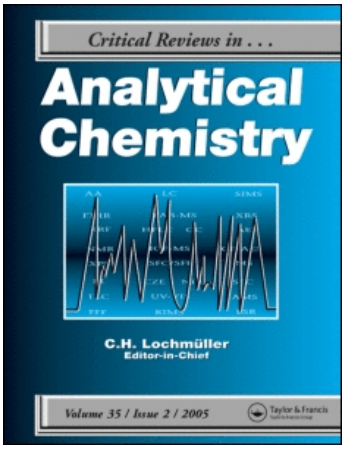

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Estrogens and Their Analytics by Hyphenated Separation Techniques

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Estrogens as biologically active compounds belong to a group of steroids hormones. The definition of these substances is a very important factor in humans. These compounds play an essential role because regulate many physiological processes, including normal cell growth, development and tissue-specific gene regulation in the reproductive tract and in the central nervous and skeleton systems. The much higher concentration of these matters is key in growth of sexual organs and development of secondary sexual features in women. Synthetic compounds which initiate or enhance the effect of estrogens are called xenobiotics. Xenobiotics are found in the human body in much higher concentrations than are usual. They can modify the functions of the endocrine, reproductive, nervous and immune system. For this reason it is significant to determine these compounds. Their physico-chemical properties and complex structure cause many analytical problems. Chromatography, especially liquid chromatography (LC), is used in an extremely large range of analytical methods. Successful analysis of estrogens requires a rapid, reliable, precise method for sample preparation and cleanup to remove potential interfering components. An applicable procedure is the use of solid-phase extraction (SPE), especially with molecularly imprinted polymer (MIP), therefore SPE-MIP. MIPs are taylor-made synthetic materials capable of selectivity rebinding a target analyte based on a combination of recognition mechanisms including size, shape and functionality. Combination SPE-MIP and LC with various detection techniques present an excellent method in quality control of estrogens.

Keywords Estrogens, xenobiotics, metabolomics, SPE, MIP, HPLC, detection

INTRODUCTION

Estrogens (natural estrogens) are a group of sexual hormones which are endogenously produced in fish, amphibians, birds, mammals and humans. In humans, they are produced naturally in both sexes, but women have much higher concentrations. Most of the substances are produced in the ovaries, less in the adrenal cortex, brain, testicles and, during pregnancy, also in the placenta. They are important in the development of female sexual organs and secondary sexual characters, as well as in the maintenance and regulation of the female reproduction system and they are also involved in the development of the male reproduction system (1, 2). Naturally occurring and the most important hormone from estrogens is 17β-estradiol, which is oxidized into estrone and hydrated into estriol in liver. Intermediate products of convergence estrone to estriol are 2-hydroxyestrone, 4-hydroxyestrone, and 16α-hydroxyestrone (Fig. 1). Major convergence metabolites of 17β-estradiol are also 2-hydroxy-17β-estradiol, 4-hydroxy-17β-estradiol, and 16α-hydroxy-17β-estradiol (Fig. 2).

The determination of metabolites in which estrogenic properties are not explained is an important problem of these compounds. Choice of selective method for isolation and preconcentration of these analytes from complicated matrixes such as water, blood, physiological fluids and tissue should be ground of complex determination. Accurate selection of sensitive and reproducibility final determination methods and validation are basis and obligation from a good laboratory practice point of view in life chemistry (bioanalytics, ecology, pharmacology, medicine).

The main techniques used for preparation of samples are extraction, especially liquid-liquid, liquid-solid as well as membrane technology methods, and finally multi-dimensional

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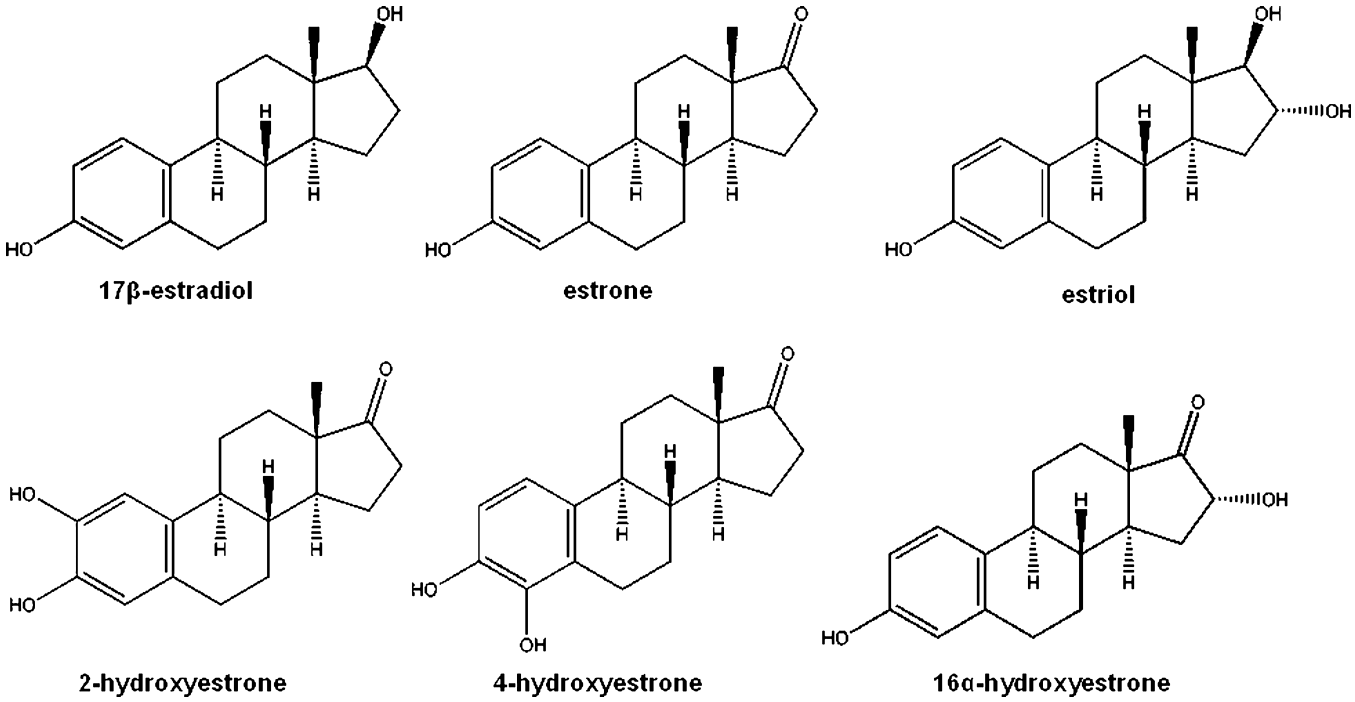

FIG. 1. Chemical formulas of the most important estrogens.

systems which are susceptible for automatization and computer aided roboting. Most of the literature and works written about these systems in which the emphasis is on "fingerprint adsorbents" like molecularly imprinted polymers.

This review describes the complex determination of estrogens by separation techniques, especially taking pains in metabolism and metabolomics of these matters. Extra emphasis is concentrated in the preparation of samples mainly with molecularly imprinted polymer (MIP) and final determination methods with taking advances for separation techniques which are connected with selective and sensitive detectors.

ESTROGENS AS XENOBIOTICS

A range of synthetic and natural substances which are known as xenobiotics have been identified that also possess estrogenic activity. Unintended side-effects of these agents or their metabolites is estrogenic stimulation. A xenobiotic is a chemical which is found in an organism but which is not normally produced or expected to be present in it. It can also cover substances which are present in much higher concentrations than are usual, as is also used in the context of pollutants such as polychlorinated biphenyls (PCBs), dioxins (polychlorinated dibenzodioxins and dibenzofurans – PCDD and PCDF), and plant protection chemicals, e.g., DDT (dichlorophenyl-trichloroethane) or polynuclear aromatic hydrocarbons (PAHs) (2). Xenobiotics may penetrate into organisms via air, water, soil, dust and food, through skin, respiratory system and alimentary tract (Fig. 3). The number of xenobiotics is still growing, which is very dangerous as they can modify the functions of the endocrine, reproductive, nervous and immune system in humans. Some xenobiotics are resistant to degradation. The degree of exposure depends, among other things, on their concentration in a given ecosystem, stability, rate of migration and potential bioaccumulation. These chemical substances are toxicants with toxic, teratogenic, mutagenic and carcinogenic properties. The information on the effects of these substances on human health is scant, so it is difficult to estimate the degree of risk they pose (3, 4).

Effects on the human body are described in mechanisms of impression. Important determinants which are recognized in the biological activities of many xenobiotics are *transporters* mediated by absorption, secretion, and reabsorption. Efflux transporters in intestine, liver, kidney, brain, testes, and placenta can efflux xenobiotics out of cells and serve as barriers against the entrance of xenobiotics into cells, where many xenobiotics enter the biological system *via* uptake transporters. The functional importance of a given transporter in each tissue depends on its substrate specificity, expression level, and the presence/absence of other transporter(s) with overlapping substrate preferences (5). Biotransformation or metabolism of these dangerous matters is defined as the chemical alteration of substances by reactions in the living organism. It occurs in several steps called Phase 1 (Phase I reactions) metabolic transformations or functionalization and Phase 2 (Phase II reactions) conjugation with natural body constituents (Fig. 4). The purpose is converting lipophilic to hydrophilic compounds and facilitating excretion. Changes in pharmacokinetic characteristics, detoxification, and metabolic activation are consequences of this metabolism. The Phase 1 is established to improve water solubility functional groups which

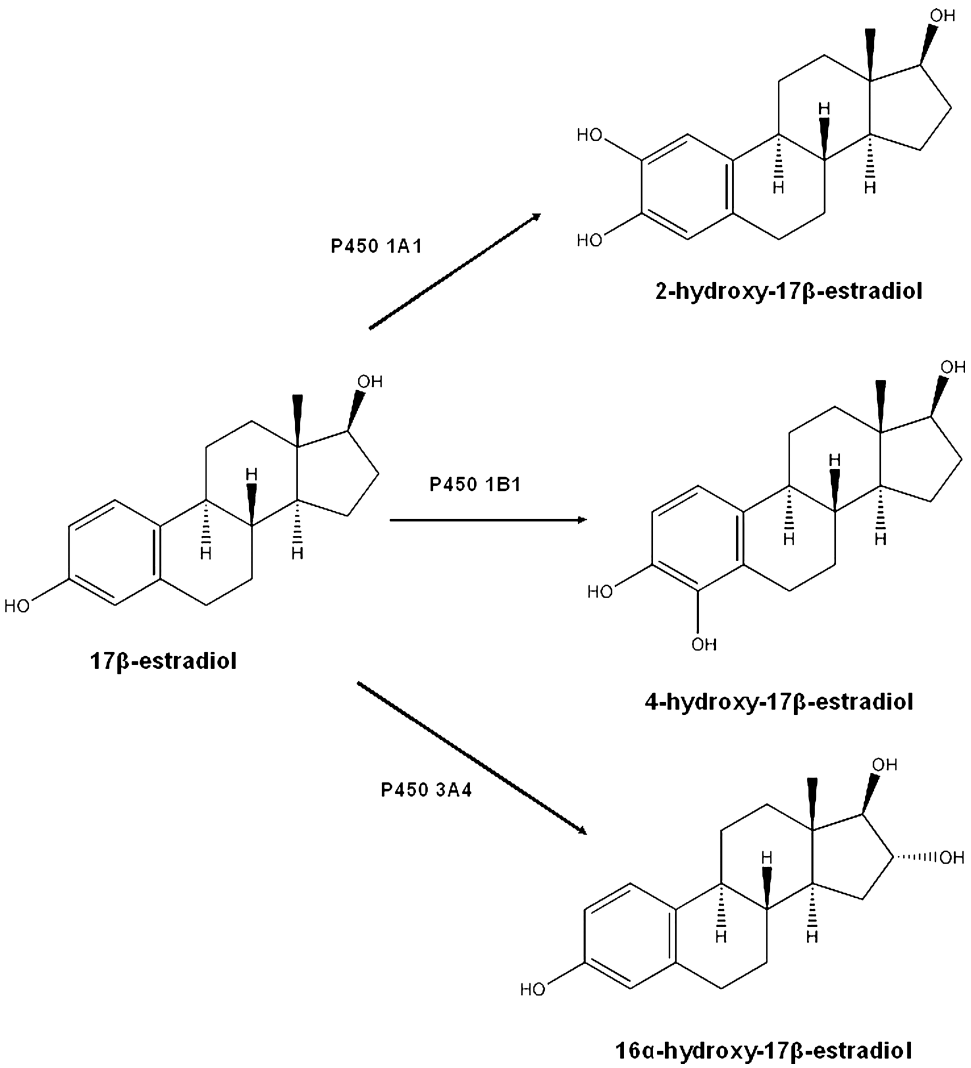

FIG. 2. Structures of estradiol and major metabolites.

are added or exposed to the chemicals in steps such as hydrolysis, oxidation and reduction. In the Phase 2 hydrophilic conjugating species can be added into these functional groups that can either be electrophilic (epoxides, carbonyl groups) or nucleophilic (hydroxyls, amino and sulfhydryl groups, carboxylic groups). Once chemicals undergo functionalization, the electrophilic or nucleophilic species can be detrimental to biological systems. Electrophiles can react with electron-rich macro-molecules such as proteins, DNA and RNA by covalent interaction whilst nucleophiles have the potential to interact with biological receptors. Many chemicals, when exposed to certain metabolizing enzymes, can induce those enzymes, a process called enzyme induction. There are two groups of enzymes in Phase 1 — oxidoreductases and hydrolases. Oxidoreductases introduce an oxygen atom into or remove electrons from their substrates. The major oxidoreductase enzyme system is called the P450 monooxygenases. Other systems include flavin-containing monooxygenases (FMO), cyclooxygenases (COX) and monoamine oxidases (MAO). Hydrolases hydrolyze esters, amides, epoxides and glucuronides.

Phase 1 functionalization pathways are:

- *Cytochrome P-450*
- *FMO reactions*
- *COX reactions*
- *Amine Oxidase reactions*
- *Hydrolases*
- *Ethanol Catabolism* (6).

Most of the Phase II enzymes are located in the cytosol except UDP-glucuronosyltransferases (UGT), which are microsomal. Phase II reactions are typically much faster than Phase I reactions, therefore the rate-limiting step for biotransformation of a compound is usually the Phase I reaction. Phase II metabolism can deal with all the products of Phase I metabolism, be they

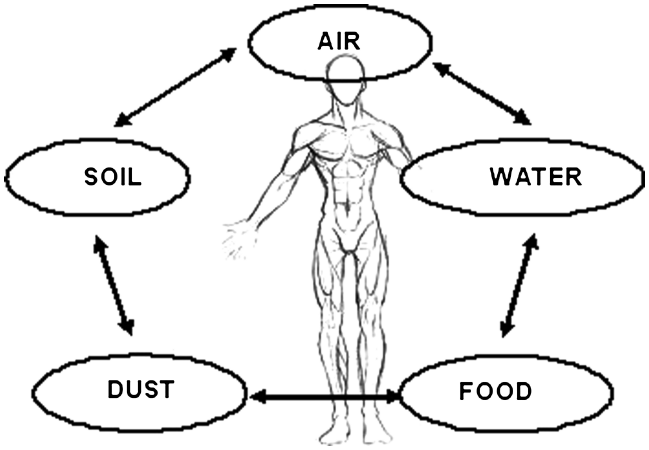

FIG. 3. Impact xenobiotics to humans by ecosystem.

reactive (Type I substrate) or unreactive/poorly active (Type II substrate) compounds. As many substrates and/or their metabolites are chemically reactive, their continued presence may lead to toxicity.

Phase 2 conjugation pathways are:

- *Glucuronidation*
- *Cytosolic sulfonation of small molecules*
- *Acetylation*
- *Methylation*
- *Glutathione conjugation*
- *Amino Acid conjugation.*

The body can remove xenobiotics by *xenobiotic degradation*. It consists of the deactivation and the secretion of xenobiotics and happens mostly in liver. Secretion routes are urine, feces, breath, and sweat (2, 6).

Many human diseases are caused by or are a consequence of an abnormal metabolic state such as the high glucose concentration in blood of diabetes patients and the high urine amino-acid level resulted from liver or renal disorders. Metabolic processes are also heavily involved in xenobiotics degradation and drug clearance (7). Drug safety is often linked to inhibition of metabolic processes (8, 9).

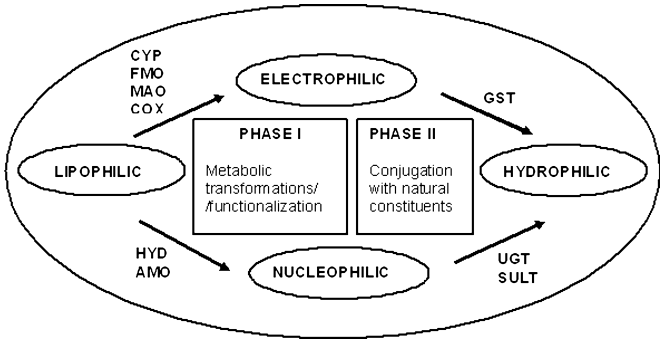

FIG. 4. Xenobiotics metabolism.

Detecting an unusual level of certain specific metabolites in a patient's blood or urine has long been established as an effective method to identify biomarkers for diagnosing particular diseases. Recent rapid developments of advanced metabolomics technology is opening up new horizons, as hundreds or even thousands of metabolites can be measured simultaneously, providing a much more comprehensive assessment of a patient's health status (10, 11).

Although metabolomics is a young science discipline, it is used to identify the function of genes, describe the effects of toxicological, pharmaceutical, nutritional and environmental interventions, and to build integrated databases of metabolite concentrations across human and research animal populations. The interest of metabolomics is nutrition. It is an invaluable tool for determining the distributions of metabolite concentrations in humans, the relationship of these metabolite concentrations to disease, and the extent to which nutrition can modulate metabolite concentrations (12).

Metabolomics is complementary to transcriptomics and proteomics. Results of gene expression are as "downstream", changes in the metabolome are amplified relative to changes in the transcriptome and the proteome. Metabolic fluxes are not regulated only by gene expression but by post-transcriptional and post-translational events and, as such, the metabolome can be considered closer to the phenotype. Metabolomics detects and quantifies the low molecular weight molecules, known as metabolites (constituents of the metabolome), produced by active, living cells under different conditions and times in their life cycles in different metabolomic approaches (Fig. 5).

Metabolite target analysis is the quantitative analysis of metabolites that participate in a specific part of the metabolism (e.g., particular enzyme system affected by abiotic or biotic perturbation). *Metabolite profiling* is focused on a specific group of metabolites (*e.g.*, lipids, carbohydrates and amino acids) or those associated with a specific pathway. *Metabolomics* is comprehensive analysis of the whole metabolome under a given set of conditions. *Metabolic fingerprinting* is used to classify

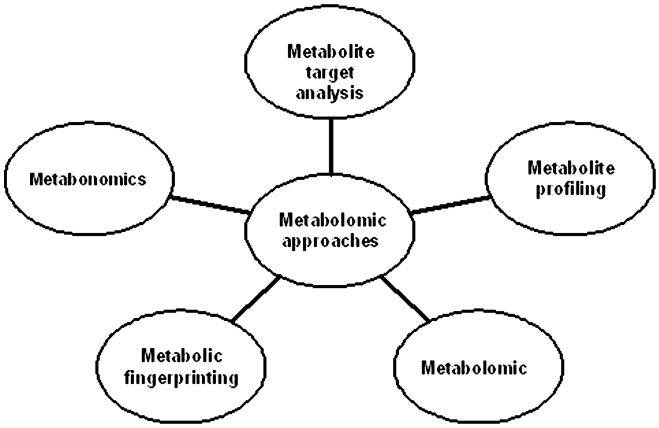

FIG. 5. Classifications of metabolomic approaches.

samples based on provenance of either their biological relevance or origin by using a fingerprinting technology that is rapid but does not necessarily give specific metabolite information. Spectra from either nuclear magnetic resonance spectroscopy (NMR) or mass spectrometry (MS) analyses provide a fingerprint of the metabolites that are produced by a cell. *Metabolic profiling* is often used interchangeably with "metabolite profiling"; metabolic fingerprinting is commonly used in clinical and pharmaceutical analysis to trace the fate of a drug or metabolite. *Metabonomics* is a measure of the fingerprint of biochemical perturbations caused by disease, drugs and toxins (13–15). The most sensitive and precise analyses are typically those for single metabolites. Targeted methods for metabolic analysis provide high-quality data on a single class of compounds using dedicated and optimized methods. Data quality encompasses overall sensitivity, accuracy, precision of quantification and metabolite identification rates, electrospray ionization mass spectrometry (ESI–MS), Fourier transform ion cyclotron resonance mass spectrometry (FTICR–MS), gas chromatography mass spectrometry (GC–MS), gas chromatography time of flight mass spectrometry (GC–TOF–MS), high performance liquid chromatography (HPLC), and liquid chromatography mass spectrometry (LC–MS) (Fig. 6) (28). Estrogens are interested in the activation or inactivation of the cell cycle, cell growth and proliferation, apoptosis, transcription/translation, the degradation/metabolism of proteins and the transport of ions, amino acids and other small molecules, depending on the cell or tissue type, too. 17β-estradiol affects a variety of physiological phenomena in development, metabolism, reproduction and behavior. These processes can be monitored with transcriptomic, proteomic and metabolomic assays (17, 18).

The three technologies used currently for metabolomic analyses of estrogens are NMR spectroscopy (19, 20), MS (21, 22) and classical chromatographic techniques (17, 23). High-resolution NMR provides many of the necessary elements for metabolomic analysis. NMR has the unusual dual properties of providing rigorous quantification of analytes, while not necessarily providing accurate qualitative identification of the analyte. As NMR spectra are increasingly linked to individual metabolites, NMR will become a progressively more powerful technology for broad-scope, high-throughput screening of samples (24–26). The potential MS provide a breadth of analytical capabilities necessary for metabolomics. MS-based metabolomics is already employed in a clinical setting for screening newborns for inborn errors of metabolism, including amino acid disorders and organic acids. This method can be applied to determine the structures of 17β-estradiol, estrone, estriol and other important metabolites of estrogens. MS spectra of mentioned compounds are in Table 1. Classical chromatographic techniques such as gas chromatography (GC) and HPLC offer the advantage of rigorous quantitative analyses, but are often slow and ill-suited for high-throughput platforms. GC and HPLC, in combination with classical detection techniques, are useful in metabolome analysis of the most important hormone 17β-estradiol and other estrogen metabolites to provide quantitative data that are suitable for creating an integrated and seamless database of metabolite concentrations. The creation of an integrated database is essential for developing knowledge of the population distribution of all metabolites and for understanding the natural relations among metabolites (12). Clinical and metabolomic investigations of complex human fluids require cost-effective methodologies that can rapidly assess the steroid hormone milieu of individual samples. For these investigations the determination of estrogens in blood, serum, urine and other biological fluids is important. Experimental data for metabolomic studies such as common separation protocols for quantification and identification of steroids can be obtained using following separation science techniques include gas chromatography (32, 33), supercritical fluid chromatography (34,

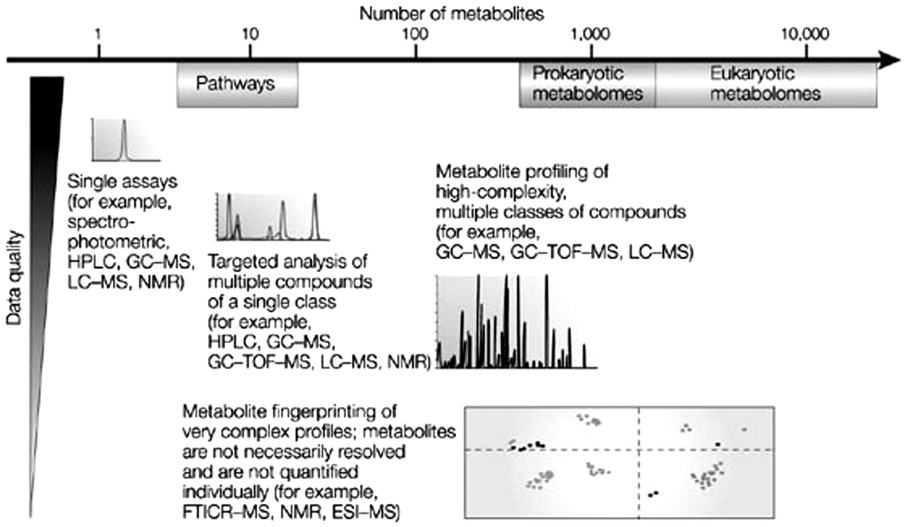

FIG. 6. The trade-off between metabolic coverage and the quality of metabolic analysis.

TABLE 1
17β-estradiol, estrone, estriol and significant metabolites of estrogens identified by MS

Compound	MS spectrum	Main character fragment ion	References
17β-estradiol	272	271–273–290	(28, 30)
Estrone	270	253–271–293	(28)
Estriol	288	107–146–160–172–185–201–213	(30, 31)
2-hydroxyestradiol	287	147–162–214–227	(27)
4-hydroxyestradiol	287	161–269–285	(27)
2-hydroxyestrone	285	162–269	(27)
4-hydroxyestrone		161–231	(27)
16α-hydroxyestrone	286		(29)
17β-estriol	288.4	289	(28)

35), liquid column chromatography (36–38), planar chromatography (39, 40) overpressured thin-layer chromatography (41, 42), as well as capillary electrophoresis (43, 44).

MEDICAL CHEMISTRY DIAGNOSIS

Estrogens exert diverse biological activity on mammals, especially females. Biologically, the most active and abundant estrogen is estradiol. Because their metabolites have divergent biological effects, it is important to quantify their occurrence in biological samples. They also have effects on bone, cardiovascular system, brain, and skin. Lipophilic form of fatty acid esters store estrogens in adipose tissue. They are also produced in the male and play an important role in spermatogenesis, cardiovascular health and bone homeostasis. The most potentially useful metabolite is 2-methoxyestradiol in the prevention of tumor growth in various cell lines. It is currently under clinical experiment. The carcinogenic effect of estrogens is also mediated through its various oxidative metabolites which may provide persistent estrogenic activity or trigger DNA damage by forming DNA adducts (45). Among the estrogen metabolites, 4-hydroxyestrone and 16α-hydroxyestrone are thought to be carcinogenic, whereas, 2-hydroxyestrone is not. 16α- and 2-hydroxyestrone are two main metabolites in humans (46). Therefore, the ratio of urinary 2-hydroxyestrone to 16α-hydroxyestrone (2/16α ratio) has been hypothesized as a biomarker of breast cancer risk and some evidence supported this hypothesis (47). For their divergent biological effects, it is important to quantify their occurrence in biological samples. Bone, cardiovascular system, brain, and skin are also in influence. Epidemiologic data demonstrate that estrogens can also provide protective effects against dopaminergic neuronal degeneration in Parkinson disease (48, 49). Several estrogenic actions are potentially relevant to Alzheimer's disease, and it is hypothesized that one consequence of estrogen deprivation after the menopause is a higher risk of this dementing disorder. These scientists are also interested in menopause estrogen deficiency (50–54). Their functions are summarized in Table 2. Estrogens also cause breast, endometrial and ovarian cancer and other hormonal cancers (about 95%). The relationship between estrogen metabolism and cancer is reported in this review below (55, 56).

FUNCTIONAL ROLE OF ESTROGEN METABOLISM

Estrogens are implicated in numerous diseases and conditions (biological effects) that affect our aging population (both women and men). Two main estradiol, estrone and their metabolites are responsible for much of the effect and that how an individual metabolizes his or her estrogen are predictive of disease risk, prognosis, and efficacy of therapy. These are consequence from direct interaction of the estrogen with an intracellular receptor that activates the gene expression by encoding proteins with important biological functions. Superpotent mitogenic action in hormone sensitive tissues such as e.g. uterus and breast are one of the most important and notable affects of estrogens (57, 58). The metabolism of estrogens includes *oxidative metabolism* (hydroxylations). The main pathways are the 2-hydroxylation and 16α-hydroxylation shown in Fig. 7 (59). A minor pathway is the 4-hydroxylation. The oxidative metabolism of estrogens is performed by cytochrome P450 enzyme families mainly in the liver (60). In humans, estrogen metabolism consists mainly of the 15 metabolites also shown in Fig. 7. The *O*-methylation of catechol estrogens is catalyzed by catechol-*O*−methyltransferase (COMT). Estrogenic hormones are eliminated from the body by metabolic conversion to hormonally inactive (or less active) water-soluble metabolites that are excreted in the urine and/or feces. This conjugative metabolism consists from glucuronidation and sulfonation (61–63).

The news from scientific research has implicated estrogens in the development of hormone dependent cancers. Estrogens may simultaneously stimulate cell proliferation and gene expression via the estrogen receptor and cause DNA damage via their oxidation products, the catechol estrogens in their dual role of ligand and substrate. The latter mechanism is based on the unique chemical structure of estrogens. Estrogens activate various types of estrogen-induced DNA damage include: (a) direct

TABLE 2
Estrogens physiologic function (48–54)

Site of action	Action	Significance
Genital tract	Development of female reproductive organs; stimulates growth of smooth muscle and development of epithelial lining of uterus and vagina	Involved in regulation of menstrual cycle in pre-meno-pausal women
Breast	Stimulates proliferation of glandular and ductal tissue in breast (trophic effect) and alveolar growth	Develops breast tissue in puberty secondary to onset of ovulation and ovarian production of estriol
Skin and muscle	Increases water and hyaluronic acid concentrations; alters collagen metabolism and decreases epithelial proliferation	Decreased estrogen induces wrinkles and vaginal atrophy in post-menopausal women
Bone	Decrease in bone reabsorption; increases bone mineral density	Increased risk of fracture postmenopausally
Liver	Stimulates production of sex hormone-binding globulin (SHBG); increases concentration of bile salts	Estrogen bound to SHBG is not bioavailable; this is a negative feedback system that maintains the status quo for the percent of bioavailable estrogen in plasma
Lipids	Increases synthesis of triglycerides, high-density lipoprotein (HDL) cholesterol, and clearance of low-density lipoprotein (LDL) cholesterol; suppresses hepatic lipase activity, which increases HDL	Improves lipid profile with undetermined effect on risk for atherosclerosis and/or cardiovascular disorders
Coagulation	Stimulates prothrombin and factors VII, VIII, IX, X; increases platelet adhesion; decreases antihrombin III	Increases proclotting factors and increases risk for thromboelomic events
Brain	CNS effects that are as yet no well documented but estrogen appears to facilitate verbal memory	

covalent binding of estrogen quinone metabolites to DNA; (b) enhancement of endogenous DNA adducts by chronic estrogen (c) free radical generation by metabolic redox cycling between quinone and hydroquinone forms of estrogens and free radical damage to DNA such as strand breakage, 8-hydroxylation of purine bases of DNA and lipid hydroperoxide-mediated DNA modification (63–66). Chemical carcinogens covalently bind to DNA to form two types of adducts: stable ones that remain in the DNA, unless removed by repair, and depurinating ones that are lost from the DNA by destabilization of the glycosyl bond. Evidence that depurinating polycyclic aromatic hydrocarbonand estrogen-DNA adducts play a major role in tumor initiation (56). Two different types of chromosomal damage have also been induced by estrogen in vivo and in cells in culture such as numerical chromosomal changes and also structural chromosomal aberrations. The estrone and estradiol can form catechol estrogen metabolites, catechol estrogen quinones, which react with DNA to form predominantly depurinating adducts. This may lead to mutations that initiate cancer (58). Experiments on estrogen metabolism (67, 68), formation of DNA adducts (69), carcinogenicity (70), mutagenicity (71) and cell transformation (72, 73) are in the centre of research.

SEPARATION SCIENCE AND LIFE CHEMISTRY

Sample preparation is an essential stage and belongs to a large field in analytical chemistry. The quality of sample preparation is a key factor in determining the success of analysis. It is used not only to extract traces of compounds (estrogens)

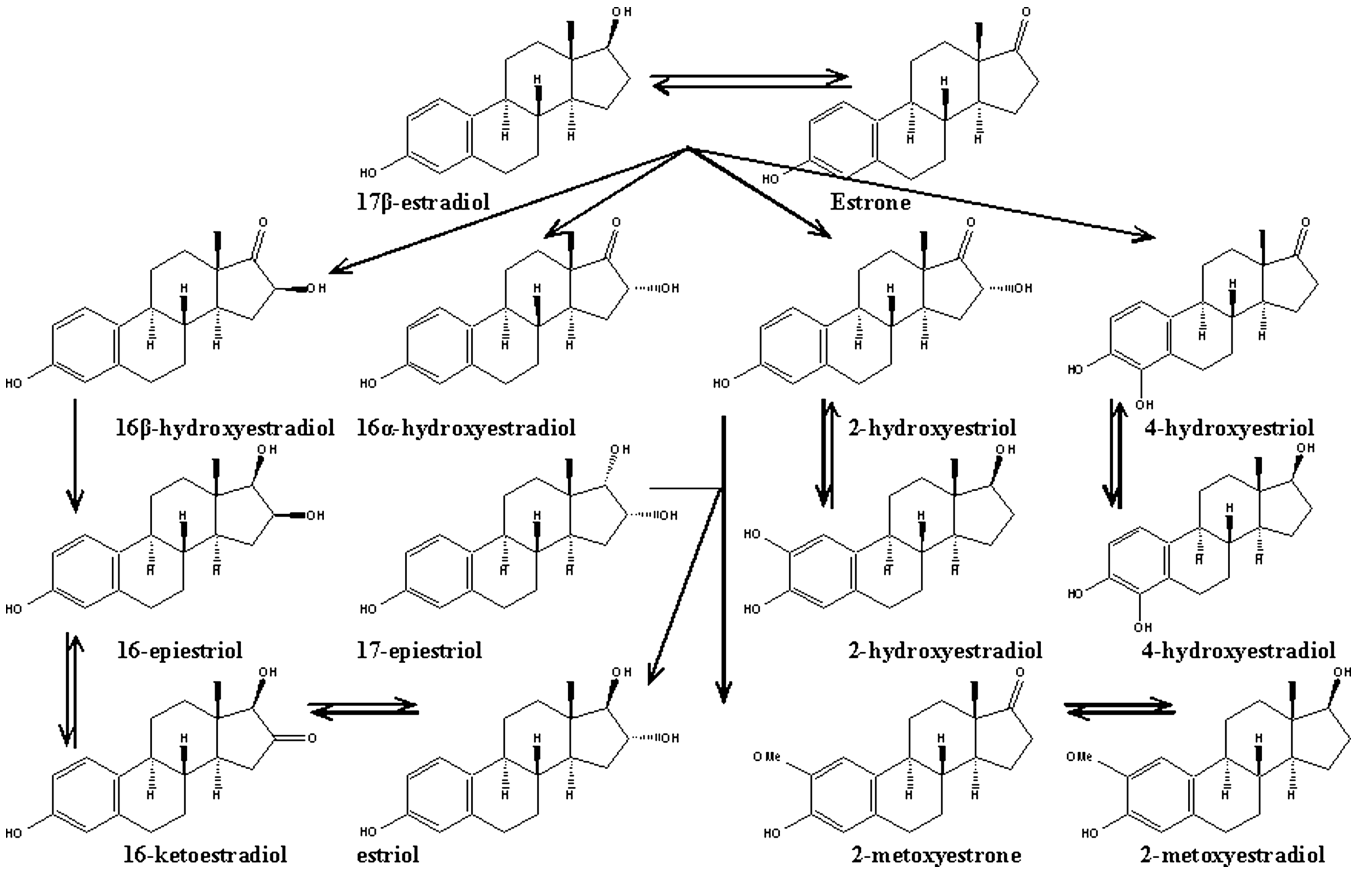

FIG. 7. The oxidative metabolism of estrogens in women ovaries.

from samples but also to remove interfering components from the matrix. An ideal sample preparation methodology should be fast, accurate, precise, and should consume little solvent. Other demands for modern extraction methods include sample integrity, high throughput, and compatibility with subsequent analysis (74–76). Solid-phase extraction (SPE) has gained in popularity and today is considered as being the technique of choice for sample work-up (77). For this reason SPE is the most widely useful preparation. This technique, in a variety of combinations, may be used to isolate and pre-treat (pre-clean) estrogens, especially 17β-estradiol, estrone, estriol and its medically significant metabolites such as 2-hydroxyestradiol, 4-hydroxyestradiol, 16α-hydroxyestradiol and etc (20, 78–80). Requirements for SPE of estrogens from different matrices such as blood, plasma, urine, and water may be certain to extend by automatization of all parts of analytical methods. The most important thing in SPE analysis of liquid biological samples is to prevent clogging of the sorbent. It should be pre-treated to disrupt of the estrogen-protein bindings to addition an equal amount of acetonitrile or methanol to the sample after the mixture is centrifuged before adding to an SPE device. Another procedure is to simply dilute the samples in a large quantity of buffer prior to sample application. Estrogens such as estradiol, estrone, and estriol, along with a few metabolites, are analyzed in urine, serum, blood, plasma, saliva and other liquid biological fluids by solvent partition, with polymer-based resins such as Amberlite XAD-2, DEAE-Sephadex and Sephadex resins bonded with various other function groups and with chemically bonded reversed-phase silicas by C_8, C_{18} and ion-exchange SPE columns for sample work-up (77, 81). The next convenient and portable solid extraction method is solid-phase microextraction (SPME) or headspace SPME which can be applied to the analyses of estrogens from complicated samples such as blood, plasma, serum, urine and aqueous samples like waste water or river water (82). SPME uses polymer coated fibers for estrogens, especially polyacrylate (PA), to extract analytes from aqueous and gaseous samples (83). Analytes are either desorbed thermally by exposing the fiber in the injection port of a gas chromatograph or chemically desorbed by liquid chromatography after extraction (84, 85). A New methods for the analysis of steroids and their significant metabolites is MIP in combination with SPE.

MIP AS DEDICATIVE SYSTEMS FOR ISOLATIONS

MIP-SPE is a class of smart sorbents for separation procedures and chemical analysis and exhibit high affinity and selectivity for the compound of interest (estrogens), which is used as template (86). Most of the biological processes are induced or governed by molecular recognition mechanisms based on weak

interactions such as immune-response, ligand–receptor interactions, etc., which involve biological hosts specifically binding to certain molecular species. Formation of a molecular print is based on the presence of specific and non-specific interactions (covalent and non-covalent imprinting). Two main approaches to molecular imprinting with a wide variety of modifications and combinations are published:

- The covalent (pre-organized) approach pioneered by Wulff and Sarhan. The complexes in solution prior to polymerization are maintained by (reversible) covalent bonds (87).
- The non-covalent (self-assembly) approach initially developed by Arshady and Mosbach (88). Pre-arrangement between the imprint antigen and the functional monomers is formed by non-covalent or metal coordination interactions.

The development of synthetic receptors capable of encapsulating target analytes with high affinity is therefore in demand, especially if natural receptors are not available, or laborious and expensive to isolate. Molecular imprinting technology (MIT) is an attractive synthetic approach to mimic natural molecular recognition (see in Fig. 8). Intermolecular forces that develop during polymerization between the template molecule, functional monomer and developing polymer matrix are responsible for creating a polymer micro-environment for the template or imprint molecule.

The mechanism of preparing MIPs is composed of several steps:

- pre-polymerization complex formation between template and functional monomers
- co-polymerization with an excess of cross-linking monomer
- extraction of template from imprinted binding site
- rebinding of template molecule to imprinted binding sites.

The resulting polymer network contains synthetic receptors that are complementary in size, shape and functional group orientation to the template molecule (89–92).

SPE materials are a promising and innovative application of MIPs. These polymers allow certain analytes to be selectively extracted from complex matrices without matrix interference. In MIP-SPE, the solid phase particle size is less critical, with main emphasis on:

- rapid and complete but separate elution of interferants and target analyte(s)
- elimination of template leaking from the MIP matrix, which is of particular importance when using MIP-SPE for pre-concentration in quantitative trace analysis.

Important factors in MIP efficiency are morphology of the synthesized MIP phase, accessibility of the recognition sites comprised within the polymer matrix, and preparation of MIPs requires careful selection of appropriate solvents.

Properties of appropriate solvents shall:

- preferably not interfere with and rather strengthen the intermolecular interactions between functional monomers and templates during the self assembly
- create porous structures within the synthesized polymer matrix
- support the preparation of MIPs as monolith or in bead format
- provide sufficient solubility for the template molecules and the involved polymeric building blocks (90, 93, 94).

These materials exhibit high physical and chemical resistance and are remarkably stable against mechanical stress, elevated temperatures and high pressures, resistant against treatment with acid, base, or metal ions and stable in a wide range of solvents. The storage life of the polymers is also very high: storage for several years at ambient temperature leads to no apparent reduction

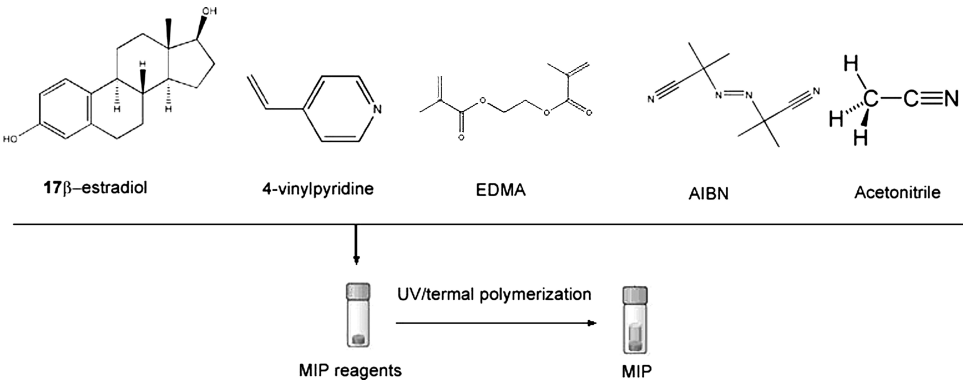

FIG. 8. The mechanism of formation of MIPs : Actual MIP polymer in which is used 17 estradiol as template, 4-vinylpyrolidine as functional monomer, ethylene glycol dimethacrylate (EDMA), as crosslinker monomer, 2,2′-azobis(isobutyronitrile) (AIBN) as initiator and acetonitrile as porogen.

TABLE 3
Molecular imprinting technology

Present Applications	References
Recognition studies Isolations (SPE)	(95–99)
Separations (chromatography)	
• Chiral	(100, 101)
• Substrate-selective	(102–106)
Antibody and receptor body mimics in	(100, 107–112)
• Competitive ligand binding assays	(109, 113–117)
• Diagnostic applications	(114, 118, 119)
Enzyme mimics in catalysis applications	(120–126)
Biosensor-like devices	(127–132)
Site-mediated synthesis	(133)

in performance. Further, the polymers can be used repeatedly, in excess of 100 times during periods of years without loss of the "memory effect." In comparison with natural biological recognition sites, which are often proteins, these properties are highly advantageous (94).

A wide range of compounds have been used as imprint antigens to investigate the feasibility of various practical applications (seen in Table 3). Applications in which 17β-estradiol and estrone are used as templates can see, in Table 4 (132–139). The newest molecular imprinting chromatography (MIC) has been the most extensively studied application area and several intriguing separations have been performed, which have exhibited high separation factors and resolutions. One advantage of imprinting for separations is that it allows the preparation of tailor-made supports with pre-determined selectivity.

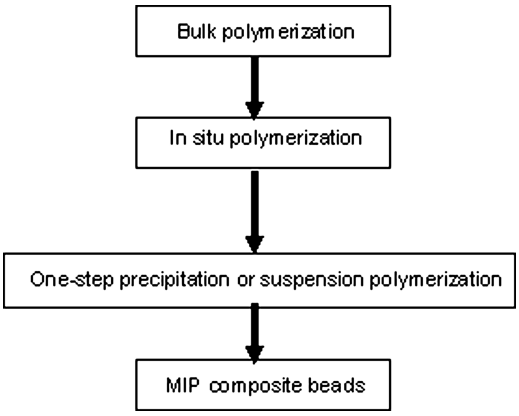

FIG. 9. MIP-HPLC polymerization methods for stationary phases.

Polymerization methods for MIP-based HPLC stationary phases are shown in Fig. 9. The future of MIT is in preparative scale separations, controlled-relaces matrices and equilibrium shifting (94).

FINAL DETERMINATION

Over recent years several techniques have been developed which interfaced continuous-flow biochemical detection with a range of analytical instruments, such as LC with different detectors: DAD (diode array detector), UV and MS. The combination of analytical technologies and continuous-flow biochemical detection has enabled biological and chemical evaluation of bioactive molecules within a single analysis and profoundly reduces the time required for compound characterization. Recent advances in this field as well as the application of continuous-flow biochemical detection for the screening of complex mixtures, such as estrogens, are reviewed in these paper (140). Steroid hormones, both natural and synthetic, can be found in

TABLE 4
MIP synthesis

Template	Monomer	Crosslinker	Porogen	References
17β-estradiol	TFMAA/MAA	TRIM	MeCN	(132)
	MAA/4-VP	EDMA	$CHCl_3$	(133)
	4-VP	EDMA	MeCN/$CHCl_3$	(134)
17β-estradiol	4-VP	EDMA	MeCN	(135)
	MAA	DVB	Acetone	(136)
	MAA	DVB	Acetone/MeCN (1:3, v/v)	
	MAA	EDMA	Toluene/MeCN (1:3, v/v)	
17β-estradiol, estrone	MAA	EDMA/TRIM	MeCN	(137–139)
	4-VP	EDMA/TRIM	MeCN	

[AIBN—2,2′-Azobis(isobutyronitrile), 4-VP—4-vinylpyridine, MAA—methacrylic acid, EDMA—ethylene glycol dimethacrylate, DVB—divinylbenzene, TRIM—trimethylolpropane trimethacrylate, TFMAA—2-(trifluormethyl)acrylic acid, MeCN—acetonitrile, $CHCl_3$—chloroform].

the environment as a result of human or animal excretion due to growing population concentration and intensive farming. Hormones, such as estradiol, estrone and ethynylestradiol, have been found in water at ng/L levels but, even at these low concentrations, some of them may induce estrogenic responses and cause adverse effects on aquatic and terrestrial organisms and on humans (141–145).

The separation methods routinely used in the laboratory include the various chromatographic and electrophoretic techniques. The techniques of LC (which are used in separation, include column chromatography (HPLC or simply GC) (34, 35, 146) and planar chromatography (thin-layer chromatography—TLC), over pressured thin-layer chromatography (OPLC) (39–42), and electro-migration techniques such as capillary electrochromatography (CEC) and capillary zone electrophoresis (CZE) (43, 44, 144) are also used in separation of biologically active compounds. The purpose of the current review is to present an overview of the available methodological developments for estrogen analysis with the main emphasis on HPLC methods.

HPLC is the most popular technique for the qualitative and quantitative analysis of a wide range of analytes. The determination of biologically active substances, especially 17β-estradiol, estrone, estriol and its medical important metabolites, often responsible for the proper function of natural living systems, is particularly important. Physico-chemical properties play an important role in governing the biological performance. However, these properties influence not only biological activity of a compound, but also chromatographic behavior. They are main deciding factors in the possible interactions between the analyte and the stationary phase surface and consequently the separation process. The development of chemically bonded stationary phases and column preparation techniques guarantees the high precision and efficiency of analysis. Physico-chemical properties such as matrix type, porosity, type and concentration of hydroxyl groups and metal impurities and the structure of chemically bonded ligands on the silica surfaces have been the subject of many publications. Determination of the structure and physico-chemical characteristics of adsorbents allowed the pattern of analyte molecule behavior during the chromatographic process to be predicted and provided some information about its quality (147, 148).

GC, in spite of other developments in analytical chemistry, remains one of the most frequently used analytical tools. It is applied in widely different areas such as medicine, biology, environmental sciences and, most notably, in an impressive number of industrial applications. No other analytical technique can provide the combination of resolving power with speed of analysis and sensitivity. The separation process in GC is influenced by polarity of the stationary phase, temperature, carrier gas flow, flow rate, column length, and amount of injected material.

Static headspace-gas chromatography (SHS-GC) is an indispensable technique for analyzing volatile organic compounds, enabling the analyst to assay a variety of sample matrices while avoiding the costly and time-consuming preparation involved with traditional GC (149).

LC-MS or GC-MS and other techniques have been developed for the determination of estrogens in water, sediment, tissue, plasma, urine and blood. Estradiol, estrone, estriol and its metabolites are described on a highly sensitive and specific quantification method of determination (150–155). Summary of these methods are shown in Table 5 (23, 84, 138, 142, 162–200).

Detection methods, which provide real information concerning the separated substances, are necessary in order to be able to analyze the separation result and separation performance achieved by such a system. LC detectors equipped with the flow-through cell was a major breakthrough in the development of modern liquid chromatography. They have high sensitivities often allowing the detection of nanograms of material, and the better models are very flexible, allowing rapid conversion from one mobile phase to another and from one mode to another. Almost all LC detectors are the on-stream monitors. Regardless of the principle of operation, an ideal LC detector should have the following properties: low drift and noise level, sensitivity, fast response, wide linear dynamic range, low dead volume, cell design which eliminates remixing of the separated bands, insensitivity to changes in type of solvent, flow rate, and temperature, operational simplicity and reliability, tunable, and non-destructive. The four dominant detectors are used in LC analysis: ultraviolet/visible spectroscopic detectors (UV absorbance detector, fixed and variable wavelength detectors, diode array detector), electrical conductivity detector (ECD; belonging to electrochemical detectors), and fluorescence detector (FLD) and refractive index detector (RID; deflection and reflective RI detectors). These detectors are employed in over 95% of all LC analytical applications (19, 155). For screening purposes, in stability studies, for initial method development, and for other applications where the limited sensitivity is sufficient, UV detection should be considered because of its ruggedness, ease of use and cost-efficiency. Estrogenic compounds are generally detected at 280 nm (75, 156). Electrochemical detectors are associated with low dead volumes, fast and linear response, and low cost. Electrochemical detection is not widely used in estrogen analysis despite the envisioned advantages. It has also been used, for example, in the quantification of 17β-estradiol and estrone in serum (157). An alternative detection mode for quantitation of estrogens is fluorescence detection. It is, as previously mentioned, a rather uncommon technique for quantification of medical, important metabolites of 17β-estradiol, estrone and estriol called hydroxyestrogens (158). MS detection is an analytical tool for estrogens quantitation. Two methods, atmospheric pressure chemical ionization (APCI) and electrospray ionization, are used in determination of estrogens (159). 17B-estradiol, estriol and estrone are determinated by LC-APCI-MS-MS (160, 161). The summary of applications of biologically active compounds—estrogens are in Table 5 below (162–200).

TABLE 5
Summary of estrogens applications

Matrix	Sample preparation	Method	References
Wastewater, surface water, river water	SPE	HPLC-DAD LC-MS/MS^2 RP-LC-MS/MS^2 RP-LC-ESI-MS/MS^2 RP-LC-APCI-MS/MS^2 GC-MS/MS^2 GC-EI-MS/MS^2	(162–171)
River water surface water wastewater	SPME	HPLC-DAD HPLC-FLD HPLC-ED LC-MS/MS^2 GC-MS/MS^2	(84, 172–177)
Wastewater river water surface water	MIP-SPE	HPLC-DAD HPLC-FLD LC-ESI-MS^2 LC-ECD	(138, 142, 178, 179)
Plasma serum, urine	SPE SPME MIP-SPE	HPLC-DAD LC-ECD GC-MS/MS^2 LC-MS/MS^2 LC-ESI-MS/MS^2 GC-SIM-MS GC-NICI-MS	(169, 180–192)
Blood, plasma	SPE, SPME, MIP-SPE	HPLC-DAD LC-ECD	(23, 193–195)
Tissue	SPE, SPME, MIP-SPE	HPLC-DAD LC-MS/MS^2 LC-ECD GC-ECD GC-MS	(196–200)

FINAL REMARKS

Estrogens belong to the steroid class and are produced by adrenal cortex, ovary and peripheral conversion in fat from androstenedione. Three of them are naturally produced in the female body (estradiol, estrone and estriol). Estrogens are metabolized into biologically less active or inactive forms via two mechanisms: 1) conjugation into water-soluble and no biologically active metabolites for excretion and 2) conversion into estrone or estriol, which are biologically active, but approximately ten times less potent than estradiol. The main mediators of estrogen action are estrogen receptors in human body. In summary, it is well appreciated that estrogens have profound influences in brain degeneration, osteoporosis, cardiovascular diseases, obesity and hormone-dependent cancers. It has become increasingly accepted that we must not only consider the parent estrogens, estradiol and estrone, when we evaluate disease risk but also the estrogen metabolites. For this reason it is very important to know contain in human body. The purpose of this review is to present an overview of the available methodological developments for estrogen analysis with the main emphasis on HPLC. Separated compounds can be identified indirectly and directly using photometry, electrochemistry, fluorescence or mass spectrometry. LC is considered as an equal separation technique. Presently, MIP technology is very popular in combination with HPLC. MIPs are prepared using conventional free radical bulk polymerization followed by grinding and sieving which are finally packed as stationary phase into HPLC or SPE columns. The use of MIPs for SPE and LC is at an early stage and there have been several successful approaches in bioanalysis and environmental analysis.

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