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SPECIAL FEATURE: PERSPECTIVE

Determination of estrogens and progestogens by mass spectrometric techniques (GC/MS, LC/MS and LC/MS/MS)

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Steroid sex hormones and related synthetic compounds have been shown to provoke alarming estrogenic effects in aquatic organisms, such as feminization, at very low concentrations (ng/L or pg/L). In this work, different chromatographic techniques, namely, gas chromatography/mass spectrometry (GC/MS), liquid chromatography/mass spectrometry (LC/MS) and liquid chromatography/tandem mass spectrometry (LC/MS/MS), are discussed for the analysis of estrogens, both free and conjugated, and progestogens, and the sensitivities achieved with the various techniques are inter-compared. GC/MS analyses are usually carried out after derivatization of the analytes with bis(trimethylsilyl)trifluoroacetamide (BSTFA). For LC/MS and LC/MS/MS analyses, different instruments, ionization techniques (electrospray (ESI) and atmospheric pressure chemical ionization (APCI)), ionization modes (negative ion (NI) and positive ion (PI)) and monitoring modes (selected ion monitoring (SIM) and selected reaction monitoring (SRM)) are generally employed. Based on sensitivity and selectivity, LC/ESI-MS/MS is generally the method of choice for determination of estrogens in the NI mode and of progestogens in the PI mode (instrumental detection limits (IDLs) 0.1–10 ng/mL). IDLs achieved by LC/ESI-MS in the SIM mode and by LC/ESI-MS/MS in the SRM mode were, in general, comparable, although the selectivity of the latter is significantly higher and essential to avoid false positive determinations in the analysis of real samples. Conclusions and future perspectives are outlined. Copyright © 2003 John Wiley & Sons, Ltd.

INTRODUCTION

During the last decades, a large number of chemicals with proved acute toxicity, mutagenicity or carcinogenic effects have been released into the environment, as a result of anthropogenic activities. While these compounds will forever be of concern, in the last ten years there has been increased interest in endocrine disrupting compounds (EDCs). EDCs interfere (disrupt) normal hormonal functions (development, growth and reproduction) and cause dangerous consequences to humans and wildlife, such as hermaphroditism, decreased fertility and feminization,[1] even at concentration levels as low as pg-ng/L water. As time goes on, and information on the origin, occurrence and effects of EDCs grows, the number of chemicals recognized as EDCs increases. Compounds that have been determined to be estrogenic include both natural (such as phytoestrogens, found in many plants including rice, carrots, potatoes and apples, among others) and synthetic estrogens (such as those used in birth control pills), phthalates, pesticides, dioxins, surfactants and polychlorinated biphenyls (PCBs). Among EDCs, progestogens, and especially estrogens, are of particular interest because of their high estrogenic potency and the extent of their use, not only as contraceptives, but also for therapeutic purposes, such as in the management of the menopausal syndrome or in diverse cancers, such as prostatic and breast cancer (see Fig. 1).

The analytical determination of micropollutants in the environment is not an easy task, first, because of the complexity of the environmental matrices, and second, because of the usually extremely low concentrations of the target compounds. Thus, to achieve the sensitivity and selectivity necessary for their analysis at physiologically active concentrations (pg-ng/L in water), quite laborious and time-consuming procedures are required. For many years, the environmental determination of steroid sex hormones has been dominated by the use of biological techniques, such as immunoassays, and gas chromatography/mass spectrometry (GC/MS).[2–4] However, recently,

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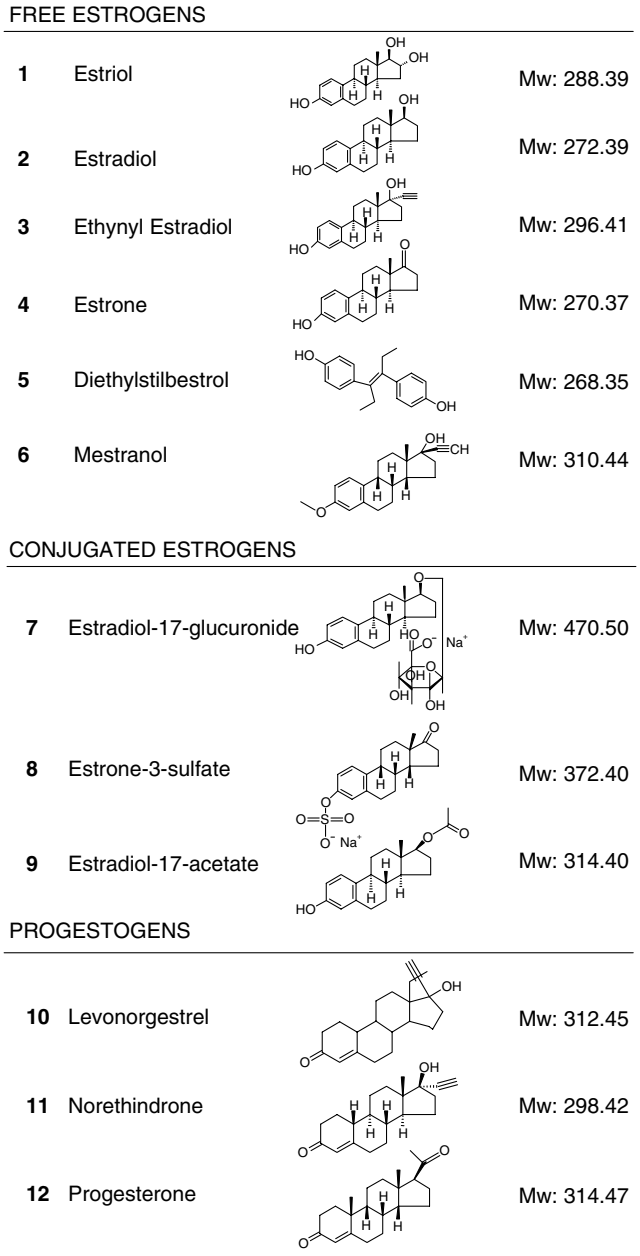

Figure 1. Molecular structures of the natural and synthetic estrogens and progestogens investigated.

the application of liquid chromatography/mass spectrometry (LC/MS) has experienced rapid growth, in part due to the instrumental developments afforded in this field.[5] The introduction of tandem mass spectrometry coupled to liquid chromatography (LC/MS/MS) has largely improved the performance of the technique by reducing the detection and quantification limits and enhancing analyte identification.

In this paper different mass spectrometric approaches for the analysis of estrogens (both free and conjugated) and progestogens are discussed. The goal is to compare LC/MS/MS in terms of sensitivity with other techniques commonly employed for this purpose, such as GC/MS and LC/MS.

Analysis by GC/MS

Methods published for the determination of steroid sex hormones in aquatic environmental samples are frequently based on solid-phase extraction, silylation and detection by GC/MS.[4] Previous to GC/MS analysis, steroids are usually derivatized. The derivatization is carried out in the case of thermolabile, polar, and low volatile compounds, such as estrogens, to avoid thermal decomposition and to improve the chromatographic separation and the sensitivity of analysis. For derivatization of the hydroxyl functional groups present in the molecular structure of steroids, diverse derivatization agents, such as *N*-methyl-*N*-(*tert*-butyldimethylsilyltrifluoracetamide) (MTBSTFA), BSTFA, and pentafluoropropionic acid anhydride (PFPA), have been used.[2] *tert*-Butyldimethylsilyl (*tert*-BDMS) and pentafluoropropionyl (PFP) derivatives are formed more quickly and are less sensitive to hydrolysis than many silyl derivatives, e.g., trimethylsilyl (TMS) derivatives.[6,7] However, when using these reagents, partial derivatization, and in many cases derivatization in only the hydroxyl group of the unsaturated ring, occurs, which, in turn, results in less improvement in the sensitivity.

Using BSTFA, all hydroxyl groups (-OH) in the estrogens investigated, except mestranol, were observed to react readily with the derivatization agent to yield the corresponding trimethylsilyl (-OSi$(CH_3)_3$) ethers, as evidenced by molecular ions at m/z 412, 342, 416, 440, and 504 for diethylstilbestrol, estrone, estradiol, ethynyl estradiol, and estriol, respectively. Mestranol, as well as progestogens, did not react with the derivatization reagent. This was not surprising in the case of progesterone, as this compound do not present any hydroxyl functional group in its structure (see Fig. 1). However, in the case of levonorgestrel, norethindrone, and especially mestranol (the 3-methyl ether derivative of ethynyl estradiol), derivatization could have taken place, as observed for ethynyl estradiol, in the 17-OH group. The inability to substitute the 17-OH group of estrogens presenting an ethynyl group at the same location has been reported by other authors using different derivatization agents (PFPA[6] MTBSTFA[7]); however, this phenomenon was common to both mestranol and ethynyl estradiol. Major ions for estrogens, with the exception of mestranol, corresponded to the derivative form, whereas the progestogen group and mestranol were detected as non-derivatized compounds. Diethylstilbestrol shows two different peaks at retention times 15.8 and 17.4 min, which probably correspond to two different isomeric forms of the compound.[8]

Table 1 compiles the detection limits achieved with the various MS techniques in the analysis of estrogens and progestogens. As can be seen, GC/MS detection limits ranged between 1 and 20 ng/mL, with the lowest values corresponding to estrogens. The fact that ions of much higher m/z with improved chromatographic peak shape are formed for the estrogens derivatives compared with the parent compounds contributes to these low detection limits.

Analysis by LC/MS and LC/MS/MS

In a previous work in our laboratory using a single-quadrupole MS instrument, ESI was found to afford detection limits for progestogens about one order of magnitude better than those achieved with APCI (both in

Table 1. Instrumental detection limits (ng/mL) achieved in the analysis of estrogens, including conjugated ones, and progestogens by different MS chromatographic techniques

		LC/MS (SIM)			LC/MS/MS (SRM)	
		ESI(NI)		APCI(PI)	ESI(NI)	APCI(PI)
Compound	GC/MS (SIM)	Single-quad	Triple-quad		Triple-quad	
Estriol	1	0.5	0.5	nd	1	nd
Estradiol	3	10	1	100	1	nd
Ethynyl estradiol	10	10	1	nd	2	nd
Estrone	1	5	1	400	1	nd
Diethylstilbestrol	3	1	0.1	nd	0.5	nd
Mestranol	7	nd	nd	nd	nd	nd
Norethindrone	5	0.4*	10*	20	10*	400
Levonorgestrel	20	0.4*	10*	10	10*	40
Progesterone	5	0.4*	20*	nd	10*	nd
Estradiol-17-gluc.			10	200	5	nd
Estrone-3-sulfate			0.1	200	0.1	nd
Estradiol-17-acetate			1	5	1	5

nd, >1000 ng/mL; * , (PI).

Table 2. Time-scheduled SIM conditions, *m/z* ions and corresponding structures used in the LC/ESI/MS analysis of estrogens and progestogens (listed in LC elution order)

Compound	Time (min)	Ion mode	M_W	m/z	Corresponding structure
Estriol	00.0	NI	288	287	$[M-H]^-$
Estradiol	18.0	NI	272	271	$[M-H]^-$
Ethynyl estradiol		NI	296	295	$[M-H]^-$
Estrone		NI	270	269	$[M-H]^-$
Diethylstilbestrol		NI	268	267	$[M-H]^-$
Norethindrone	00.0	PI	298	321	$[M+Na]^+$
Levonorgestrel	24.0	PI	312	335	$[M+Na]^+$
Progesterone	28.0	PI	314	337	$[M+Na]^+$

the PI mode), and to be the only ionization mode capable of detecting estrogens in the ng/mL range (in the NI mode).[8] However, a few works have recently reported the use of LC/APCI(PI)-MS/MS for analysis of estrogens in water with sensitivities comparable to those achieved in many cases by LC/ESI(NI)-MS/MS.[9–11] All target compounds undergo very light fragmentation in the (first) quadrupole analyzer showing only one predominant ion. In LC/ESI-MS and LC/ESI-MS/MS, this predominant ion (used as base peak in the SIM mode or as precursor ion in the SRM mode) corresponded in the case of estrogens to the deprotonated molecule, $[M-H]^-$, regardless of the instrument used; however, in the case of progestogens, it varied depending on the instrument used (Tables 2–4). Thus, in the single-quadrupole instrument, the major ion found for progestogens corresponded to adducts of the analyte molecule with a sodium atom $[M+Na]^+$ (see Table 2), whereas in the triple-quadrupole mass spectrometer the most abundant ion was the protonated molecule $[M+H]^+$ (see Table 3). The synthetic estrogen mestranol could not be detected with either ESI or APCI.

With regards to sensitivity, slightly different detection limits can be obtained from analyses carried out by LC/ESI-MS in the SIM mode depending on the instrument employed (see Table 1). Thus, for estrogens, detection limits 1–10 times lower with the triple-quadrupole operating in the SIM mode (0.1–10 μg/L) than with the single-quadrupole (1–10 μg/L) were achieved, whereas, for progestogens, the sensitivity furnished with the single-quadrupole mass spectrometer (0.4 μg/L) was better than that attained with the triple-quadrupole mass spectrometer in the SIM mode (10–20 ng/mL). This observed difference in sensitivity in the case of progestogens may also be due to the different base peaks monitored with each instrument: $[M+Na]^+$ in the single-quadrupole (an HP1100 MSD from Agilent), and $[M+H]^+$ in the triple-quadrupole mass spectrometer (a Quatro LC from Micromass).

In the analyses of the target compounds by LC/ESI-MS/MS, two different SRM transitions can be monitored for each compound: the first one in Table 3, and more abundant, for quantification, and the second one for confirmation. Figure 2 illustrates the reconstructed ion chromatograms

Table 3. Time-scheduled SRM conditions used in the LC/ESI-MS/MS analysis of estrogens, including conjugated ones (NI mode), and progestogens (PI mode)

Compound	Time (min)	SRM transitions (m/z) Precursor ion → Product ion	Cone (V)	Coll. (eV)
Estradiol-17-gluc.	00.0–12.5	447 → 113	40	30
		447 → 271	40	50
Estrone-3-sulfate		349 → 269	40	40
		349 → 145	40	40
Estriol		287 → 171	50	40
		287 → 145	50	40
Estradiol	12.5–20.5	271 → 145	50	45
		271 → 183	50	45
Ethynyl estradiol		295 → 145	50	40
		295 → 159	50	40
Estrone		269 → 145	50	40
		269 → 143	50	45
Diethylstilbestrol		267 → 222	30	35
		267 → 237	30	50
Estradiol-17-acetate	20.5–25.0	313 → 253	30	30
		313 → 145	30	45
Norethindrone	00.0–15.3	299 → 171	30	20
		299 → 145	30	20
Levonorgestrel	15.3–19.0	313 → 245	30	20
		313 → 185	30	20
Progesterone	19.0–25.0	315 → 297	30	15
		315 → 279	30	15

Table 4. Time-scheduled SRM conditions used in the LC/APCI(PI)-MS/MS analysis of estrogens, including conjugated ones, and progestogens

Compound	Time (min)	SRM transitions (m/z) Precursor ion → Product ion	Cone (V)	Coll. (eV)
Estradiol-17-gluc.	00.0–19.5	534 → 493	40	3
		534 → 330	40	3
Estrone-3-sulfate		477 → 339	20	50
		477 → 445	20	100
Estradiol	19.5–29.0	314 → 287	50	30
		314 → 263	50	100
Norethindrone		340 → 299	35	3
		—		
Estrone		312 → 207	30	3
		—		
Levonorgestrel	29.0–35.0	354 → 313	35	5
		354 → 216	35	5
Estradiol-17-acetate		356 → 315	35	10
		—		

obtained in the analysis of a standard mixture of the analytes at the SRM transitions used for quantification.

The LC/ESI(NI)-MS/MS analysis of all free estrogens reported in this study, with the exception of diethylstilbestrol, has already been addressed.[12–14] As interpreted in these reports, estradiol shows losses consistent with ring cleavages (i.e., losses of $C_5H_{12}O$ and $C_8H_{14}O$) to give major product ions at m/z 183 and 145, respectively. The $[M - H]^-$ ion from estriol at m/z 287 gives major product ions at m/z 171 and 145 upon excitation. These ions correspond to losses of $C_6H_{12}O_2$ and $C_8H_{14}O_2$, respectively, from the steroidal ring system. The $[M - H]^-$ ion from estrone at m/z 269 gives major product ions at m/z 145 and 143 (loss of $C_8H_{12}O$ and $C_8H_{14}O$) and the $[M - H]^-$ ion from ethynyl estradiol gives major fragment ions at m/z 159 and 145, from what are believed to be losses of $C_9H_{12}O$ and $C_{10}H_{14}O$, respectively.

Diethylstilbestrol was used in the past for growth-promoting purposes in livestock and as anti-abortive in

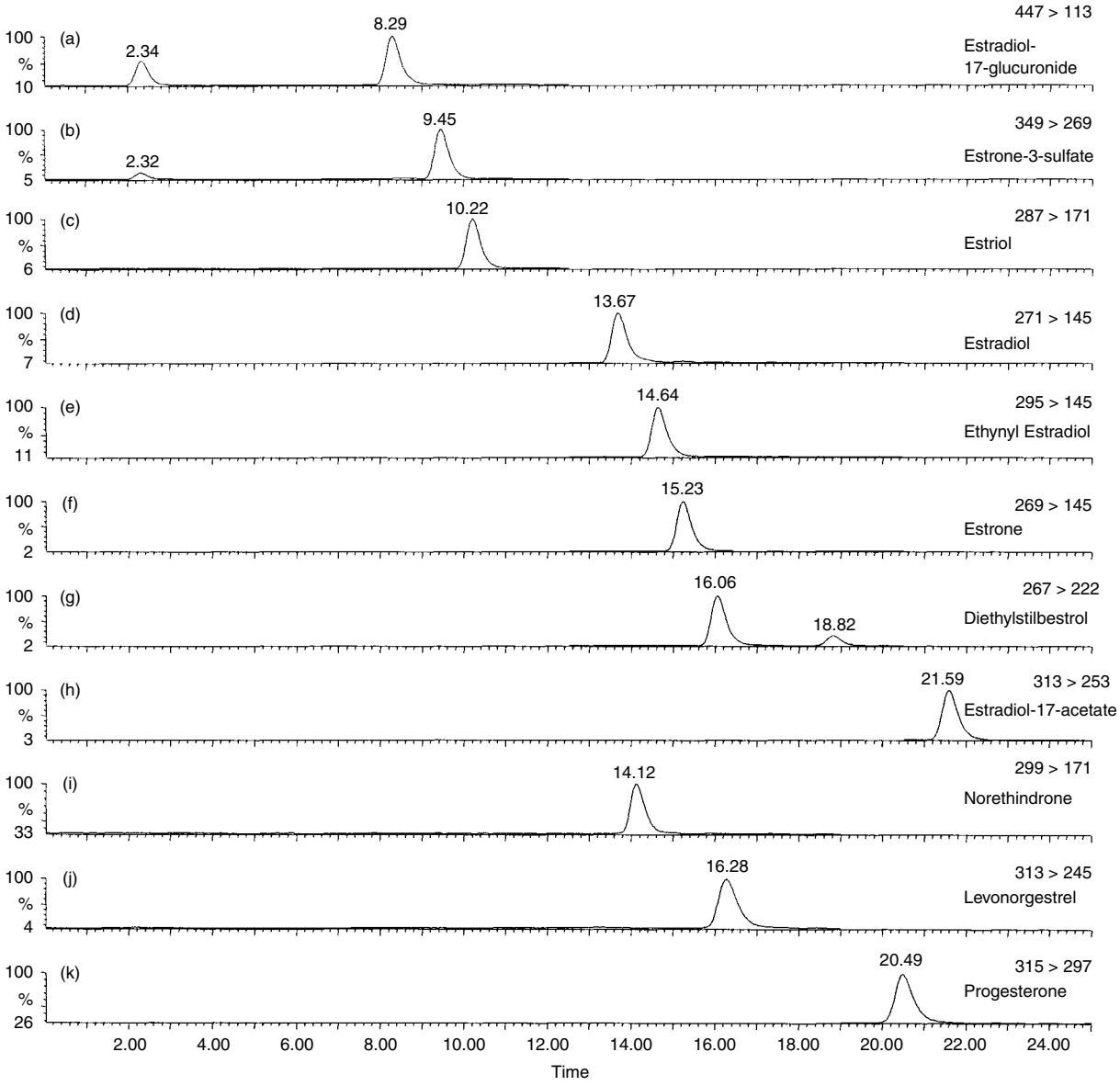

Figure 2. SRM chromatograms corresponding to the LC/ESI-MS/MS analysis of a 100 ng/mL standard mixture of estrogens (a–i) and progestogens (j–k) Column: Purospher STAR RP-18e.

humans, but the FDA banned its use in 1971. In Spain, its application has been limited in the last years to the treatment of urinary incontinence in castrated dogs. However, according to a recent study, this compound is still present in some Spanish rivers, due to either its previous or its current fraudulent use,[15] and therefore this compound was included in the list of target analytes. As indicated in Table 3, the SRM transitions 267 → 222 and 267 → 237 should be selected for quantification and confirmation of diethylstilbestrol by LC/ESI(NI)-MS/MS.

As compared to free estrogens, conjugated estrogens have been much less studied, and their analysis by LC/MS/MS has been attempted on only two occasions.[13,16] These compounds, likewise the unconjugated ones, present as base peak in the SIM mode and as precursor ion in the SRM mode, the singly charged molecular anion. Estrone-3-sulfate produced after dissociation a major fragment ion corresponding to the free estrogen ($[M - 80]^-$) at m/z 269, and another, less abundant, fragment ion characteristic for estrone at m/z 145, whereas estradiol-17-glucuronide gave major product ions at m/z 113, corresponding to a characteristic fragment of the glucuronide ring (base peak), and at m/z 271, corresponding to the free estradiol ($[M - 176]^-$). In the fragmentation of estradiol-17-acetate the major product ions observed at m/z 253 and 145 can be easily identified as the parent compound without the acetate group and as the same fragment ion previously identified in the collision induced dissociation (CID) spectrum of estradiol related to the loss of $C_8H_{14}O$ from the steroidal ring, respectively.

The base peak observed for progestogens when analyzed by ESI with the triple-quadrupole mass spectrometer corresponds as previously mentioned to the protonated molecule. After CID, the $[M + H]^+$ ion of progesterone gave major fragment ions at m/z 297 and 279, indicating the loss of one and two water molecules, respectively (see Fig. 3). Major

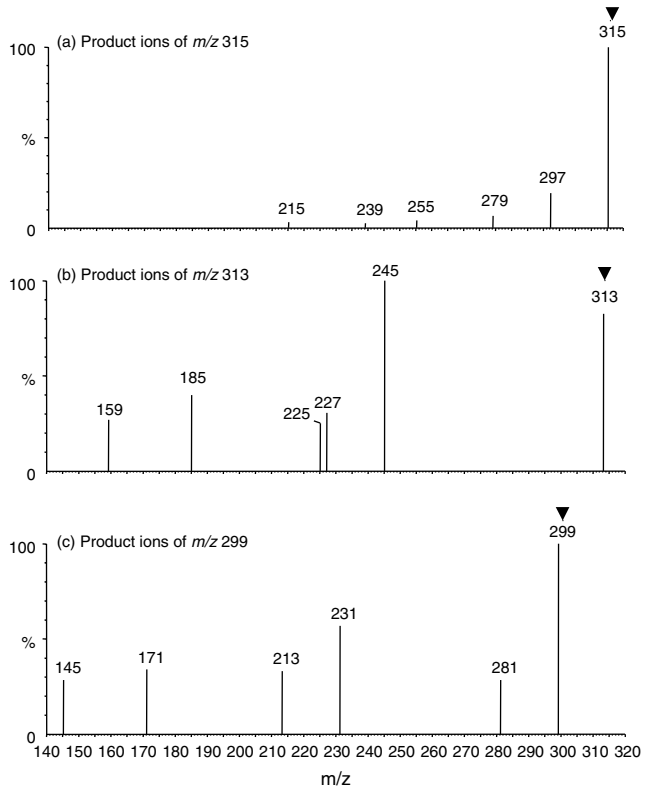

Figure 3. Product ion mass spectra obtained for (a) progesterone, (b) levonorgestrel, and (c) norethindrone by LC/ESI(PI)-MS/MS. The corresponding precursor ions are indicated with a vertical arrow.

product ions for levonorgestrel are at m/z 245, which corresponds to the steroidal ring without substituents, and at m/z 185, which relates to loss of $C_8H_{16}O$ from the steroidal ring system. The spectrum for norethindrone showed losses consistent with ring cleavages to give major product ions at m/z 171 and 145.

Application to real-world environmental samples

Under these selected SRM conditions, detection limits calculated for estrogens, including conjugated ones, varied between 0.1 and 5 ng/mL, and were somewhat higher for progestogens (10 ng/mL, see Table 1). These detection limits are similar to, or differ very little from, those achieved in the SIM mode with the same instrument (0.1–10 ng/mL for estrogens, 10–20 ng/mL for progestogens), i.e., the instrumental sensitivity achieved in both monitoring modes (SIM and SRM) is approximately the same. However, this does not mean that LC/MS methods can compete in terms of sensitivity and selectivity with LC/MS/MS methods in the analysis of real, complex samples, such as the environmental ones. In this context, LC/MS/MS is markedly superior, as is well known, to the former. To set an example, in a recent monitoring study carried out by our research group to determine the presence of both free and conjugated estrogens in different river waters from the area of Catalonia, eight samples out of eleven were found to contain traces of these compounds when analyzed by LC/ES(NI)-MS. However, further analysis of the positive samples by LC/ESI(NI)-MS/MS indicated that only two of them did, in fact, contain estrogens (unpublished data). In order to avoid false positives it is very important to monitor two different, characteristic SRM transitions for each compound and to consider the following confirmation criteria:

1. LC retention must be within 1–2% of the retention time of the standard compound.
2. The relative abundances of at least two selected precursor ion-product ion transitions must be within 20% of the ions ratios obtained for the standards.

With regards to LC/APCI(PI)-MS/MS analysis, even after careful optimization of the experimental conditions, only a few compounds can be detected and at considerably high concentrations (see Table 4). The base peak observed for all detected compounds, except estradiol-17-glucuronide and estrone-3-sulfate, corresponds to the solvent adduct ion $[M + H + CH_3CN]^+$ at m/z $[M + 42]^+$. Major ions for estradiol-17-glucuronide at m/z 534 ($[M + 105]^+$) and for estrone-3-sulfate at m/z 477 ($[M + 64]^+$) were identified as the solvent adduct ions $[M + Na + 2CH_3CN]^+$ and $[M + Na + CH_3CN]^+$, respectively. These ions, however, do not agree with those reported in previous works, where the base peaks used for quantification in SIM mode, or as precursor ion for CID in the SRM mode, were $[M + H - H_2O]^+$ for estriol, estradiol, and ethynyl estradiol, and $[M + H]^+$ for estrone, even though gradient acetonitrile/water was used also as mobile phase.[9–11] These discrepancies in the most abundant ions observed, also pointed out for progestogens when analyzed by LC/APCI-MS (base peak $[M + H]^+$) and by LC/ESI-MS with the single quadrupole (base peak $[M + Na]^+$) and the triple quadrupole (base peak $[M + H]^+$), indicate that not only the ionization technique (ESI or APCI), but also the design of the interface (orthogonal in the single quadrupole, Z-spray in the triple quadrupole) and the source designed by different manufacturers (the single quadrupole was from Agilent and the triple quadrupole from Micromass) may drive the formation of different base peaks.

In the SRM mode, only four compounds, namely, estrone-3-sulfate, estradiol-17-glucuronide, estradiol, and levonorgestrel, could be characterized with the required two SRM transitions. The sensitivity achieved with APCI in the SIM mode is either equal to or better than that attained in the SRM mode, and in any case it was comparable to that achieved with ESI. In this respect, it should be pointed out that in the methodologies previously published reporting considerably low levels of detection with APCI, both the concentration factor of the method (2000) and the volume of extract injected (100 μL) were considerably higher than those commonly used in LC/MS and LC/MS/MS methods.

CONCLUSIONS AND FUTURE PERSPECTIVES

The complexity of the analysis of progestogens and estrogens indicates that LC/ESI-MS/MS is the method of choice. The analysis of estrogens (both free and conjugated) is better achieved by using the negative ionization mode whereas progestogens should be better analyzed in the positive ionization mode. GC/MS allows sensitivities (1–20 ng/mL) comparable to or slightly higher than those of LC/ESI-MS/MS in SRM mode (0.1–10 ng/mL), but presents the

drawback of derivatization. The detection limits achieved by LC/ESI-MS (SIM mode) vary slightly depending on the compound and the instrument used: 0.4–10 ng/mL with the single-quadrupole and 0.1–20 ng/mL with the triple-quadrupole instrument. Instrumental sensitivities achieved by LC/ESI-MS in the SIM mode and by LC/ESI-MS/MS in the SRM mode are in general comparable, although the selectivity of the latter is much higher and almost essential to avoid false positive results when real samples are analyzed. The analysis of these analytes in real-world environmental samples requires, apart from an excellent analytical determination like the use of MS/MS, also appropriate sample preparation. This is due to the fact that steroid sex hormones are the most potent endocrine disrupting compounds and are already active at the very low limits of the ng/L level. This implies the use of sophisticated analytical procedures. The introduction of new LC/MS capabilities, like the new generation of LC/ion trap mass spectrometers, may be of help to allow the routine determination of low ng/L levels of steroid sex hormones in real-world aquatic environmental samples.

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REFERENCES

1. Colborn T, vom Saal FS, Soto AM. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ. Health Perspect.* 1993; **101**: 378.
2. López de Alda MJ, Barceló D. Review of analytical methods for the determination of estrogens and progestogens in wastewaters. *Fresenius J. Anal. Chem.* 2001; **371**: 437.
3. Petrovic M, Eljarrat E, López de Alda MJ, Barceló D. Analysis and environmental levels of endocrine-disrupting compounds in freshwater sediments. *Trends Anal. Chem.* 2001; **20**: 637.
4. Petrovic M, Eljarrat E, López de Alda MJ, Barceló D. Recent advances in the mass spectrometric analysis related to Endocrine disrupting Compounds in aquatic environmental samples. *J. Chromatogr. A* 2002; **974**: 23.
5. López de Alda MJ, Díaz-Cruz S, Petrovic M, Barceló D. A review. LC-MS and LC-MS-MS analysis of selected emerging pollutants (steroid sex hormones, drugs and alkylphenolic surfactants) in the aquatic environment. *J. Chromatogr. A* 2003; **1000**: 503.
6. Mol HGJ, Sunarto S, Steijger OM. Determination of endocrine disruptors in water after derivatization with N-methyl-N-(*tert*-butyldimethyltrifluoroacetamide) using gas chromatography with mass spectrometric detection. *J. Chromatogr. A* 2000; **879**: 97.
7. Lee HB, Peart TE. Determination of 17β-estradiol and its metabolites in Sewage effluent by solid-phase extraction and gas chromatography/mass spectrometry. *J. AOAC Int.* 1998; **81**: 1209.
8. López de Alda MJ, Barceló D. Determination of steroid sex hormones and related synthetic compounds considered as endocrine disrupters in water by liquid chromatography-diode array detection-mass spectrometry. *J. Chromatogr. A* 2000; **892**: 391.
9. Seifert M, Brenner-Weiß G, Haindl S, Nusser M, Obst U, Hock B. A new concept for the bioeffects-related analysis of xenoestrogens: hyphenation of receptor assays with LC-MS. *Fresenius J. Anal. Chem.* 1999; **363**: 767.
10. Laganà A, Bacaloni A, Fago G, Marino A. Trace analysis of estrogenic chemicals in sewage efluent using liquid chromatography combined with tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* 2000; **14**: 401.
11. Rose J, Holbech H, Lindholst C, Norum U, Povlsen A, Korsgaard B, Bjerregaard P. Vitellogenin induction by 17β-estradiol and 17α-ethinylestradiol in male zebrafish (Danio Rerio). *Comp. Biochem. Physiol. C* 2002; **131**: 531.
12. Baronti C, Curini R, D'Ascenzo G, Di Corcia A, Gentili A, Samperi R. Monitoring natural and synthetic estrogens at activated sludge sewage treatment plants and in a receiving river water. *Environ. Sci. Technol.* 2000; **34**: 5059.
13. Gentili A, Perret D, Marchese S, Mastropasqua R, Curini R, Di Corcia A. Analysis of free estrogens and their conjugates in sewage and river waters by solid-phase extraction then liquid chromatography-electrospray-tandem mass spectrometry. *Chromatographia* 2002; **56**: 25.
14. Croley TR, Hughes RJ, Koenig BG, Metcalfe CD, March RE. Mass spectrometry applied to the analysis of estrogens in the environment. *Rapid Commun. Mass Spectrom.* 2000; **14**: 1087.
15. López de Alda MJ, Gil A, Paz E, Barceló D. Determination of estrogens and progestogens in river sediments by liquid chromatography/mass spectrometry. *Analyst* 2002; **127**: 1279.
16. Isobe T, Shiraishi H, Yasuda M, Shinoda A, Suzuki H, Morita M. Determination of estrogens and their conjugates in water using solid-phase extraction followed by liquid-chromatography-tandem mass spectrometry. *J. Chromatogr. A* 2003; **984**: 195.