Determination of endocrine disrupters in sewage treatment and receiving waters

Rachel L. Gomes, Mark D. Scrimshaw, John N. Lester

The presence of numerous endocrine-disrupting compounds (EDCs) in surface waters and sediment has been primarily attributed to their incomplete removal in the sewage-treatment process. Determination of these chemicals is required in order to assess their environmental impact. An overview is given on the biological and analytical methodologies used, both individually and in combination, to determine estrogenicity and quantification for several EDCs present in sewage and surface-water matrices.

Keywords: Endocrine-disrupting compounds; Extraction; Quantification; Surface water; Wastewater

Abbreviations: ACN, Acetonitrile; APCI, Atmospheric pressure chemical ionisation; APE, Alkylphenol ethoxylate; BEHP, Bis(2-ethyl-hexyl)phthalate; BPA, Bisphenol A; CID, Collision-induced dissociation; DAD, Diode array detection; E1, Estrone; E2, 17β-estradiol; E3, Estriol; EE2, 17α-ethinylestradiol; EC, Electrochemical; EDC, Endocrine-disrupting compound; EEq, Estradiol Equivalent; EI, Electron impact; ELISA, Enzyme-linked immunosorbent assay; ER, Estrogen receptor; ESI, Electrospray ionisation; GC, Gas chromatography; GC/MS(/MS), Gas chromatography/mass spectrometry (tandem); GPC, Gel permeation chromatography; HPLC, High-performance liquid chromatography; IC_{50}, Median immobilisation concentration; LC, Liquid chromatography; LC/MS(/MS), Liquid chromatography/mass spectrometry (tandem); LOD, Limit of detection; MS, Mass spectrometry; MeOH, Methanol; $MeCl_2$, Methylene chloride (dichloromethane); NCI, Negative chemical ionisation; NP, Nonylphenol; NPE, Nonylphenol ethoxylate; NP-HPLC, Normal phase-high performance liquid chromatography; NS, Not stated; oaTOF, Orthogonal accelerating time-of-flight; PBDE, Polybrominated diphenyl ether; PFB, Pentafluorobenzoyl; qTOF, Quadrupole time-of-flight; PCB, Polychlorinated biphenyl; RIA, Radio immunoassay; RP-HPLC, Reversed phase-high performance liquid chromatography; SFE, Supercritical fluid extraction; SPE, Solid phase extraction; TBT, Tributyltin; TIE, Toxicity identification and evaluation; TOF, Time-of-flight; UV, Ultraviolet; VTG, Vitellogenin; YES, Yeast estrogen screen

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1. Introduction

The endocrine-disrupting phenomena is a relatively new area of concern, first brought to light during the 1980s when deformities in fish were observed in certain stretches of UK rivers. The mode of action may be anti-estrogenic as expressed by PCBs, organotins and certain pesticides or estrogenic. Focus is predominantly on natural and anthropogenic compounds, which mimic E2 in producing an estrogenic or feminising effect [1]. Such an approach has been driven by observations in wildlife, and epidemiological studies have also found increased incidence of cancer and reproductive abnormalities (e.g. hypospadias and decreasing sperm counts) [2]. Whilst linking such effects in humans to the influence of EDCs is contentious, what is unequivocal are the feminising effects observed in biota in waters that receive sewage effluent [3]. Both industrial and domestic effluents have been identified as sources of EDCs entering the aquatic environment. Depending on the EDC, effects to biota have been observed down to 0.1 ng/L for EE2 [4].

EDCs embrace many chemicals that possess diverse characteristics. Determination of EDCs must therefore be carried out in many compartments of the treatment process and receiving waters. In the sewage-treatment process (Fig. 1), influent and effluent are the matrices most often investigated. When composite samples are obtained, the removal efficiency for the EDC of interest can then be calculated. The hydrophobic properties of many EDCs allow for partitioning to

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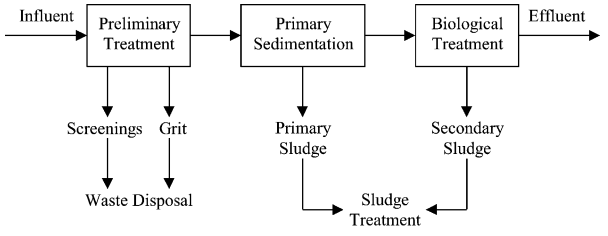

Figure 1. A conventional sewage-treatment process.

occur. The degree of sorption depends on suspended solid removal and particle characteristics (density, size, flocculate capacity). The biodegradation and sorption that many EDCs undergo necessitate sampling of solids during the treatment process, which can be:

1. biological (secondary) treatment, which is generally activated sludge or trickling filters; or,
2. primary and secondary sludges, which are generally treated by anaerobic digestion.

In surface waters, sampling has often been at the sewage outfall and upstream and downstream of the outfall. Such an approach allows determination of the range and the effect that EDCs have on the water and the biota within. The sediment phase also constitutes an important compartment and has been reflected in the sampling. Consequences of EDCs in the sediment phase are [5]:

1. prolonged persistence of the EDCs in the aquatic environment;
2. a fluvial sediment phase that can act as an effective transport system introducing contamination to previously unaffected areas;
3. effects on transformation processes, such as the degree of hydrolysis or the availability of an EDC for biodegradation; and,
4. the bioavailability of EDCs to benthic organisms and demersal fish resulting in endocrine-disrupting effects to the organism and bioaccumulation in the food chain

The choice of method for determination of EDCs depends on the required outcome. Biological methodologies ascertain the endocrine-disrupting activity exhibited by a chemical or sample, whilst chemical techniques identify known chemical(s) and quantify the concentration within that sample. Linking cause and effect — identifying the chemical(s) responsible for the estrogenic activity - relies on a combination of both approaches. On determining the endocrine-disrupting capability of a sample by biological methods, a measurement of that potency against a standard (commonly E2) and identification of the causative chemical(s) by analytical techniques is required before risk can be assessed.

2. Biological methodologies

Biological methods are primarily used to determine the endocrine-disrupting activity of a chemical or sample and the effects, if any, these may have on organisms. Methods include *in vitro* and *in vivo* assays and whole animal studies. Assessing whether a chemical is an EDC necessitates *in vitro* testing, which can have a variety of endpoints, such as estrogen-receptor binding or cell proliferation. To determine whether an organism has been exposed to EDCs (such as fish in rivers downstream of a sewage effluent outfall) can be achieved by examining at the biochemical or histological level.

2.1. In vitro *assays*

In vitro assays are rapid, cost effective tools requiring smaller concentration factors and can achieve lower detection limits than chemical analysis because of their specificity. They are able to determine the endocrine-disrupting activity of a chemical via a specific mode of action and can also investigate synergistic or additive effects [6,7]. Biological activity present in wastewater, receiving waters and the biota metabolism may transform EDCs, the degradative products or metabolites being more or less estrogenically active than the parent EDC. The incorporation of a metabolic transformation step in the assay can determine the endocrine-disrupting activity of the metabolite. Microsomal incubation has illustrated that metabolites of methoxychlor and PBDEs are more estrogenic than the parent, the inverse being true for the metabolites of E2, NP and BPA [8,9].

The general model for the mechanism of action is that EDCs act as ER ligand mimics, able to bind to the receptor. However, binding to the ER is not a measure of endocrine-disrupting activity alone. Potency also relies on binding affinity and maintaining receptor occupancy [10]. EDCs may also act through mechanisms independent of the ER. An overview of *in vitro* assays and associated limitations is given in [10], and they include:

1. inaccurate determination of endocrine-disrupting activity possessed by the test chemical;
2. poor correlation of endocrine-disrupting activity between *in vitro* assays; and,
3. matrix interferences.

In vitro assays can overestimate the relative activity of an EDC to reference chemical (usually E2). This may

be because of cross-reacting chemicals producing false positives caused by interactions from other chemicals. Such chemicals may be similar in structure, metabolites or co-extractives, and their presence can be minimised by sample preparation [11]. Correlation between assays can be poor with the relative potency of a test chemical varying depending on the *in vitro* assay utilised (Table 1). For example, the ER-CALUX screen is 20 times more sensitive to E2 than the recombinant yeast screen [8], which will affect the subsequent inter-assay ranking of chemicals.

Sample matrices have generally been river, ground and drinking water. When assessing more complex samples from biota (e.g. liver) or the environment (e.g. sewage effluent, sediment), sample preparation and extraction become important steps as other chemicals in samples may prove toxic to the assay cells [15]. The solvent used for extraction must be compatible with the cell system so that no effect is induced by the solvent alone and that the extracted material is distributed to the cells [16].

The limitations of *in vitro* screens necessitate confirmation by other *in vitro* or *in vivo* screens/whole animal studies (for an estrogenic response) or chemical methods (for quantification). *In vitro* assays can be used for quantification, generally as EEqs which express estrogenic activity of the sample in concentration units of E2. Definitive analysis using assays can also be determined with the aid of HPLC detection for retention time identification of the EDCs of interest (see Section 4.1). Despite disadvantages to *in vitro* assays, they remain the definitive method for screening chemicals for endocrine-disrupting activity [10].

2.2. In vivo *assays and whole animal studies*

In vivo screens utilise phenotypical changes, such as protein expression or effects on organ weight. If VTG induction/increase or histological responses, such as gonad abnormalities, are observed, ascertaining the chemical responsible then requires isolation and analytical identification. VTG is an egg-yolk precursor protein, normally identified in only fertile females, and induction in male fish is a recognised response to the presence of estrogenic chemicals [3]. VTG can be measured by several methods: RIA; ELISA; and, Western Blot. Numerous laboratory and field studies using VTG as a biomarker are tabulated in [17].

In vivo life-cycle studies are especially pertinent when determining the endocrine-disrupting effect on biota of wastewater entering surface waters or the presence of contaminated sediment [18]. These studies can directly assess the impact of environmentally relevant concentrations of EDCs on its surrounding environment utilising several endpoints (Table 2). In comparison, *in vitro* assays require extrapolation to the field situation and fail to account for habitat niche, diet, position in the food web and duration of exposure [3]. The sensitivity of some *in vitro* assays can also be significantly less

Table 1. Estrogenic potencies of selected EDCs citing the *in vitro* assay used [E1; E2; E3; EE2; BPA; NP; YES]

EDC	Class	Relative Estrogenic Potency (E2 = 1)	Assay	Reference
EE2	Synthetic steroid estrogen	0.5	YES	[12]
		0.91	E-screen	[13]
		1.25	MVLN cells	[14]
		1.25	E-screen	[14]
		5.71	HGELN cells	[14]
E1	Natural steroid estrogen	0.01	MVLN cells & E-screen	[14]
		0.056	HGELN cells	[14]
		0.096	E-screen	[13]
		0.3	YES	[12]
E3	Natural steroid estrogen	0.002	YES	[12]
		0.083	MVLN cells	[14]
		0.071	E-Screen	[14]
		0.4	HGELN cells	[14]
NP	Surfactant	0.0000125	MVLN cells	[14]
		0.000013	E-Screen	[14]
		0.000023	ER-CALUX	[8]
		0.00008	HGELN cells	[14]
		0.0001	E-screen	[13]
		0.001	YES	[12]
BPA	Organic oxygen compound	0.000025	MVLN cells	[14]
		0.000025	E-Screen	[14]
		0.000053	E-screen	[13]
		0.00006	YES	[12]
		0.00006	YES	[12]
		0.00019	HGELN cells	[14]

Table 2. Examples of *in vivo* life-cycle studies with different endpoints [E2; NP; TBT; VTG]

Endpoint	Sample	Organism	Observation	Reference
Reproductive	Sewage effluent	Trout, flounder, roach	VTG induction	[18,20,21]
	Pulp-mill effluent	Whitesucker	Intersex	[22]
Developmental	NP	Barnacle	Increased size of gonads	[23]
	TBT	Dogwhelk	Retarded/disrupted larval development	[24]
Behavioural	E2	Japanese medaka	Decreased fecundity	[25]

than *in vivo* assays, and that may lead to false negatives *in vitro* [19]. Depending on the experimental set up, the detection of estrogenicity during the life cycle and the bioavailability of that EDC(s) to the test subject can be investigated *in vivo*.

Upon determining the endocrine-disrupting activity of a chemical or sample by *in vitro*, *in vivo* or in combination, chemical analysis becomes the method of choice for detecting presence and concentration in the environment.

3. Chromatographic methodologies

Whereas the biological approach identifies the estrogenic potency of a compound or mixture, chemical techniques identify the chemical of interest and quantify the concentration. In order to preserve the integrity of the sample and EDCs within, sampling and handling are important considerations. The complexities of the matrices, especially with respect to sewage or sediment, often require extensive extraction and clean-up procedures before the sample is fit for determination. Extraction methods and analysis for numerous EDCs pertaining to sewage and surface-water samples have been tabulated [26,27]. Summarised below are the extraction and analytical approaches for two groups of EDCs with very different characteristics and method requirements. The natural and synthetic steroid estrogens were identified during the late 1990s as the main contributors to the endocrine-disruption phenomena [28]. Comparatively, PBDEs are now emerging as EDCs [9,29].

3.1. Sample collection and handling

For steroid estrogens, determination is at the trace level (ng/L) and requires concentration of the aqueous sample to reach these levels. Depending on the sensitivity and the selectivity of the chemical technique, this can necessitate large volume collection of typically 1 L and up to 20 L [30]. Upon aqueous sample collection, preservatives, such as formaldehyde (1% v/v) [31] and MeOH [28], may be added to halt microbial activity whilst solid samples are refrigerated or stored at −18°C [32]. The hydrophobic nature of many EDCs ensures that their presence will be concentrated in the solid phase, thus requiring smaller sample volume. Typical concentrations for PBDEs range from several thousand to below 500 ng/g for sediment and sewage sludge, respectively, and sample sizes in the range 0.5–50 g [33]. Comparatively, E1 and EE2 have been identified in sediment at levels below 23 ng/g with sample collection of 5–30 g [34,35]. Solid samples, such as sediment or sludge, are usually dried and homogenised to ensure a representative sample [33] or undergo lyophilisation prior to extraction [36]. When determining historical profiles from sediment core, sampling must ensure that sediment at different depths is not intermixed.

3.2. Sample preparation

Upon collection, aqueous samples are usually filtered to remove particles that may interfere with the extraction procedure. Washing of particles with a solvent, such as MeOH, may be used to extract any EDCs associated with the particles. Extraction is generally carried out within 48 hours of collection and may even be carried out on site, negating the need for preservatives and ensuring that EDCs within are not adversely affected by storage conditions. The extent of manipulation to isolate the analyte(s) of interest from their matrix depends on:

1. contamination of the sample with co-extractives;
2. quantity of analyte present in the sample; and,
3. the analytical tool to be utilised.

3.2.1. Extraction. SPE is the main method used for steroid extraction from aqueous samples, whilst solid samples have been freeze dried prior to ultrasonic extraction (Fig. 2). SPE utilises either disks or, more commonly, cartridges.

Disks reduce sample clogging and have a large surface area for sample contact compared to cartridges. However, with disks, larger volumes of solvents are required for analyte elution increasing the overall method duration whilst the eluted sample is blown down. C18 bonded silica is the most widely used SPE adsorbent, and graphitised carbon and SDB have also been utilised [37]. Solvent choice for elution of retained analytes depends on the SPE adsorbent and whether disk or cartridge. Numerous solvents have been utilised, including MeOH, acetone and ethyl acetate, individually, in combination or as sequential steps. Steroid estrogen extraction from solids has used MeOH and acetone extraction, either in combination or sequentially [36,38].

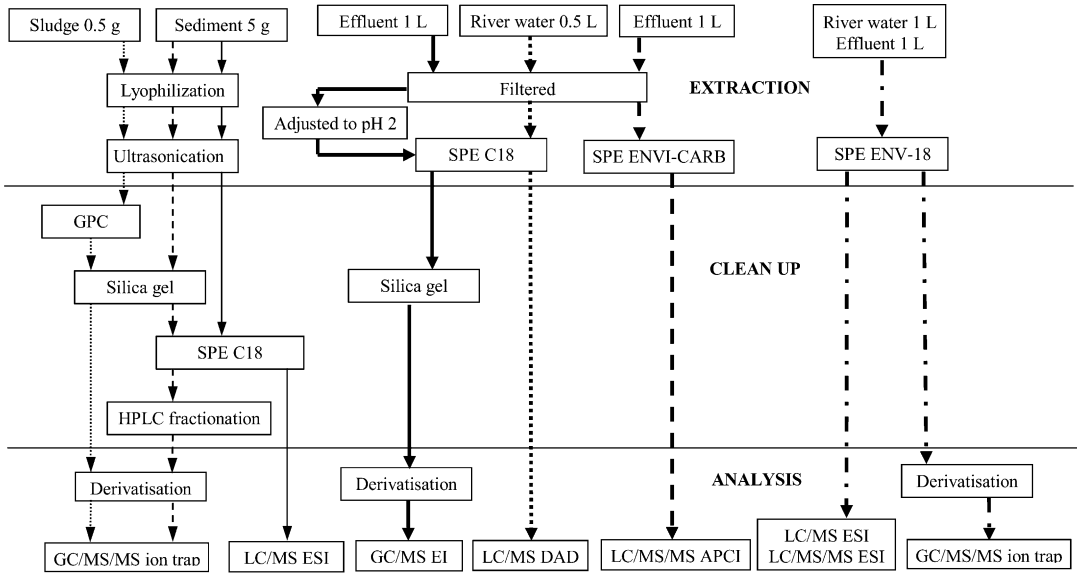

Figure 2. Several methodologies for steroid estrogens in solid and aqueous environmental samples [APCI; DAD; EI; ESI; GC/MS; GPC; LC/MS; SPE]. ------→ [38]; ——→ [36]; ·····→ [38]; ——→ [39]; ·····→ [37]; – – → [40]; · – · → [41].

The similarities between PBDEs and PCBs mean that PCB methods are also applicable to PBDE extraction and analysis. The hydrophobic character and low solubility of PBDEs dictate that they will predominantly be present in the solid phase. Using dried sediment or sewage sludge samples, Soxhlet is the preferred extraction technique with duration periods of 4–24 h [26]. Solvents utilised for extraction include acetone and hexane, individually or in combination [42,43], hexane and diethyl ether [44] and toluene [45]. Compared to a 20-h Soxhlet extraction, cleaner extracts were produced by SFE with solid phase trapping negating the need for clean-up prior to determination by GC [46]. Solid phase trapping acted as a second extraction procedure after SFE, increasing selectivity.

3.2.2. Clean-up. Sample clean-up removes further co-extractives utilising mainly adsorption chromatography or SPE [27]. For aqueous samples, clean-up is not necessarily required. However, for steroid estrogens in sediment and sludge samples, clean-up is a requirement and may be a multi-step procedure. Following MeOH/acetone extraction, clean-up of steroid estrogens in sewage sludge was by GPC followed by silica gel [38].

For biota samples, clean-up prior to PBDE determination has involved acidified silica gel after GPC [47]. Intensive clean-up by HPLC followed by elution over silica gel and sulphuric acid treatment has been utilised [43]. Sulphuric acid is used for lipid removal although it may adversely effect the analyte. GPC, alumina and florisil columns are gentler ways to remove lipids. Sulphur present in sludge and sediment samples can interfere with GC determination. Removal of sulphur interferences can be achieved with the addition of copper either prior to extraction or during the clean-up stage [48].

3.3. Quantification

Identifying the EDC of interest relies on an extraction procedure and an analytical method, which are selective for that compound. To allow investigation into the study of compounds and their interaction with their environment, column chromatography is able to achieve the objectives of separation, identification and quantification.

3.3.1. GC. PBDE determination by GC is the preferred analytical technique, according to [49], having utilised GC with EC detection and GC/MS with NCI or EI detection. For PBDEs, lower LODs are achievable using NCI whereas EI provides superior structural information and selectivity. No structural information on the degree of bromination is determined by NCI and source temperature impacts on the degree of fragmentation in the NCI mode, lower temperatures generating greater fragmentation [47,48]. Using EI detection, the dominant peak varies, depending on the congener, $[M]^+$ for mono- to tetra- congeners and $[M\text{-}Br_2]^+$ for penta- to hepta-congeners [48,50,51]. For biota samples, selectivity was improved using EI detection when compared to EC detection [52]. PBDE concentrations in sewage sludge, sediment and fish have been determined by EC negative ionisation MS [53,54]. Determination of the higher molecular mass PBDE congeners is limited because of the instability of deca-PBDE at high temperatures, which are necessary for GC determination [55]. Hence, shorter columns (typically 10–15 m), as

opposed to the regular 25–30 m, are needed to compensate for the large molecular mass and heat sensitivity of deca-PBDE [50,53,54].

A prerequisite for analysis by GC is that the chemical of interest is volatile and thermally stable. When this is not the case, derivatisation can be used to overcome this limitation [56]. Traditionally, GC necessitating the use of derivatives has determined steroid estrogens. Disadvantages to derivatisation are that it is labour intensive and can reduce analyte recovery. Several derivatives under varying conditions (solvents, bases, reagent gases) were investigated for trace detection of a range of EDCs, including steroid estrogens. Some of these derivatives led to either decomposition of E3 and/or partial to complete conversion from EE2 to E1 [57].

GC/MS NCI, a method that is selective because only electron-capturing species produce a signal, has used PFB derivatives to determine steroid estrogens. For natural steroid estrogens, complete derivatisation of all -OH groups was achieved. However, because of the steric hindrance of EE2, only mono derivatives may be formed [58].

Apart from making chemicals amenable to GC/MS analysis, derivatisation can increase sensitivity using LC/MS. LC/MS APCI has also used PFB derivatives to determine steroid estrogens in the negative mode, producing an intense [M-PFB$^-$] ion with on-column detection limits of 0.2 pg for E1 [59].

3.3.2. LC. Analysis of environmental samples utilising LC/MS has increased whilst GC/MS and LC with UV or fluorescence detection have declined [60]. Comparison of sensitivity between different analytical techniques was in the order LC/MS/MS > GC/MS/MS > LC/MS [41]. The time-consuming derivatisation step and the possible loss of analyte has meant that LC techniques are preferred for determining steroid estrogens. GC/MS(/MS) requires derivatisation of polar compounds so they become amenable to analysis. LC/MS(/MS) negates such an approach and allows detection of the analyte directly rather than detection of a modified analogue. UV, EC and fluorescence detection relies on retention time of the compound for comparative work, whereas MS provides both structural and quantitative analysis and increased sensitivity. By contrast to GC/MS, ionisation of the analyte occurs prior to entering the MS with the two main sources being ESI and APCI. ESI provides superior detection limits compared to APCI and, for maximum sensitivity, is carried out in the negative mode [M-H]$^-$ [37]. For complex matrices, such as sewage samples, ionisation suppression of the analyte can occur when analysing by ESI.

Environmental analysis by GC/MS necessitates hydrolysis of conjugates to the free steroid followed by derivatisation, compounding errors from the limited efficiency of the hydrolysis step and the recoveries of the extraction and quantification stages [61]. Direct determination of the analyte of interest is especially pertinent when dealing with conjugated steroid estrogens and allows assessment of fate and determination according to conjugate moiety. Environmental analysis of steroid conjugates is beginning to follow the biological approach utilising LC/MS to measure these precursor EDCs directly [62,63].

4. Combination of techniques

Linking biological and chemical methodologies allows for the endocrine-disrupting effect (e.g. estrogenicity) produced by a compound(s) in a sample and structural elucidation of the compound(s) and concentration present in the sample. Such an approach, termed TIE, has been applied to several environmental matrices (Table 3). The general scheme involves fractionation of the sample, bioassay determination of the estrogenic fractions and then analysis by GC/MS, LC/MS or tandem MS to elucidate chemical structure and concentration responsible for the estrogenicity (Fig. 3).

Extraction and fractionating conditions vary between studies; hence, estrogenic activity has been associated with different polar fractions (Table 3). To compare inter-study TIE, a full methodology is required and standardisation of this multidisciplinary approach is needed [26]. Because of some of the problems encountered with *in vitro* methods, confirmation of endocrine activity using *in vivo* studies may be sought [64]. When determining the chemicals responsible for the estrogenicity, GC/MS or tandem MS has been the traditional approach [28]. However, care must be taken to ensure that all chemicals present in the active fractions are amenable to GC analysis, so derivatisation is a prerequisite [27].

The use of LC/MS extends the polarity range at the identification stage [65]. However, compared to GC/MS, the unavailability of spectral libraries for LC/MS means identification of previously unknown compounds is more difficult. Ion-trap and quadrupole LC/MS provide similar resolution, which is generally insufficient to elucidate molecular structure. However, LC/MS/MS is able to generate further data from the daughter ions after CID. Better suited to the TIE procedure is the use of oaTOF-MS, which is able to determine formula with a high sensitivity [65]. Selectivity can be further increased with the use qTOF, which allows tandem MS to be performed [60,66] and has been utilised for structural elucidation of unknown compounds in a genotoxic fraction of industrial wastewater [67].

Combination of biological and chemical techniques can also be used to increase the sensitivity and the selectivity in the quantitation step. Interferences from a

Table 3. Several methodologies using TIE [APEs/NPEs; ACN; BEHP; EI; IC_{50}; $MeCl_2$; MeOH; NP; NP-HPLC; NS; RP-HPLC; SPE]

Sample	Extraction	Fractionation	Estrogenic Identification	Quantification	Fraction estrogenic	Chemicals Responsible	Reference
Sewage effluent 20 L	5g + 2g C18 SPE in series MeOH/H_2O mix 0–100% MeOH Then low to non-polar solvent elution	HPLC UV 210nm Spherisorb ODS2 C18 250 x 10 mm x 5µm MeOH/H_2O gradient Fine fractionation with RP-HPLC using shallower gradient	Yeast-based screen with ERE	GC/MS Ion-trap EI mode LOD: 0.2 ng/L	Mid-polar	E2 and E2 identified up to 50 ng/L and 80 ng/L, respectively	[28]
Sewage effluent 5 L Surface water 5 L	90mm SDB SPE Empore disks 15 mL acetone, 25 mL $MeCl_2$, 10 mL hexane sequential elution	NP-HPLC: Phenomenex Luna silica 250 x 4.6mm x 5µm. Three-step isocratic: $MeCl_2$:hexane (30:70), $MeCl_2$, MeOH Three fractions of polarity obtained: F1 non-polar to F3 polar	RIA (E2 & EE2) LODs: E2 107 pg/L, EE2 53 pg/L	RP-HPLC ODS 250 x 4.6 mm x 5µm. Fluorescence 229 nm excitation 310 nm emission ACN/H_2O gradient	Mid-polar to polar	E2 & EE2 only tested with RIA. Cross reactivity to compounds structurally similar to E2 (E1 11.2%)	[68]
Landfill leachate volume NS Textile effluent volume NS	2 x SPE LiChrolut EN 3 mL SPE 1: 5 mL ethyl acetate elution (GC/MS and assay) SPE 2: 5 mL MeOH (HPLC fractionation)	RP-HPLC LiChrosphere C18 244 x 10 mm x 10 µm UV 220 nm and 245 nm MeOH/H_2O gradient F1-F5, increasing hydrophobicity	Ephippia (dormant eggs) of *D. magna* to induce hatching 24 h and 48 h IC_{50}	GC/MS GC-ECD	Fraction 5 (of F1-5) equates to log K_{ow} 2.81–5.16	NP, BEHP, dibutyl and diethyl phthalates	[69]
Sewage effluent 0.3–2 L Surface water 2–6 L	C18 SPE discs 47mm 15–30 mL MeOH elution	HPLC 250mm 5µm Alltima C18 UV 220 nm and 280 nm MeOH/H_2O or ACN/H_2O isocratic	ELISA polyclonal	GC/MS/MS Ion-trap EI mode LOD: 0.1 ng/L LOD: 0.05ng/L	NS	E2 and EE2 (EE2 quantified by ELISA only)	[61]
Surface water 20 L	3 SPE in series: C8 5g, Isolute ENV+ 1g, ENVI-Carb 10 mL MeOH elution	RP-HPLC Econo-Prep C18 300 x 10 mm x 5 µm Guard column 50 x 10mm x 5 µm	YES with hER-α Detects all known estrogens and xenoestrogens	GC/MS Ion-trap LOD: 15 ng EEq/L	non to mid polar	Majority E2, also NP, BEHP and androsterone up to 24 ng EEq/L	[70]
Sediment 5 L (to obtain pore water)	Centrifuge then decant to obtain pore water 3 SPE in series: C8 500 mg, ENV+ 100 mg, non-porous carbon 500 mg 10 mL MeOH elution	UV 210 nm MeOH/H_2O gradient 30 x 5 mL fractions at 1 min intervals				Could not be identified up to 4.7 µg EEq/kg wet weight	

sample of sewage effluent were removed using an immunosorbent technique to limit the ionisation suppression that can occur with the analysis of complex matrices by LC/MS, particularly in ESI mode [71]. Monoclonal antibodies to E1 and E2 were utilised to produce an immunosorbent, which acted in place of traditional SPE to concentrate and to extract steroid estrogens. The use of monoclonal antibodies, rather than polyclonal antibodies, allowed for greater selectivity and reproducibility. Using 1-L samples of effluent, after concentration the limit of quantitation was in the sub ng/L range. Using the immunosorbent for extraction, quantification of an effluent sample spiked to 10ng/L for E1 and E2 and the internal standard at 50ng/mL was achieved. Comparatively, without the use of the immunosorbent, none of the spiked chemicals was identified. The high selectivity of the immunosorbent was exhibited for EE2, giving recoveries of only 2%. Other concepts for combining biological and chemical techniques include the following [72].

1. Receptor affinity chromatography followed by LC/MS/MS — Similar concept to the above, utilising ERs fixed on a receptor affinity column in place of SPE. Passing through a sample, then washing and elution would result in receptor-relevant chemicals, which can then be identified and quantified by LC/MS/MS, so is consistent with previous numbered bullet points.
2. Homogeneous receptor LC/MS/MS — Using homogeneous binding assays with separation and detection of free ligands and ligand-receptor complexes by LC/MS. EEqs can be determined by the ratio of the bound to the free fraction assayed by MS and structural elucidation of the bound chemicals.

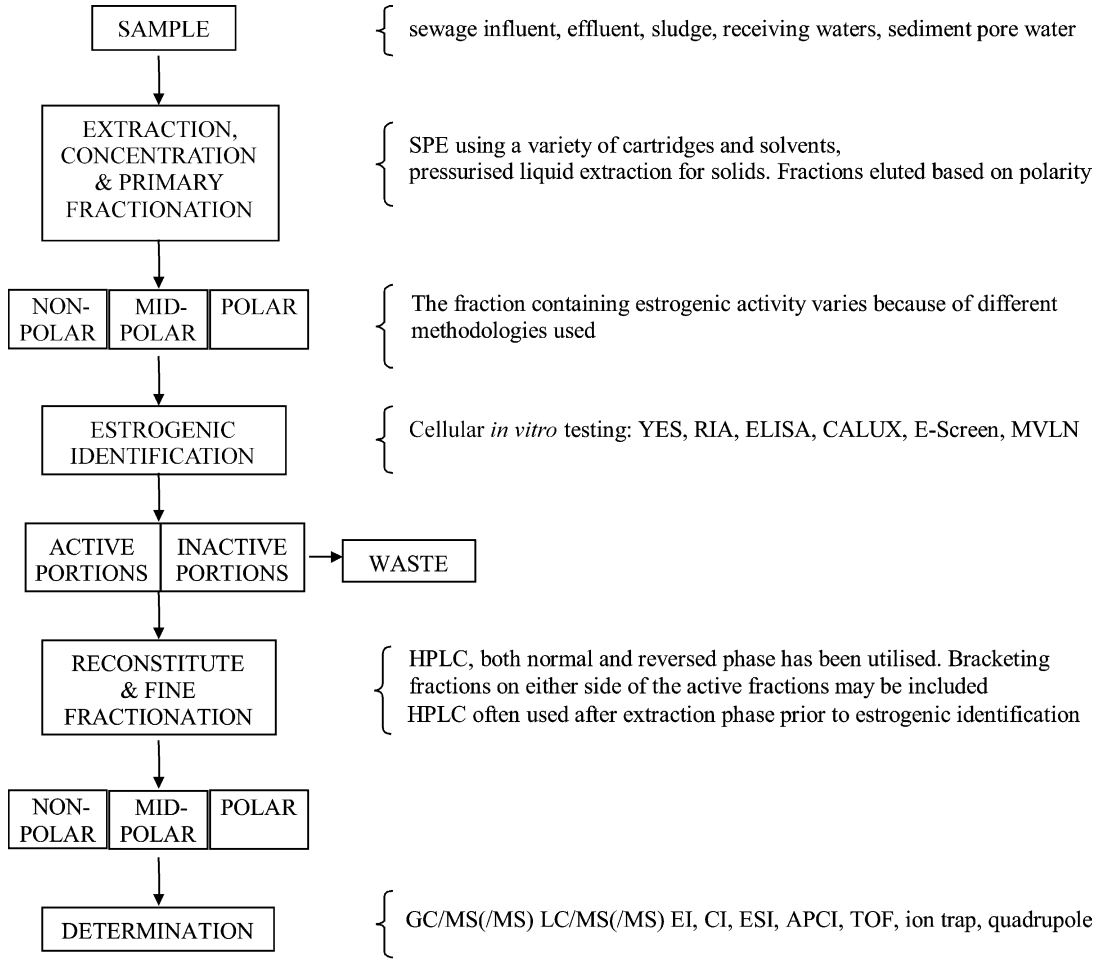

Figure 3. TIE approach to determine compounds responsible for estrogenic effects. [APCI; CI; EI; ESI; ELISA; GC/MS(/MS); HPLC; LC/MS(MS); RIA; SPE; TOF; YES].

4.1. Comparison of biological and chemical approaches

Biological and chemical techniques possess advantages and disadvantages specific to each approach (Fig. 4). When bioassay and chemical analysis on the same sample have separately determined quantification of EDC concentration, then a comparison between the two can be assessed. After SPE, HPLC fractionation was utilised for clean-up and concentration of sewage effluent and surface waters, prior to estrogenicity identification by ELISA. Cross reactivity for other steroid estrogens using the ELISA containing polyclonal antibodies was reported by the manufacturers to be less than 1% [61]. Retention times for E2 and EE2 standards were determined by UV detection with a sample analysed after each standard to confirm the fraction of interest according to the retention time. Quantification by ELISA was determined using a calibration of six standards relating the absorbance signal to estrogen concentration. Confirmation analysis was carried out by GC/MS/MS, which gave higher levels for E2 (GC/MS/MS 0.38 ± 0.21 ng/L; ELISA 0.08 ± 0.02 ng/L). The reason for the disparity was attributed to possible problems with GC/M/MS quantification at concentrations close to the method LOD. This is likely as other samples gave similar results for E2 when quantified by ELISA and GC/MS/MS methods (3.68 ± 1.27 ng/L and 3.90 ± 1.40 ng/L, respectively). Comparing *in vivo* applications and chemical analysis has demonstrated [34] a correlation between VTG induction in feral male carp and alkylphenol concentrations in surface water and sediment (r=0.83-0.84) and VTG induction and E3 and E1 concentrations in surface water (r=0.78 and r=0.94).

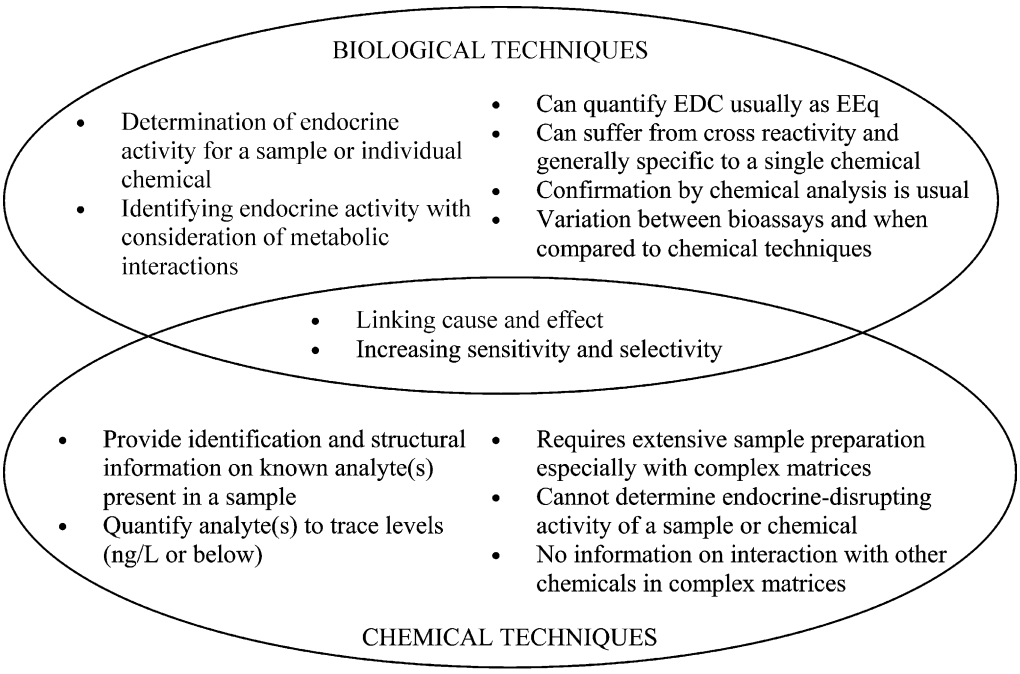

Figure 4. Overview of several advantages and disadvantages to EDC determination using chemical and biological methodologies [EDC; EEq].

5. Summary

EDCs and their interaction and impact on the surrounding environment are a growing topic of concern for both the environment and public health sectors. Numerous chemicals, some of which have been in use for some time, have emerged or are now being identified as EDCs. Determining their presence in the environment, behaviour and exposure routes are prerequisites to risk assessment. The choice of method for determining EDCs depends on what requires elucidation, as follows.

1. the endocrine-disrupting activity of a sample or a particular chemical;
2. identification of a chemical causing the activity in a sample; or,
3. quantification levels of known EDC(s) in the sample

In vitro assays are used as a preliminary screening tool to determine endocrine-disrupting activity and require verification *in vivo*. Similarly, quantification by biological techniques should be validated by chemical analysis, just as a combination of both techniques first linked endocrine-disrupting activity to certain chemicals in sewage wastewater. In order to identify emerging EDCs and ascertain environmental risk, future applications may increasingly combine biological methods to provide initial screening of large numbers of samples followed by the more complex, quantitative chemical approach [27].

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References

[1] T.-J.S. Kledal, M. Jorgensen, F. Mengarda, N.E. Skakkebeak, H. Leffers, Andrologia 32 (2000) 271.
[2] J.W. Birkett, in: J.W. Birkett, J.N. Lester (Editors), Endocrine disrupters in wastewater and sludge treatment processes, CRC Press, Boca Raton, Florida, USA, 2003, p. 1.
[3] C.R. Tyler, S.R. Jobling, J.P. Sumpter, Crit. Rev. Toxicol. 28 (1998) 319.
[4] C.E. Purdom, P.A. Hardiman, V.J. Bye, N.C. Eno, C.R. Tyler, J.P. Sumpter, Chem. Ecol. 8 (1994) 275.
[5] R.L. Gomes, J.N. Lester, in: J.W. Birkett, J.N. Lester (Editors), Endocrine disrupters in wastewater and sludge treatment processes, CRC Press, Boca Raton, Florida, USA, 2003, p. 219.
[6] W. Körner, V. Hanf, W. Schuller, C. Kempter, J. Metzger, H. Hagenmaier, Sci. Total Environ. 225 (1999) 33.
[7] E. Silva, N. Rajapakse, A. Kortenkamp, Environ. Sci. Technol. 36 (2002) 1751.
[8] J. Legler, M. Dennekamp, A.D. Vethaak, A. Brouwar, J.H. Koeman, B. van der Burg, A.J. Murk, Sci. Total Environ. 293 (2002) 69.
[9] I.A.T.M. Meerts, J.J. van Zanden, E.A.C. Luijks, I. van Leeuwen-Bol, G. Marsh, E. Jakobsson, A. Bergman, A. Brouwer, Toxicol. Sci. 56 (2000) 95.
[10] T. Zacharewski, Environ. Sci. Technol. 31 (1997) 613.
[11] J. Gascon, A. Oubina, D. Barcelo, Trends Anal. Chem. 16 (1997) 554.

[12] H. Tanaka, Y. Yakou, A. Takahashi, T. Higashitami, K. Komori, Water Sci. Technol. 43 (2001) 125.
[13] W. Korner, P. Spengler, U. Bolz, W. Schuller, V. Hanf, J.W. Metzger, Environ. Chem. Toxicol. 20 (2001) 2142.
[14] B. Gutendorf, J. Westendorf, Toxicol. 166 (2001) 79.
[15] S.A. Snyder, D.L. Villeneuve, E.M. Snyder, J.P. Giesy, Environ. Sci. Technol. 5 (2001) 3620.
[16] J.P. Giesy, K. Hilscherova, D. Jones, K. Kannan, M. Machala, Marine Poll. Bull. 45 (2002) 3.
[17] P.D. Jones, W.M. De Coen, T.L. Keith, J.P. Giesy, Water Sci. Technol. 42 (2000) 1.
[18] S. Jobling, M. Nolan, C.R. Tyler, G. Brighty, J.P. Sumpter, Environ. Sci. Technol. 32 (1998) 2498.
[19] L.C. Folmar, M.J. Hemmer, N.D. Denslow, K. Kroll, A. Chen, H. Richmn, H. Meredith, E.G. Grau, Aquatic Toxicol. 60 (2002) 101.
[20] J.E. Harries, D.A. Sheahan, S. Jobling, P. Matthiesson, P. Neall, J.P. Sumpter, T. Tylor, N. Zaman, Environ. Toxicol. Chem. 16 (1997) 534.
[21] C.M. Lye, C.L. Frid, C.E. Gilli, D. McCormick, Marine Poll. Bull. 34 (1997) 34.
[22] K.R. Munkittrick, G.J. Vanderkraak, M.E. McMaster, C.B. Portt, M.R. Vandenheucel, M.R. Servos, Environ. Toxicol. Chem. 13 (1994) 1089.
[23] Z. Billinghurst, A.S. Clare, M.H. Depledge, J. Exp. Marine Biol. Ecology 257 (2001) 255.
[24] J. Oehlmann, U. Schulte-Oehlmann, E. Stroben, B. Bauer, C. Bettin, P. Fiorni, Endocrinically Active Chemicals in the Environment, Umwelt Bundes Amt, Berlin, Germany, 1995, p. 111.
[25] A.C. Nimrod, W.H. Bensen, Aquatic Toxicol. 44 (1998) 141.
[26] M. Petrovic, E. Eljarrat, M.J. Lopez de Alda, D. Barcelo, J. Chromatogr. A 974 (2002) 23.
[27] N. Voulvoulis, M.D. Scrimshaw, in: J.W. Birkett, J.N. Lester (Editors), Endocrine disrupters in wastewater and sludge treatment processes, CRC Press, Boca Raton, Florida, USA, 2003, p. 59.
[28] C. Desbrow, E.J. Routledge, G.C. Brighty, J.P. Sumpter, M. Waldock, Environ. Sci. Technol. 32 (1998) 1549.
[29] I.A.T.M. Meerts, R.J. Letcher, S. Hoving, G. Marsh, A. Bergman, J.G. Lemmen, B. van der Burg, A. Brouwer, Environ. Health Persp. 109 (2001) 399.
[30] A.C. Belfroid, A. Van der Horst, A.D. Vethaak, A.J. Schäfer, G.B.J. Rijs, J. Wegener, W.P. Cofino, Sci. Total Environ. 225 (1999) 101.
[31] C. Baronti, R. Curini, G. D'Ascenzo, A. Di Corcia, A. Gentili, R. Samperi, Environ. Sci. Technol. 34 (2000) 5059.
[32] R. Jeannot, H. Sabik, S. Sauvard, T. Dagnac, K. Dohrendorf, J. Chromatogr. A 974 (2002) 143.
[33] T. Hyotyläinen, K. Hartonen, Trends Anal. Chem. 21 (2002) 13.
[34] M. Petrovic, M. Sole, M.J. Lopez de Alda, D. Barcelo, Environ. Toxicol. Chem. 21 (2002) 2146.
[35] R.J. Williams, A.C. Johnson, J.J.L. Smith, R. Kanda, Environ. Sci. Technol. [in Press (2003)].
[36] M. Petrovic, E. Elijarrat, M.J. Lopez de Alda, D. Barcelo, Trends Anal. Chem. 20 (2001) 637.
[37] M.J. Lopez de Alda, D. Barcelo, J. Chromatogr. A 892 (2000) 391.
[38] T. Ternes, H. Andersen, D. Gilberg, M. Bonerz, Anal. Chem. 74 (2002) 3498.
[39] P. Spengler, W. Korner, J.W. Metzger, Environ. Chem. Toxicol. 20 (2001) 2133.
[40] A. Lagana, A. Bacaloni, G. Fago, A. Marino, Rapid Commun. Mass Spectrom. 14 (2000) 401.
[41] T.R. Croley, R.J. Hughes, B.G. Koenig, C.D. Metcalfe, R.E. March, Rapid Commun. Mass Spectrom. 14 (2000) 1087.
[42] C.R. Allchin, R.J. Law, S. Morris, Environ. Poll. 105 (1999) 197.
[43] J. de Boer, A. van der Horst, P.G. Wester, Organohalogen Compd. 47 (2000) 85.
[44] K. Nylund, L. Asplund, B. Jansson, P. Jonsson, K. Litzen, U. Sellstrom, Chemosphere 24 (1992) 1721.
[45] H. Hagenmaier, J. She, T. Benz, N. Dawidowsky, L. Dusterhoft, C. Lindig, Chemosphere 25 (1992) 1457.
[46] K. Hartonen, S. Bøwadt, S.B. Hawthorne, M.L. Riekkola, J. Chromatogr. A 774 (1997) 229.
[47] K. Akutsu, H. Obana, M. Okihashi, M. Kitagawa, H. Nakazawa, Y. Matsuki, T. Makino, H. Oda, S. Hori, Chemosphere 44 (2001) 6.
[48] E. Eljarrat, S. Lacorte, D. Barcelo, J. Mass. Spectrom. 37 (2002) 76.
[49] S.D. Richardson, Anal. Chem. 74 (2002) 2719.
[50] S. Huber, K. Ballschmiter, Fresenius' J. Anal. Chem. 371 (2001) 882.
[51] J.K. Huwe, M. Lorentzsen, K. Thuresson, A. Bergman, Chemosphere 46 (2002) 635.
[52] M. Alaee, D.B. Sergeant, M.G. Ikonomou, J.M. Luross, Chemosphere 44 (2001) 1489.
[53] K. Öberg, K. Warman, T. Öberg, Chemosphere 48 (2002) 805.
[54] U. Sellstrom, A. Kierkegaard, C. De Wit, B. Jansson, Environ. Toxicol. Chem. 17 (1998) 1065.
[55] J. de Boer, H.J. de Geus, U.A.Th. Brinkman, Organohalogen Compd. 45 (2000) 1.
[56] K. Blau, J.M. Halket (Editors), Handbook for Derivatives for Chromatography, John Wiley & Sons Ltd, Chichester, UK, 1993, p. 369.
[57] O. Lerch, P. Zinn, J. Chromatogr. A 991 (2003) 77.
[58] X.U. Xiao, D.V. McCalley, J. McEvoy, J. Chromatogr. A 923 (2001) 195.
[59] G. Singh, A. Gutierrez, K. Xu, I.A. Blair, Anal. Chem. 72 (2000) 3007.
[60] T. Reemtsma, J. Chromatogr. A 1000 (2003) 477.
[61] C.-H. Huang, D. Sedlak, Environ. Toxicol. Chem. 20 (2001) 133.
[62] A. Gentilli, D. Perret, S. Marchese, R. Mastropasque, R. Curini, A. Di Corcia, Chromatographia 56 (2002) 25.
[63] L.D. Bowers, Sanaullah, J. Chromatogr. B 687 (1996) 61.
[64] E.J. Routledge, D. Sheahan, C. Desbrow, G.C. Brighty, M. Waldock, J.P. Sumpter, Environ. Sci. Technol. 32 (1998) 1559.
[65] T. Reemtsma, Anal. Chim. Acta 426 (2001) 279.
[66] M.J. Lopez de Alda, S. Diaz-Cruz, M. Petrovic, D. Barcelo, J. Chromatogr. A 1000 (2003) 503.
[67] I. Bodeldijk, P.G.M. Stoks, J.P.C. Vissersc, E. Emke, A. van Leerdam, B. Muilwijkb, R. Berbeed, T.H.M. Noij, J. Chromatogr. A 970 (2002) 167.
[68] S.A. Snyder, T.L. Keith, D.A. Verbrugge, E.M. Snyder, T.S. Gross, K. Kannan, J.P. Giesy, Environ. Sci. Technol. 33 (1999) 2814.
[69] S. Galassi, E. Benfenati, J. Chromatogr. A 889 (2000) 149.
[70] K.V. Thomas, M.R. Hurst, P. Matthiessen, M.J. Waldock, Environ. Chem. Toxicol. 20 (2001) 2165.
[71] P.L. Ferguson, C.R. Iden, A.E. McElroy, B.J. Brownawell, Anal. Chem. 73 (2001) 3890.
[72] M. Seifert, G. Brenner-Weiss, S. Haindl, M. Nusser, U. Obst, B. Hock, Fresenius' J. Anal. Chem. 363 (1999) 767.

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