection in the low ppb range. Fast ToF results were consistent with the HRMS results within the 95% confidence interval limits. Focant et al. [142] developed a new method for the simultaneous analysis of PCBs and persistent pesticides using fast GC-isotope dilution-ToF-MS. Fig. 7 illustrates the capability of the method to simultaneously consider the PCBs and the persistent pesticides present in the same sample. This represents a significant increase in the analytical power.

During GC-ToF analyses, full scan spectra are always acquired. The sensitivity during such a quadrupole full scan run is however 10–20 times lower than achieved with the GC-ToF. The acquisition of full scan spectra during the analysis opens the possibility of screening for ''unknown'' pollutants present in the extracts. A SIM-GC–MS run always

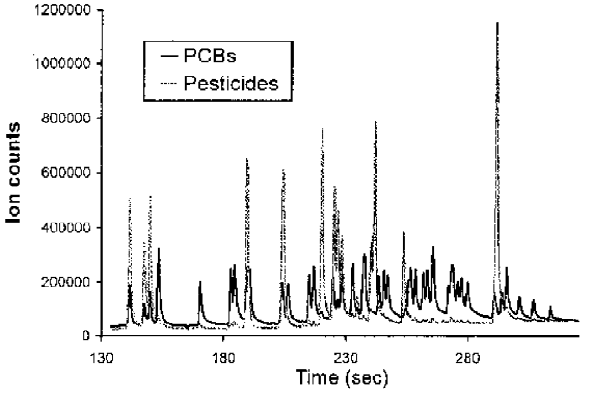

Fig. 7. Chromatogram (5.6 min) including the 38 PCBs and 13 persistent pesticides. Reprinted with permission from the authors [142].

needs input on the masses to be monitored, and thus this kind of quantitative analysis is restricted to compounds known to be present in the extracts. By using GC-ToF, new compounds can be identified by their mass spectra while doing quantitative analysis of known compounds at the same time.

7.3. PBDEs

Regarding PBDE determinations, several methods for qualitative and quantitative analysis have been developed involving GC-negative chemical ionisation (NCI)-MS and GC–EI-MS. Most of the analyses have concentrated on only a few specific major PBDE congeners. However, a reliable method for the separation and ultra-trace quantification of individual congeners is required to determine the extent of environmental exposure, the risk associated with specific congeners and their fate in the environment.

Until recently, quantitative work has been carried out using technical PBDE products, i.e. Bromkal 70-5DE (BK70), due to the lack of pure reference standards for most BDE congeners. The major three components in BK70 have been identified as BDE 47, BDE 99 and BDE 100 [143], and only these congeners could be quantified. Since more than 30 BDE congeners now are available, it has become possible to analyse for additional BDEs. Moreover, the availability of some ^{13}C-labeled standards allows the development of a methodology based on the quantification by the isotopic dilution method. Some authors, such as Ryan and Patry [144] and Lebeuf and Trottier [145] have used the ^{13}C-BDE surrogate standards. Previous reports had used other ^{13}C- or ^{12}C-labelled PCB and other organochlorine surrogates that, in general, give poorer precision and accuracy in the determination of analytes.

Eljarrat et al. [146] optimised the congener specific analysis of 40 different PBDEs by GC–MS. Two different MS approaches were used: the NCI-MS and the EI-MS. Operating parameters such as electron energy and source temperature were optimised in order to obtain the maximum sensitivity in the EI-MS study. For NCI-MS analyses, the effect of moderating gas (methane or ammonia), source temperature and system pressure were studied. The quality parameters of the two approaches tested were compared. The NCI-MS gave detection limits between 30 fg and 1.72 pg, whereas EI-MS gave detection limits between 0.53 and 32.09 pg. Thus, NCI gave an approximately 15 times higher response for PBDEs than EI. Comparison of detection limits for PBDEs clearly indicates that NCI offers better sensitivity than does EI.

The main advantage of EI-MS is that it provides better structural information. No structural information on the degree of bromination was obtained by NCI. The mass spectra of all PBDEs were dominated by the ion $[Br]^-$ and did not show any molecular ion. However, the EI provided better structural information, giving the molecular ions and the sequential losses of bromine atoms. As an example, Fig. 8 shows the co-elution of the pentaBDE≠126 with the hexaBDE≠155 observed in the NCI conditions. However, the EI-MS-SIM mode allowed the separation of these two compounds by monitoring selected ions of each bromination group. Moreover, the use of EI-MS allowed the use of an isotopic dilution method for quantification, making the analysis more reliable at trace levels.

8. Conclusions and future perspectives

Due to the diversity of chemical compounds that are responsible for endocrine disruption, tailor-made specific analytical protocols are required for their determination. As EDCs are chemically extremely heterogeneous and range from very polar and well soluble to very hydrophobic, the range of instrumental techniques is also very wide. However, within modern analytical techniques, only GC and LC combined with MS and tandem MS, respectively, provide sufficient selectivity and inherent sensitivity in analyzing EDCs in complex samples.

The analysis of eco-toxicologically-relevant concentrations (often low ppt levels) of EDCs in complex environmental matrices has been made possible mainly due to the availability of MS instrumentation. However, further improvements in chemical analysis to lower the limits of detection for some EDCs (e.g. steroid estrogens, particularly ethynylestradiol and other compounds from the same group) are needed. Since these compounds are affecting the aquatic organism at levels as low as 1 ng/l, the chemical

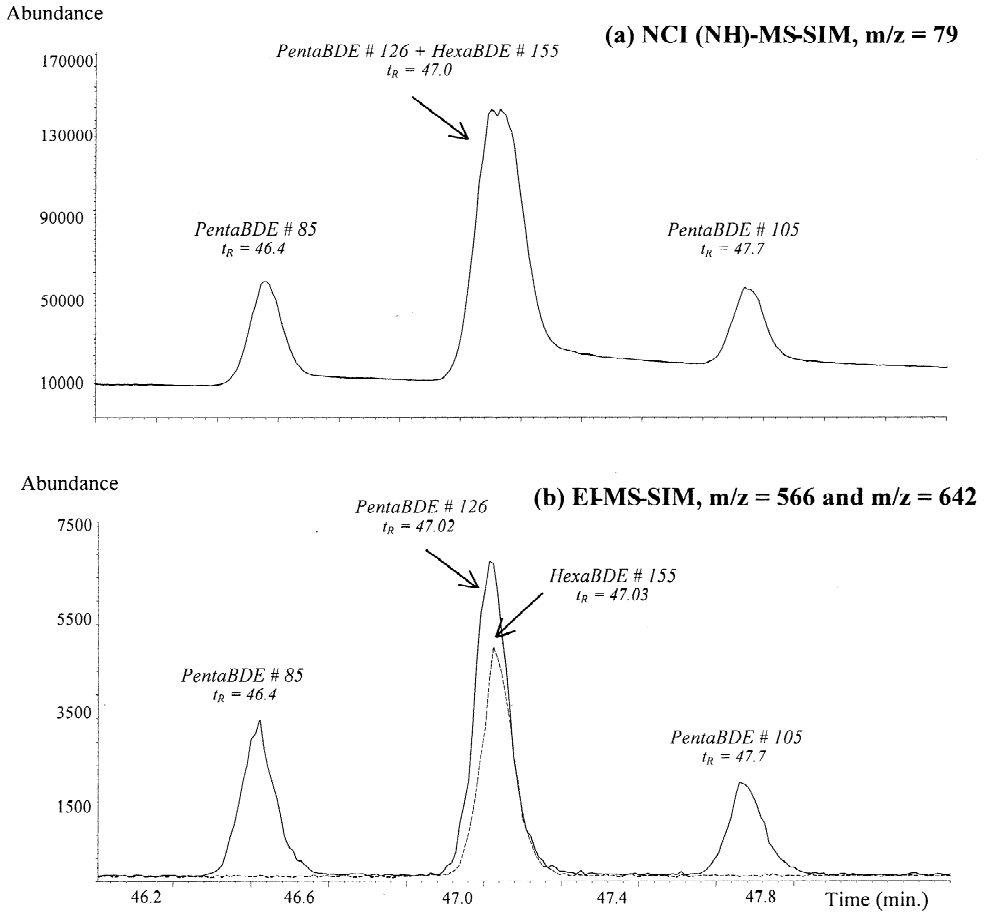

Fig. 8. PentaBDE≠126 and hexaBDE≠155 chromatogram in (a) NCI-MS-SIM and (b) EI-MS-SIM conditions. Reprinted with permission from [146] John Wiley & Sons Limited (Copyright 2000).

analysis of such analytes should be able to quantify these molecules at 0.1–1 ng/l, which is quite difficult at present.

Application of advanced sample preparation strategies in combination with tandem MS is expected to provide the means to unambiguously solve this problem. The application of advanced extraction techniques, such as PLE, subcritical hot water extraction, continuous-flow sonication, integrated in completely automated, on-line systems and the availability of more selective sorbents, such as MIPs (currently under development for alkylphenols and steroid sex hormones), and immunoaffinity cartridges might greatly improve the determination of trace levels of EDCs. Further development and application of dual-column systems for integrated purification of extracts and analysis will increase sample throughput and reduce operating costs and contamination risks.

With the recent advances in mass spectrometry, e.g. introduction of ToF-MS, a new powerful identification tool has become available, although environmental applications, especially for EDCs, are still scarce. The advantage of these instruments is that they can achieve a resolution, between 5 and 7000 and they can be used simultaneously for full scan and SIM determination. So they can be used for screening of environmental samples for EDCs thus permitting to achieve exact mass determination. It is expected that some of these instruments will replace GC–HRMS instruments due to easier operational

procedures, although at present their cost is too high compared to GC-ITD-MS.

Furthermore, the introduction of new GC techniques, such as fast GC, GC using narrowbore columns or GC×GC, improved the analysis of polychlorinated and polybrominated compounds, while the introduction of more widely applicable API interfaces allowed, by now routine use of LC–MS in the analysis of polar and semi-polar EDCs. The added power of MS–MS, applying a variety of scan functions and modes, improved analytical performances (reliability and sensitivity) and allowed a gradual shift from the detection of parent compounds to the analysis of metabolites and transformation products.

However, it is obvious that by using conventional LC–MS only one part of the analytes could be identified as compounds causing the endocrine disruption. The potential of tandem MS and especially combination of a quadrupole instrument and an orthogonal acceleration ToF-MS (Q-ToF) could enable accurate mass measurement and structural elucidation that might be used to identify unknown compounds responsible for observed estrogenicity.

In this respect, the availability of compound databases and mass spectra libraries is very relevant, and their lack often present an obstacle for efficient structural elucidation of unknown EDCs through effect-related detection. Another crucial problem is lack of standards, especially when dealing with metabolites and/or transformation products. Although LC–MS–MS or accurate measurement systems like LC-Q-TOF can be used for compound identification, we still need authentic standards for the final and definitive confirmation as well as for quantitation.

However, the main drawback of the conventional approach of the endocrine disrupter problem is target compound monitoring, which is often insufficient to assess the estrogenic effects that they may cause in living organisms. Effect-related analysis seems to be a more appropriate way to tackle the complex problem of endocrine disruption. Such an integrated approach, combining analytical chemistry and Toxicity Identification Evaluation (TIE) to identify the causes of the observed endocrine disrupting effects, is recommended for monitoring EDCs. In this line, Fig. 9 shows a strategy, based on advanced mass

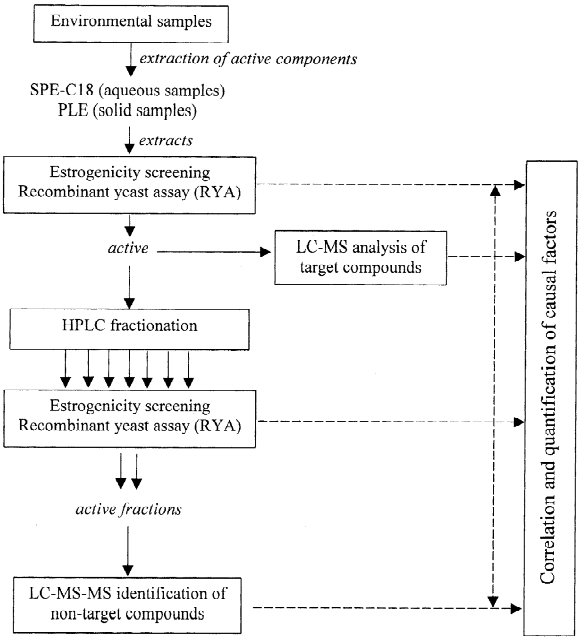

Fig. 9. Scheme used for the fractionation of environmental samples based on yeast assay in combination with TIE using LC–MS and LC–MS–MS.

spectrometric techniques and molecular biology, that should be considered. Among the different biological assays that are being considered for such purpose, in-vitro screens based on genetically engineered yeast in combination with TIE are one of the proposed approaches [147]. There are at present many different yeast assays being used and one of the problems to be resolved is the standardisation and inter-comparison of the different effects measured.

Finally, the complex issue of fate and behaviour of EDCs in the environment needs further study, especially in order to gain more insight into the factors that determine their bioavailability and release. Combined exposure to mixtures of EDCs (even at concentration levels lower than the no-observed effect level) that may produce additive effects still has to be assessed. Furthermore, improvements in analytical protocols will allow additional research, which is needed to evaluate the environmental presence and impact of some EDCs, especially in the field of flame retardants and steroid sex hormones where there is a lack of data.

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