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Topic 2.1

Analysis of endocrine active substances in food and the environment*

Patrick T. Holland

Cawthron Institute, 98 Halifax Street E, Nelson, New Zealand

Abstract: A critical review is made of techniques for analysis of residues of endocrine active substances (EASs) including sampling, extraction cleanup and determination based on GC/MS, LC/MS, ELISA, and bioassays. The growing importance of receptor-based in vitro bioassays is highlighted for integrated monitoring of environmental levels of certain classes of EASs and for establishing exposures. Some recent advances in methods of analysis for each of the key classes of EASs are summarized including for organochlorines, PCBs, dioxins and dioxin-like substances, polybrominated diphenyl ethers, phenolic xenoestrogens, phthalates, organotin compounds, steroidal hormones, and phytoestrogens. The issues raised in interpreting complementary chemical and bioassay data at an effects level are briefly discussed.

INTRODUCTION

This chapter provides a critical review of analytical methods that can detect, chemically characterize, and quantify levels of endocrine active substances (EASs) in foods and environmental matrices including water, effluents, sediments/soil, and wildlife. Biological monitoring is particularly important for the field of EASs. The concept is defined by IUPAC as: "The continuous or repeated measurement of potentially toxic substances or their metabolites or biochemical/physical effects in tissues, secreta, excreta and expired air in order to evaluate occupational or environmental exposure and health risk by comparison with appropriate reference values based on knowledge of the probable relationship between ambient exposure and resultant adverse health effects." Recent reviews have discussed aspects of this topic [1].

Risk analysis for endocrine-disrupting chemicals (EASs—here taken as proven or potential) in the environment or humans requires quantitative exposure estimates. These should be based on analytical data covering all compounds that may be expected to give rise to significant additive, antagonistic, or synergistic biological effects. EASs are represented in many chemical structure classes covering a range of polarities and other physical properties and including both synthetic organic chemicals and natural products. Continuing discoveries of subtle endocrine activities for many persistent organic pollutants (POPs) emphasize their particular significance as bioaccumulative EASs that require careful monitoring and control [2]. The biological activities of EASs with closely related structures may vary by several orders of magnitude with ppt-ppb levels of some chemicals being significant for endocrine effects while related compounds may only begin to exert effects at ppm levels. Therefore, general chemical screening for EASs must be based on methods that have detection capability for many specific com-

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ponents, high sensitivity, and a wide dynamic range. This is a demanding task, made more difficult for food and many environmental matrices by their complex natures. In many cases, recourse must be had to several chemical assays each covering sub-sets of the required analytes. These studies may also extend to precursors of EASs such as the alkylphenol-ethoxylate surfactants or to metabolites such as hydroxylated PCBs, which may be more active than the parent.

Modern analytical chemistry has available a range of powerful technologies which are capable of accurately determining EASs to low levels in various food and environmental matrices. More rapid and less solvent-dependent techniques have been developed for extraction of analytes including accelerated solvent extraction (ASE) for solids and solid phase extraction (SPE) for aqueous samples. Preparative HPLC (silica, reversed phase, or size exclusion) provide universal clean-up systems that can be automated. Combined mass spectrometry with gas chromatography (GC/MS) or liquid chromatography (LC/MS) provide multi-residue capability, automation, and a high degree of confidence in the identification and quantification of EASs in complex matrices. LC combined with atmospheric pressure ionization has revolutionized the trace analysis of polar compounds that may be unstable in GC/MS [3].

Biochemical techniques such as receptor-linked expression assays for endocrine receptor agonists or immunoassays for biomarker proteins provide an alternative to chemical screening that allow biological monitoring of the integrated effects of diverse EASs with common modes of action [4]. They are increasingly also being used to provide estimates for environmental or food-monitoring purposes of the effective concentrations of particular classes of EASs (e.g., dioxins [5]) and to guide analyses in research to determine the specific chemicals causing adverse effects in an environment [6–8]. Assays in wildlife and man for biomarkers that are directly linked to EAS exposure levels (e.g., ELISA for the estrogenicity biomarker protein vitellogenin [9]) can also be used to establish the lower concentration ranges for particular EASs that are significant for biological effects. These bioassay results can assist setting of appropriate scope and limits of detection for chemical screening methods. Biosensors, where activation of a selective bioassay element is coupled by transducers to provide a concentration dependent electrical signal, are a significant research area. They provide analytical systems for continuous monitoring of temporal concentrations. Biosensors for EASs have been reported based on response elements for estrogens [10] and cytochrome P-450 [11].

The following sections summarize some of the key techniques for analysis of EASs and then review recent advances in methods of analysis for each of the most important classes of EASs.

OVERVIEW OF ANALYTICAL TECHNIQUES

Environmental sampling and sample preparation

Protocols for sampling of *water* emphasize the need for cleanliness of sample containers and adequate flushing of bores and sample lines where water is being pumped from wells or other remote locations [12]. Relatively large samples (0.5–2 l) are generally taken where possible to minimize walls effects in the sample container and provide laboratory flexibility. Sample preservation has mainly depended on cooling for multi-residue methods although specific compounds such as estrogens may tolerate low pH (2–3). Field extraction using SPE columns was shown to be a practical approach to sampling and preservation of labile surfactant and estrogen residues [13]. Field sampling of *soils* or *sediments* must ensure that that each sample is representative of the area of interest (plot, field, lake, estuary, etc.) and that cross-contamination is avoided. Where transport of EASs through soil or distribution in sediments is being studied, extreme care must be taken to avoid contamination of sub-surface core sections with upper sections where the majority of residues are typically found. Field sampling and laboratory preparation of organisms and wildlife are specialized activities, but biologists involved need to be aware of analytical requirements for representative and uncontaminated samples.

Extraction

Sample homogeneity is the major limitation to miniaturization of analytical methods for soil or sediment samples. Where analytes are stable to drying and fine-grinding, 2–5 g sub-samples may be adequate. However, overall cost-effectiveness of sample preparation and extraction will often dictate use of 10–50 g sub-samples of coarse-sieved (1–2 mm) moist field samples. ASE using elevated pressures and temperatures is an efficient and automated alternative to soxhlet extraction for intractable weathered residues of nonpolar contaminants such as organochlorine insecticides [14,15]. ASE is the basis for U.S. EPA SW-846 Method 3545 for extraction of contaminants from solid samples. The related technique of microwave-assisted solvent extraction also gave high recoveries of these contaminants, although degradation of some organophosphate compounds was observed [16,17]. Supercritical fluid extraction (SFE) using carbon dioxide requires expensive equipment but greatly reduced use of solvents [18]. Subcritical water (150 °C) has been shown to be an efficient extractant for POPs and some non-hydrolyzible pesticides [19]. Simple blending/ultrasonication/shaking methods using mixtures of water miscible (methanol, acetone, acetonitrile) and nonpolar (pentane, hexane) solvents either on field moist samples or with added water are effective for extraction of compounds of medium to low polarity from solids or biological specimens [20]. Water aids extraction by displacing analytes from polar binding sites and swelling clays. Buffers may be added to assist extraction of acidic compounds [21].

SPE techniques using C18 or divinylbenzene-styrene polymeric adsorbents have largely displaced liquid–liquid partitioning for extraction of water samples, even for samples with high bio-solids such as raw sewage waste [22]. Proprietary graphitized carbon columns have proved effective for extraction of polar compounds, including estrogens and surfactant degradates [23].

Clean-up

Humic substances, lipids, and pigments are among the many coextractives that can cause problems in analysis of EASs including direct interferences, matrix effects, and contamination of GC injectors. SPE columns in normal, reversed-phase, or ion-exchange formats are widely used in clean-up systems developed for specific classes of environmental contaminants [12b]. Careful optimization is required for multiresidue applications, particularly where HPLC-UVD is used for determination. Size-exclusion chromatography (gel permeation, GPC) clean-up systems have many advantages for multi-methods based on HPLC or HRGC determination. Automated GPC systems recover a wide range of low- to medium-MW compounds with high reproducibility and reliably remove higher molecular weight coextractives such as humic acids and pigments. Laboratory-packed Biobeads SX-3 columns (450×10 mm) are robust and of low cost [24], while rigid-bead commercial columns have some advantages in speed and resolution [25]. GPC followed by automated HPLC on silica was used to clean up low-level chlorobornane residues and remove PCBs prior to HRGC/MS (NCI) determination [26]. Ultra-trace analysis of dioxins and dioxin-like compounds requires specialized clean-ups with carbon-based column fractionation of the coplanar compounds [27]. Immuno- or receptor-based affinity columns have been used for highly selective cleanups for particular analyte classes [28].

GC/MS determination

GC with high-resolution capillary columns (HRGC) coupled to MS detection has developed into a primary technique for identification and quantitative determination of many EASs using small bench-top instruments with sophisticated data systems. Electron impact is the most common ionization method, although chemical ionization (positive or negative mode) has benefits for unstable or electron-capturing compounds, respectively. Single-quadrupole instruments rely on selected-ion monitoring (SIM) to achieve picogram sensitivities [29] while triple-quadrupole instruments can use MS/MS with selected reaction monitoring to enhance specificity. Ion-trap instruments can achieve high sensitivities for full-

scan spectra and have MS/MS modes [30]. HRGC/MS with optimized capillary column separation could determine residues of 567 low- to medium-polarity compounds, including many EASs, within a 42-min run [31]. The very high effective scan rate of time-of-flight (TOF) instruments has enabled rapid GC/MS analysis of PCBs and other environmental contaminants [32]. The high selectivity of MS detection can reduce the need for sample clean-up, although coextractives may affect GC injector performance. Programmed temperature vaporizer (PTV) injection gave superior performance to splitless injection for trace analysis by HRGC/MS [33]. The direct sample introduction system of Amirav [34] allowed rapid screening with low detection limits when coupled to HRGC/MS/MS (ion-trap) for 22 pesticides in crude plant extracts [30] and alkylphenol polyethoxy-carboxylates (n = 1–9) in water [35]. The incorporation of isotope-labeled internal standards and surrogates increases the precision and accuracy of MS determination, although few labeled EASs are available. High-resolution MS techniques have become indispensable to achieving the lowest detection limits (low ppt) with high accuracy and precision for POPs such as dioxins [36] that occur as complex mixtures of congeners in association with other polychlorinated residues.

LC/MS determination

Advances in LC/MS interfacing (electrospray-ESI and atmospheric pressure chemical ionization-APCI) have enabled levels of sensitivity and reliability that are suitable for routine determination of EASs, particularly more polar compounds that would require derivatization for GC/MS. LC/MS can reduce clean-up requirements over HPLC-UVD based methods, although care must be taken with matrix effects on ESI responses that may affect quantitation. Spectra are often dominated by molecular cations or anions. The MS/MS (tandem-MS) modes available on ion-trap and triple analyzer instruments are valuable for confirmation of identity and to reduce typically high background signals. They utilize mass analysis of the collisional activation products of the predominant molecular anions or cations. The increased structural information and higher specificity of LC/MS/MS has been demonstrated for herbicide residues [37] and surfactants [38]. The techniques of GC/MS/MS, LC/MS (SIM), and LC/MS/MS were compared for determination of estrogenic hormones in environmental matrices and gave complementary information, but LC/MS/MS gave low detection limits without the need for derivatization [39].

Immunoassays

Radio-immunoassys have been the front-line technique for hormone determination in endocrinology for many years, and these assays have been adapted for food and environmental use. Enzyme-linked immunoassay (ELISA) has become an accepted technology for analysis of steroidal hormones and pesticide residues. Although it is beyond the capacity of most laboratories to develop their own antibodies for immunoassays, commercial kits are available for many compounds. ELISA can achieve remarkable sensitivities as well as high selectivity and adequate precision for screening of specific compounds [40]. A relatively simple extraction/clean-up is often adequate. IUPAC guidelines have been published for validation and operation of immunoassays for pesticide residue analysis [41]. The development of ELISAs for detection of EASs has been reviewed [42]. A high-sensitivity ELISA was shown to be a suitable basis for monitoring studies of specific estrogenic hormones at LODs of 0.05–0.1 ng/l for waste water and surface water respectively [43]. Results from an interlaboratory study comparing HPLC-UVD/FL with ELISA for determination of surfactants in wastewaters concluded that ELISA was an effective monitoring technique [44]. Immuno-affinity columns can provide very efficient extraction and clean-up of specific compounds from complex samples for standard instrumental analysis. The development of sol-gels as carriers for monoclonal antibodies in affinity columns provided greatly improved storage stability, high flows, and high specific binding capacities for s-triazine herbicides and dinitrophenol compounds [45]. Affinity media based on molecularly imprinted polymers (MIPS) [46]

are also a development toward more stable, specific, and regenerable molecular recognition substrates for extraction/clean-up or biosensor applications.

Receptor-based bioassays

The wide range of potential EASs has led to the use of in vitro assays to initially characterize the type and levels of exposure on a more generic effects basis. This approach has been very successful for compounds with dioxin-like or estrogenic activities. Bioanalytical screening methods for dioxins and dioxin-like compounds have recently been critically reviewed [5,47] including those utilizing in vitro activation of Ah receptors such as CALUX assays (AhR coupled to luciferase gene expression) and the EROD assay (7-ethoxyresorufin-*O*-deethylase activity through cytochrome P-450 induction). The E-screen system for estrogens is based on proliferation of MCF-7 human breast cancer cell lines [48,49] and has been used to monitor estrogenic activities in STW [50]. The ER-YES-screen uses stable expression in recombinant yeast cells of the human estrogen receptor and plasmids for estrogen response elements and LacZ reporter leading to galactocidase production [51,52]. The estrogen receptor has also been coupled to the CALUX reporter gene [53]. The quantitative performance of the E-screen, YES screen, and in vitro estrogen receptor competitive binding assays were compared for detecting and identifying estrogenic EASs [54] with the conclusion that these assays provided complementary information [55]. In a comparison of in vitro bioassays for detection of estrogens in waste- and surface-waters, the ER-CALUX assay was more sensitive then the ER-YES screen [56]. Transformed yeast systems have also been produced to detect androgenic chemicals through expression of an androgen receptor with a response element coupled to a reporter plasmid [57]. Chemicals with anti-aromatase activity were also detectable by incorporating plasmids to create both aromatase and androgen receptor [58].

Bioactivity-guided fractionation and analysis

The identification of specific EASs in the environment has been guided by bioassays and receptor-assays. A common strategy was HPLC fractionation of extracts using preparative C18 reversed-phase chromatography. Collected fractions were bioassayed, and those from regions of significant activity were pooled and analyzed by GC/MS (following derivatization) or LC/MS. The ER-YES screen assay guided GC/MS identification of 17β-estradiol, estrone and 17α-ethynyl-estradiol in streams and sewage treatment waste (STW) at 1–50 ng/l [7]. These excreted estrogens were confirmed as significant EASs to fish in the streams using the vitellogenin biomarker [8]. The ER-YES screen was also used to guide the identification of nonylphenol and related compounds from wool-scouring effluent as significant estrogens in STW [59]. An androgen receptor-mediated transcription assay was used to guide identification of androstenedione in paper mill effluent and related to masculinization of fish in a river downstream of the mill [60]. Similarly, the ER-YES screen was used to guide the identification of estrogenic chemicals in STW and paper products by ELISA [61] or chromatographic analyses [62]. Changes in estrogenic and androgenic activities during wastewater treatment were monitored by YES screens [63]. The cystostatic and antiestrogenic metabolites of dietary indole-3-carbinol were isolated with the guidance of the MCF-7 cell proliferation assay [64].

Quality assurance for analysis of EASs

As with any analytical work, there is a need for QA/QC of the methods and results for EASs in food and the environment. This becomes even more important as the testing moves from an area of research to becoming a regulatory activity. QA/QC systems are well developed in areas such as pesticide residues and priority pollutants. However, the basic tenets of method validation, internal quality control, and interlaboratory sample exchange have not yet been rigorously applied in many other areas of EAS analysis, including several of those that are discussed in more detail below. In many cases, re-

search reports lack basic information to support their quantitative findings such as the accuracy and precision of the methods used for the analytes and matrices under study. There are many related issues such as the handling, storage, and extraction of samples that require more investigation before there can be full confidence in quantitative data that will form the basis for risk assessments. These issues apply equally to the in vitro bioassay systems that are increasingly being used to provide quantitative estimates of EAS activities in complex matrices.

METHODS OF ANALYSIS FOR ENDOCRINE ACTIVE SUBSTANCES

Organochlorine insecticides (OCs)

Multiresidue analyses are well established for these POPs and extensive data is available on their environmental distribution, fate and bioaccumulation in wildlife, e.g., in bald eagles [65]. A recent study on bioaccumulation of pollutants in marine zooplankton illustrates some current analytical methodologies for wide-ranging screening of low-level OCs [66]. Recent trends in methods for toxaphene/chlorobornane analysis provide some paradigms for trace analysis. GC/ECD screens are of high sensitivity [67] although GC/MS (HRMS/SIM) [68] or GC/MS (NCI) [26] may be required to give greater certainty of determination, particularly at low levels where complex mixtures of PCBs and other OCs may dominate [69]. GC/MS was used to detect OCs and PCBs in human amniotic fluid with LODs of 0.01 ng/ml and 0.1 ng/ml respectively [70]. A simplified protocol for OCs in serum used SPE (disk) [71].

Polychlorinated- and polybrominated-biphenyls (PCBs, PBBs)

These POPs comprise complex mixtures of congeners with widely varying biological activities and environmental persistence. Recent reviews have emphasized that adequate risk characterization for PCBs requires exposure data for a wide range of congeners [72]. Although HRGC/ECD is suitable for congener specific analyses covering the majority of significant congeners in sediments or fatty tissues, mass spectrometric detection has become more common, especially for the dioxin-like non- and mono-ortho congeners. The hydroxylated metabolites of PCBs are potent thyroxin receptor agonists and were determined in the blood and fat of mammals using GC/ECD of the methyl derivatives [73]. Ion-trap GC/MS of the trifluoroacetyl derivatives has been optimized for their determination at trace levels [74]. Methylsulfonyl metabolites of PCBs also have significant endocrine activities and were determined in milk and human and marine mammal tissues by GC/MS after novel multiresidue clean-ups [75].

Dioxin and dioxin-like compounds

A variety of polyhalogenated environmental and food contaminants have been found to be potent AhR agonists and thus require careful monitoring and control. The most significant compounds are the polychlorinated dibenzodioxins and dibenzofurans (PCDDs, PCDFs), and coplanar congeners of PCBs. Accurate risk assessment for the complex mixtures requires congener specific analyses so that toxic equivalency factors (TEFs) relative to 2,3,7,8-TCCD can be applied for summing of activities to provide a toxic equivalency quotient (TEQ) [76]. There are a large number of other halogenated compounds that might require consideration in some situations [5]. For example, polychlorinated napthalenes were the main contributor to TEQs in wide-ranging analyses of sediments from the Detroit Basin [77] and Tokyo Bay [78]. The U.S. EPA ultra-trace (ppt LODs) analytical protocols for PCDDs and PCDFs (e.g., SW386 Method 8290 for solid waste) are based on highly selective clean-up followed by HRGC/HRMS (SIM). There is a trend to multiresidue protocols for extraction, and clean-up to streamline determination of PCBs and other chlorinated POPs as well as PCCDs and PCDFs, e.g., in food [79]. The use of isotope-labeled internal standards and well-developed quality control criteria [80]

provide a paradigm for other instrumental protocols in environmental trace analysis. Ion-trap MS/MS detection provides an alternative to HRMS for screening of PCCDs and PCDFs with equivalent accuracy but higher limits of detection [81]. The most interesting development is bioassay techniques to estimate TEQs directly with adequate sensitivity for most screening purposes. ELISAs, AhR receptor-based assays, and cytochrome P-450 induction assays have been validated for dioxins and dioxin-like compounds [82,45]. The strategies have been explored for screening food and environmental samples using such bioanalytical tools and relying on the congener specific instrumental analyses only for confirmation. The AhR-CALUX assay was used to screen 1380 food and feed samples from recent contamination incidents in Brazil and Belgium. About 10 % of samples were positive (>5 pg TEQ/g) and half of these were tested by GC/MS with 50 % (24 samples) being confirmed as due to dioxins [83]. It should be noted that EAS endpoints such as relative estrogenic effects of dioxins may not be adequately represented by TEFs derived from AhR activity.

Polybrominated diphenyl ethers (PBDEs)

PBDEs are high-volume chemicals still in use as fire retardants. Current commercial products are highly brominated and contain less than 10 of the possible 209 congeners. They have some of the characteristics of other POPs, including EAS activity, and there is concern about the rising levels in fatty tissues of wildlife and humans [84]. Extraction and clean-up procedures similar to those for residues of persistent organochlorine compounds have been found to be effective [20c]. Methods have been reported with determination by HRGC with ECD [19a], MS (NCI) [20c], and HRMS [85]. Analysis of the highly brominated congeners is complicated by their high MWs and tendency to decomposition by light or heat. Many earlier methods only determined lower congeners. These difficulties were confirmed by an interlaboratory study where results for PBDEs in sediment and biota samples were very variable, especially for the deca-BDE (#209) [86].

Phenolic xenoestrogens and phthalates

Degradation of alkylphenol-ethoxylate surfactants and related high-volume industrial chemicals result in environmental contamination by phenolic degradates, the most prevalent being 4-nonylphenol (4NP, a mixture of 22 isomers). These compounds are also ubiquitous in food [87]. Analytical methods have been reviewed [88]. Bisphenol A, derived from plasticizers, also has significant estrogenicity and environmental persistence. Phenolic xenoestrogens were determined in aqueous samples by GC/MS following SPE (divinylbenzene-styrene polymer packing) and methylation [22]. ASE plus GC/MS [89] and microwave-assisted extraction plus LC/MS [90] have been used to determine alkylphenols in sediments. Steam distillation/solvent was used for extraction of 4NP from foods [87]. Isomer-specific analysis of 4NP was accomplished by capillary GC/MS and was enhanced by using derivatives (acetate, PFB, HFB, TMS) [87,91]. Alkylphenols have also been determined in human plasma samples by HPLC with electrochemical detection [92]. GC/MS formed the basis for detection of bisphenol A in hazardous landfill leachates [93].

Recently, there has been extensive research into the pathways and rates for breakdown of parent surfactants in the environment or during sewage treatment. Degradates of 4NP-ethoxylates (NPEs) with ecotoxic characteristics include 4NP, chain-shortened nonylphenol ethoxylates (mono- NP1EO and di-NP2EO), and carboxylate oxidation products. The high selectivity of tandem MS using precursor ion scanning and multiple reaction monitoring enabled a simple flow injection analysis of sewage treatment plant wastes for parent surfactants with LODs to 50 ng/l [94]. Generic extraction protocols for alkylphenols and intermediate polar degradation products used SPE for aqueous systems [95,96] and sonication for sediments [95]. LC/MS with ESI or APCI formed the basis for methods to determine NPEs and degradates in aqueous samples [38a,95–98], sediments [95], sewage sludge [99], and fish tissues [100]. A quantitative LC/MS method for metabolites of alkylphenol-ethoxylates in water used surrogate and

isotope-labeled internal standards to correct for recovery and matrix effects during ESI [96]. HPLC with fluorescence detection formed the basis for simple and sensitive methods for 4NP, NP1EO, and NP2EO in aqueous samples [101], sediment [102] and aquatic biota [103]. Dicarboxylated metabolites of NPEs were analyzed in sewage waste using SPE (Carbopak) extraction and LC/MS and were found to be the predominant NPE degradates leaving sewage treatment plants [23,104]. Carboxylated metabolites have also been determined by GC/MS following methylation [105]. Urinary metabolites of 4NP in trout were determined by GC/MS following radio-HPLC isolation and derivatization and were found to be different from 4NP biotransformation products in sewage sludge or trout bile [106]. The matrix solid-phase dispersion technique was very efficient in extraction and clean-up of surfactants from biological tissues [107].

Well-established analytical protocols for phthalate ester plasticizers (PEPs) in environmental and food matrices are generally based on GC/MS, e.g., methods for PEPs in sewage sludge [62,108]. Their ubiquitous nature tends to result in high and variable laboratory blanks. Solid-phase microextraction (SPME) for analysis of aqueous samples has the advantage in this respect of minimizing sample handling and can achieve sub-ppb detection limits using GC/MS detection [109]. The monoester metabolites are the PEP compounds of greatest concern as EASs [110], and their excretion in urine is also a useful biomarker of overall human exposure to PEPs [111]. Seven of these compounds were determined in urine by an isotope dilution assay based on enzymatic deconjugation, SPE, and LC/MS/MS [112]. Monoesters were determined in river waters by GC/MS following SPE and methylation [113].

Organotin compounds

The high toxicity to mollusks of alkylated tin compounds, particularly tributyl-tin (TBT), necessitates sensitive and selective analyses in water and sediments. Total organic tin measurements can be reliably provided by graphite furnace atomic absorption spectroscopy (GF/AAS) or atomic emission spectroscopy (ICP/AES). However, speciation is required to understand the fate, exposures, and effects of tin compounds in marine environments. GC separation of mono-, di- and tri- alkyl tins can be achieved following peralkylation using $NaBH_4$ [114] or a Grignard reagent (commonly pentyl-magnesium bromide) [115]. Selective GC determination of the derivatives has been achieved using flame-photometric [116], atomic emission [104,117], or MS detection [115b,118]. ASE effectively extracted organotin compounds from sediments [119]. High laboratory blanks from ubiquitous tin compounds in plastics and other sources must be overcome to achieve high precision at the low concentrations in waters or sediments that are significant for ecotoxicity. Sulfur interferences must also be eliminated [120].

Steroidal hormones

Naturally occurring and synthetic steroid hormones are significant estrogenic components of animal and human waste that can act as EASs in the environment at very low concentrations. In-depth reviews of estrogen analysis, including sampling and extraction, has recently been published [22b,121]. Sensitive immunoassays in RIA [122] or ELISA [43] format are a convenient technique for screening of large numbers of samples for specific steroids. Chemical methods have been validated for steroid speciation in aqueous samples involving SPE, cleanup and GC/MS or GC/MS/MS determination of the steroids after derivatization [39,123–125]. Detection limits for 17β-estradiol, estrone, estriol and 17α-ethynylestradiol were 0.1–5 ng/l. The mono *tert*-butyl-dimethysilyl derivatives offer advantages of speed of reaction, stability, and good MS characteristics [112]. A comprehensive method using SPE and GC/MS (TMS derivatives) has been used to determine steroidal estrogens, phytoestrogens, and xenoestrogens in STW and the chemical data was correlated with E-Screen estrogenicity [126]. LC/MS (ESI or APCI; +ve or –ve ion) was found to be of superior specificity to HPLC/DAD for determination of estrogenic hormones in aqueous samples [127]. Others studies using LC/MS/MS (ESI-) gave sub-ng/l detection limits [13b,23b], comparable to the best GC/MS methods. Isotope labeled internal standards

enhanced the accuracy and precision of MS-based methods by correcting for losses of the readily sorbed estrogens during extraction and workup of samples. Androstenedione was identified as a significant androgenic component in paper mill effluent using LC/MS [60].

Phytoestrogens

Many plants, particularly legumes, produce a range of sterols, isoflavonoids, and lignans with moderate to weak or antagonistic estrogenic activities. The *Fusarium* mycotoxin zearalenone and its synthetic relative zeranol are also estrogenic. A key focus of current research is to establish the protective or adverse effects of specific phytoestrogens on human health. Chemical assays provide complementary data to effects-based studies, particularly to establish exposure levels and metabolism. The main concern has been mammalian dietary intake in foods or feeds, but environmental issues arise with some compounds such as β-sitosterol, a significant by-product of pulp and paper production. Methods of analysis for food have been reviewed [128]. Most attention has been given to the isoflavone class, but lignans have also been studied [129]. Many of these compounds have native fluorescence, and HPLC with fluorescence detection provides the basis for specific and sensitive assays. HPLC with UV detection forms the basis for standard methods for soy-based foods [130], with either acid hydrolysis to fully release the aglycones or mild base hydrolysis to release ester forms of glycosides. Coulombic-array detection based on electrochemical oxidation of the phenolic groups provided very sensitive and selective detection of phytoestrogens [131]. Comprehensive protocols based on enzymatic and acidic hydrolysis to release the aglycones followed by GC/MS of the TMS derivatives have been used to elucidate the full suite of isoflavones and lignans present in diverse foods/feeds and where more detailed knowledge of metabolism and excretion was required [128,132]. LC/MS is also increasingly being applied to flavonoid determination. Derivatization is not required, and tandem MS techniques provide excellent selectivity with structural information, including on glycosidic linkages [133]. A study in mice showed substantial metabolism of estrogenic isoflavonoids to hydroxylated or dehydrogenated forms, some of which were more estrogenic than the parent [134]. A sensitive (5 nM) and rapid immunoassay has been developed for determination of enterolactone, genestein, and daidzein in urine using time-resolved fluorimetry to detect europium-labeled phytoestrogen tracers [135]. An estrogen receptor assay in microtitration plate format has been developed for rapid screening of soy-based foods for phytoestrogen content (genestein equivalents) and gave good correlation to HPLC data [136]. Affinity column separation and LC/MS/MS were used to establish the low estrogenic potential of some triterpene glycosides [137].

CONCLUSIONS

An array of chemical analytical methods (including ELISA) have been developed that provide adequate detection limits and precision for analysis residues of the most important classes of EASs in food and the environment at levels of biological significance. Many challenges remain to make many of these methods less complex and more robust.

The high degree of complexity in interpreting potential biological effects from chemical monitoring alone has been illustrated using organohalogen compounds as an example [4c]. The development of stable and highly sensitive recombinant receptor-based assays (EROD, CALUX, YES) has been very significant for EAS research. As well as providing in vitro test methods to assess endocrine activities of different chemicals, the assays have high utility for food and environment monitoring through their ability to integrate exposures at an effects level in a rapid and relatively inexpensive format. Obviously continued development of such assays into other endocrine activities beyond estrogen, ArH and androgen receptors, or into more specialized formats (e.g., ER-b) will continue to provide major benefits to studies of EAS fate and exposures.

Despite the benefits of integrating bioassays, there will always be a need for chemical speciation both for fundamental research to identify and understand the modes of action of particular EASs and for management and mitigation of problem chemicals. The receptor screens have proved powerful in some situations for directing isolation of specific chemicals causing adverse effects. However, the comparison of results between biological and chemical monitoring is providing interesting fundamental questions relating to a valid concept for joint toxicity. Receptor activities and other relatively short-term in vitro effects of chemical mixtures must be related to various longer-term toxicological endpoints. On the other hand, the integration of chemical concentration data through use of TEFs must use relative biological potencies that are relevant to particular endocrine effects (e.g., estrogenicity of dioxins). For example, CALUX-guided risk assessment of dioxins must incorporate the significant AhR activities of some PAHs. These issues have been carefully reviewed recently in relation to AhR responses [5,138] and are of very practical consequence. Discrepancies between AhR assay data and dioxin TEQs for biological or food samples have generally not been large [5]. However, measurements in contaminated sediments of alkylphenols, PAHs, OCs, and PCBs and associated estrogen receptor and AhR activities have shown that much of the in vitro bioassay responses could not be accounted for and were attributed to undetected compounds [139]. Similar conclusions have been drawn from studies on estrogenicity of STW, although the proportions of unaccounted for activity were generally lower [56,126,59] or attributed to antiestrogenic compounds [52]. Thus, chemical and biological analyses are both required to understand and manage the risks posed by particular chemicals in complex mixtures of contaminants (natural and man-made).

Some recommendations for future research arising from this review include:

- A high priority is required for study of contaminated sites, waste treatment processes, bioaccumulated mixtures of POPs in mammals, and EASs in human diets.
- Better integration of test methods for diverse classes of EASs.
- More automated and selective clean-up procedures, e.g., immuno-affinity columns.
- Methods should include more metabolites of xenobiotics as potential EASs,
- Increased use of LC/MS to supplant GC/MS methods that require derivatizations. This will require better confirmatory techniques such as tandem-MS and greater use of isotope-labeled internal standards to correct for matrix effects.
- Increased availability of ELISA and reporter gene assays in kit formats with well-defined specificity for selected AESs or classes of AESs.
- Development of bioanalytical instrumentation where separation, spectroscopic, and biological detector elements are linked to provide information on effects and chemical properties.
- Quantitative correlations of bioresponse and chemical data are powerful tools, but require careful consideration of all confounding factors.
- Greater conformity to the principles of laboratory quality assurance in terms of method validation, in-use performance verification, confirmatory tests, and interlaboratory studies.

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