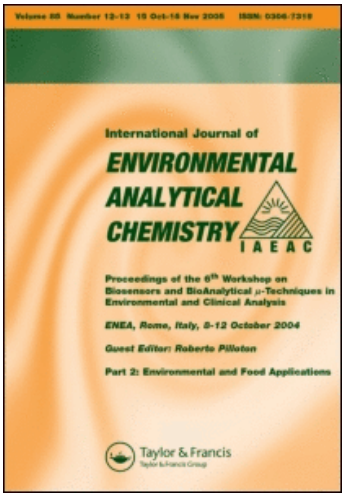

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A new method for monitoring oestrogens, *N*-octylphenol, and bisphenol A in wastewater treatment plants by solid-phase extraction-gas chromatography-tandem mass spectrometry

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A new method for monitoring oestrogens, *N*-octylphenol, and bisphenol A in wastewater treatment plants by solid-phase extraction–gas chromatography–tandem mass spectrometry

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A new analytical method, based on solid-phase extraction with Oasis HLB sorbent and gas chromatography tandem mass spectrometry, has been described for the simultaneous determination of various oestrogens (oestrone, oestradiol, ethynyloestradiol, and diethylstilboestrol) and other compounds considered as endocrine disrupters (bisphenol A and 4-octylphenol) in wastewater. The usual derivatization step is avoided so as to provide a faster and easy method, which is very suitable for routine monitoring. The injection of high sample volumes (8 μL) allowed determination limits in the range 2–20 ng L^{-1}. Recovery percentages ranged from 75 to 99% (except for 4-octylphenol, 30%) with RSDs < 9%. The method was applied to water samples, from raw influents and treated effluents, from July 2003 to April 2004. Bisphenol A (<1.7 μg L^{-1}) and oestrone (<0.2 μg L^{-1}) were usually found, indicating partial elimination during wastewater treatment.

Keywords: Endocrine disruptors; Wastewater; Gas chromatography–tandem mass spectrometry; Oestrogens; Sewage-treatment plants; Bisphenol A

1. Introduction

The number of compounds in the environment that have been recognized as endocrine disrupting chemicals (EDCs), have been growing fast [1–3] in recent years. These compounds can affect the endocrine system of humans and wildlife. Although there is laboratory and experimental evidence that chemical exposure can be associated with adverse developmental and reproductive effects on fish and wildlife [4, 5], the relationship of human diseases and exposure to environmental contaminants is still poorly understood and scientifically controversial [6]. However, recently it has been hypothesized that the decrease in sperm counts over the last decades, increasing

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incidents of testicular cancer, and other disorders regarding male infertility may be caused by the intake of oestrogens via food or drinking water [7].

From the wide variety of pollutants considered as potential EDCs (pesticides, PAHs, phthalate plasticizers, certain polychlorinated biphenyls, dioxins, furans, alkylphenols polyethoxylates, and steroids), we have directed our attention to a selected group of them, because of their endocrine-disrupting potential and their occurrence in natural waters, as reported in previous studies [8–10]. This group includes both natural oestrogens (17β-oestradiol, oestrona) and synthetic oestrogens used in medicine, as contraceptives and in hormonal therapies (17α-ethynyloestradiol), or in veterinary applications, such as growth promoters in farm animals (diethylstilboestrol). In addition, chemicals from household or industrial processes, such as alkylphenols polyethoxylates (4-octylphenol) and bisphenol A, have also been selected.

One of the main sources of natural water pollution by these EDCs is the sewage-treatment plant (STP). It has been reported that, because of incomplete removal or formation of an active form during the process of sewage treatment, endocrine disrupters are released in surface water or adsorbed onto sewage sludges or sediments [11]. The occurrence of these compounds in STP samples has recently raised the attention of the regulatory agencies (including the US EPA) about their impact in the aquatic environment. APEOn have recently been included as priority substances in the field of water policy, and octylphenols will be subject to a review for identification as possible 'priority hazardous substance' (Decision No. 2455/2001/EC) [12]. By contrast, no European water-quality guidelines have yet been developed for BPA and oestrogens.

The very low concentrations (in the ng L^{-1} range) in which these compounds show activity as endocrine disrupters [5] have stimulated the development of sensitive and specific methodologies for their determination. In most cases, SPE and, to a lesser extent, SPME are used to isolate and concentrate alkylphenolic compounds and steroid sex hormones from different aqueous matrices [13, 14].

Although some papers have reported extremely sensitive methods using LC-MS with ESI or APCI detection [15], or LC-MS-MS [16], EDCs are generally determined by GC-MS [13, 14] in environmental analyses. However, owing to the poor volatility of some compounds and the presence of various polar groups in the molecule, derivatization steps, aimed at producing more volatile products and improving the sensitivity of subsequent GC analyses, have been applied. Thus, the advantages of a better sensitivity are sometimes largely offset by sample loss during the additional manipulation [17]. To our knowledge, all the analytical methods proposed in the literature apply derivatization procedures before GC-MS analysis.

In this study, a method that obviates this tedious and critical step, based on SPE and direct analysis of the extract by GC-MS/MS, has been developed. To achieve lower quantitation limits, large-volume injections have been used. The use of tandem mass spectrometry also provides the additional specificity necessary when analysing complex samples, and methods based on MS/MS detection have been reported to be approximately 10 times more sensitive than MS detection for treated effluent [18].

With the application of the proposed method, a monitoring programme was performed in two STPs in Almería (a province in the south of Spain) in order to evaluate (1) the occurrence of the targeted compounds in influent and effluent sewage water samples and (2) the effectiveness of the treatment applied in the STPs.

2. Experimental

2.1. *Chemicals*

The compounds selected in this study were 4-octylphenol, bisphenol A, the natural oestrogens oestrone and oestradiol, and the synthetic oestrogens diethylstilboestrol and ethynyloestradiol. Pure standards of these compounds (>98%) were purchased as powders from Sigma-Aldrich (Saint Quentin Fallaviers, France). Individual stock standard solutions (250 mg L^{-1}) were prepared in ethyl acetate, or a mixture of ethyl acetate : methanol in the case of oestrone, and stored at −20°C. Working standard mixtures, at different concentrations, were prepared by appropriate dilution of the stock solutions in ethyl acetate.

Analytical-grade methanol, dichloromethane and ethyl acetate were purchased from Panreac (Barcelona, Spain) and HPLC-grade water from Merck (Darmstadt, Germany).

2.2. *Sample collection and preparation*

All the wastewater samples used in this study were collected from two municipal STPs located in south-eastern Spain (Almeria). The main STP is connected to a sewage system servicing a municipal area with ~200 000 inhabitants. The other covers a more restricted area of ~62 000 inhabitants. Both plants use pre-treatment for solid removal, a primary treatment carried out in circular sedimentation tanks to eliminate suspended material, an activated sludge biological treatment, and a final clarification. Two series of daily composite samples of raw influents and final effluents were taken over five consecutive days, for two consecutive months. Integrated samples were representatives of one day's work in the STP and were taken at hourly intervals. Sampling was carried out by an automatic sampler (0.5 L/3 h). Discrete samples were also taken during the period July 2003 to April 2004. In this case, samples were collected from the influents and effluents of the treatment plants by using pre-rinsed amber glass bottles. After collection, samples were filtered through a 0.7 μm glass fibber filter (Teknokroma, Barcelona) prior to analysis to remove particles that could interfere during the SPE procedure. Filters and samples were stored in the dark at 4°C and extracted within 48 h in all the cases.

A solid-phase extraction procedure was applied to the wastewater samples using commercial Oasis^{TM} HLB (divinylbenzene/*N*-vinylpyrrolidone copolymer) cartridges (200 mg, 6 cm^3) from Waters (Mildford, MA). An automated sample processor ASPEC XL fitted with an 817 switching valve and an external 306 LC pump from Gilson (Villiers-le-Bel, France) was used for this purpose. A conditioning step was performed with 5 mL of ethyl acetate, 5 mL of methanol, and 5 mL of LC-grade water at a flow rate of 1 mL min^{-1}. Wastewater samples (100 mL influent and 200 mL effluent) were loaded at a flow rate of 10 mL min^{-1}, followed by a washing step with 6 mL of water. Then, the cartridges were dried by a nitrogen stream for approximately 15 min and finally eluted with 2 × 4 mL of ethyl acetate at 1 mL min^{-1}. The extracts thus obtained were evaporated by a gentle nitrogen stream to yield a final volume of 500 μL for direct analysis by GC-MS-MS.

To evaluate the presence of the targeted compounds in the suspended solids, the particulates retained in the filters were extracted with 10 mL of dichloromethane.

These extracts were dried by a gentle nitrogen stream and reconstituted in 500 μL of ethyl acetate.

2.3. *GC-MS-MS analysis*

GC-MS-MS analyses were carried out using a Trace^{TM} 2000 gas chromatograph (Thermoquest CE Instrument, Austin, TX) interfaced to a GCQ^{TM} ion trap mass spectrometer (Finnigan, Austin, TX). Automatic injection was performed into a split/splitless injector working in splitless w/Surge mode. An empty liner was filled with 0.5 cm Carbofrit (Restek, Bellefonte, PA) placed at the bottom of the liner. The injector operating conditions were as follows: injection volume 8 μL; injector temperature 250°C; initial surge pressure 207 kPa (1.0 min). Analytes were separated in a cross-linked 5% phenyl–95% dimethylpolysiloxane (ZB-5 MS, Phenomenex, Torrance, CA) capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness). The temperature programme was from 105°C (1 min) to 200°C (1 min) at 20°C min^{-1}, then to 220°C (2 min) at 10°C min^{-1} and finally to 290°C (1 min) at 3°C min^{-1}. Helium was used as the carrier gas at a constant flow of 1 mL min^{-1}. The external ion source worked in EI mode at a temperature of 200°C and a source pressure of 30 mTorr. The transfer line was set at 275°C, and the electron multiplier voltage (1425 V) and other typical ion-trap mass spectrometer parameters were automatically optimized by the system software. Characteristic MS-MS conditions were individually optimized for each analyte. The product ion mass spectra resulting from fragmentation were scanned from m/z 60 to two masses over the mass of the precursor ion selected. Precursor and product ions used for identification and quantification are also listed in table 1.

2.4. *Validation studies*

All validation studies were performed using wastewater samples taken from WWTP effluents. Because of the difficulty in obtaining blanks, the samples were previously analysed and considered for the presence of the target compounds. The linearity in the response was studied using matrix-matched calibration solutions prepared by spiking sewage SPE extracts at six concentration levels, ranging from the limit of determination to 500 ng L^{-1}. Integrated peak area data of the selected quantification

Table 1. Quantification (in bold) and diagnostic ions used for the GC-MS-MS analysis of EDCs in wastewater and MS-MS fragmentation conditions.

		GC-MS-MS[a]		
Compound	MW	Precursor (RA, %)	Main product ions (RA, %)	Fragmentation voltage (V)
4-Octylphenol	206	**206** (17)	**107** (100)	0.8
Bisphenol A	228	213 (21)	**198** (100), 119 (98), **165** (95)	1.2
Diethylstilboestrol	268	**268** (65)	**239** (100), 145 (27%)	0.8
Oestrone	270	270 (20)	**185** (100), 157 (54), 170 (40)	0.95
17β-Oestradiol	272	272 (29)	**213** (100), **188** (85), **186** (75)	0.9
17β-Ethynyloestradiol	296	213 (15)	**157** (100), 128 (38), 133 (40)	1.0

[a]Isolation time, 16 ms. Isolation window, 2.

masses (see table 1) were used to construct the curves. Precision of the chromatographic method, determined as relative standard deviation (RSD), was obtained from the repeated injection (five times) of a spiked extract during the same day (repeatability) and on different days (reproducibility). A practical limit of determination was calculated experimentally from the injection of spiked wastewaters and calculated using a signal-to-noise ratio of 10. When the analyte of interest was originally present in the samples, the limit of determination was calculated in terms of sensitivity considering as a limit of determination the amount of analyte that produced an increase in the analyte response of 30%. Confirmation criteria applied to the target analytes in the sewage samples were: (a) peak retention times within a 2% window, (b) presence of two parent ion/product ion transitions with a signal-to-noise ratio higher than 10%, and (c) the ion ratio of each parent/product peak combination falling within 30% of established references.

3. Results and discussion

Most of the GC-based analytical methods developed for the analysis of the target compounds in environmental waters apply a derivatization technique prior to injection in the chromatographic system. This procedure increases the specificity of the analysis and provides an enhanced sensitivity necessary to reach the very low levels required in this kind of determination. However, derivatization techniques are labour-intensive and time-consuming; require the use of expensive deuterated internal standards; reduce the lifetime of the analytical columns; and are susceptible to reaction inversion, thus limiting the possibility of performing automatic analysis sequences and consequently constituting a source of inaccuracy [19–21]. To overcome these difficulties, the proposed method uses a direct analysis of the underivatized compounds. This method combines the high selectivity provided by tandem mass spectrometry and the higher sensibility obtained by the use of large-volume injections.

3.1. *GC-MS-MS method optimization*

The application of the chromatographic conditions described in the experimental section allowed complete separation of the analytes in a total time of analysis of 35 min. Even in the absence of derivatization, all the peaks showed a very good peak shape.

Figure 1 shows the selected ion chromatograms corresponding to the GC-MS/MS analysis of a spiked wastewater extract at $50\,ng\,L^{-1}$. Retention times obtained for each compound are also included. Although the last analyte eluted at 25 min, the analysis was maintained, and the final temperature increased up to 290°C to ensure complete elimination of the matrix components from the analytical column.

Injection of sample volumes of 8 μL in a conventional split/splitless injector was possible by adjusting the injector operating conditions. A programmable pressure injector was used. This allowed a higher injector pressure to be fixed at the beginning of the analysis to allow (1) a reduction in the solvent vapour volume generated after the injection and (2) a faster introduction of sample into the column. In this way, difficulties such as possible losses of the analytes, peak tailing, and poor peak shape were avoided [22]. It was also important to use injection-port liners filled with a packing

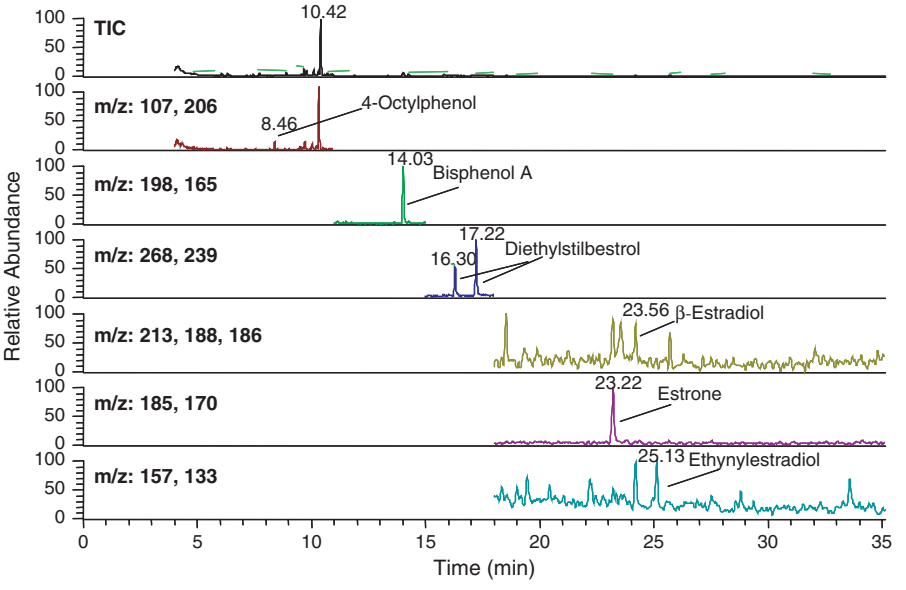

Figure 1. Typical extracted ion current GC-MS-MS chromatogram corresponding to a spiked wastewater sample at 50 ng L^{-1}.

material (carbofrit) in order to maximize the trapping of non-volatile components present in the extracts and to avoid extensive matrix effects. A frequent maintenance of the injector and the analytical column is necessary, however.

MS/MS operation parameters were optimized individually for each analyte by using spiked SPE extracts of wastewater samples, at a concentration level of 10 μg L^{-1}. The use of spiked samples was required because the presence of matrix had an effect upon the intensity ratios that needed to be evaluated. Information about the precursor ion isolated, resonance excitation voltage applied, and main product ions obtained with their relative intensities is included in table 1.

As a general criterion, precursor ion dissociation conditions were selected in order to obtain a balance between maximum sensitivity, minima spectral interferences and sufficient structural information for unequivocal identification. The base peak on the EI spectrum was initially selected as the precursor ion with a mass isolation window of 2 and isolation time of 16 ms. 4-Octylphenol, however, presented a base peak at *m/z* 107, resulting from the fragmentation of the alkyl chain $[M\text{-}C_7H_{15}]^+$. Further fragmentation of this ion did not provide any structural information of interest, yielding one solitary and uncharacteristic product ion at *m/z* 77 $[C_6H_5]^+$. In addition, this low-mass ion was not selective, thus increasing the probability of matrix interferences. The molecular ion, at *m/z* 206, was then selected even at the expense of a loss in response. The molecular ion is considered the most characteristic ion for each compound, and it coincided with the base peak in the case of diethylstilboestrol (*m/z* 268), oestrone (*m/z* 270), and oestradiol (*m/z* 272). For bisphenol A and ethinyloestradiol, however, the major ion appears at *m/z* 213. In the first case, this ion corresponded to the loss of a methyl group $[M\text{-}15]^+$, a very common loss that did not affect the molecule moiety, and could be considered as a characteristic fragment also able to

yield characteristic product ions. However, in the case of ethinyloestradiol the fragment at m/z 213, $[M\text{-}C_5OH_7]^+$ corresponded to an unspecific moiety of the molecule common to other members of this family of compounds (oestrone, oestradiol, etc.). As a consequence, this ion delivered fragment ions, which were not characteristic of the analyte structure, thus introducing uncertainty in the confirmation. When a more characteristic ion was selected, like the molecular ion (m/z 296), the sensitivity was considerably reduced (around 10 times lower), thus avoiding the concentration levels usually present in the samples. Considering both aspects, the fragment at m/z 213 was finally selected, provided that a good separation between ethinyloestradiol and other related compounds could be obtained, and a good reproducibility in the retention times could also be attained.

The resonance excitation voltage used to fragment the parent ions selected was adjusted in order to prevent its complete disappearance. Thus, the parent ions were present in the MS/MS spectra with a relative abundance of 15–30%. Only in the case of diethylstilboestrol was the precursor ion present at a relative abundance of 65% because the application of higher voltages decreased the sensitivity and did not produce any additional fragmentation. Under these conditions, two or more fragments were present for most of the compounds, thus allowing a reliable confirmation. 4-Octylphenol, however, presented a limited confirmation capability, because the single MS/MS transition $206 \rightarrow 107$ obtained could be insufficient to distinguish the analyte from either matrix interferences or from similar, but not identical, compounds. In this case, and because of the impossibility of obtaining any further fragmentation without considerable loss of sensitivity, the retention time match criterion was strictly applied in order to prevent any false positive results.

3.2. *Performance of the analytical method*

The analytical method was evaluated to prove its identification and quantification capability. The calibration curves, obtained as described in section 2.4, were linear over the entire range studied, with correlation coefficients higher than 0.991 in all cases (see table 2). These curves were used to quantify the analytes in the samples. The limits of determination calculated were in the $\mathrm{ng\,L^{-1}}$ range, which guarantees correct evaluation of the EDCs in the sewage samples. Lower limits of determination can be obtained by increasing the preconcentration factor, but then the use of clean-up steps should be recommendable. Under the conditions described, the high sensitivity and selectivity provided by the GC-MS-MS mode obviate the

Table 2. Validation studies of the analytical methods in matrix matched standards.

Compound	Recovery, % (RSD, %)[a]	LOD ($\mathrm{ng\,L^{-1}}$)	Repeatability (RSD, %)	Reproducibility (RSD, %)	Linearity R^2
4-*tert*-Octylphenol	30 (5)	20	6	14	0.995
Bisphenol A	90 (7)	2	2	10	0.991
Diethylstilboestrol	75 (9)	2	6	9	0.999
Oestrone	99 (4)	2	10	13	0.997
17β-Oestradiol	95 (5)	12	10	9	0.997
17α-Ethynyloestradiol	91 (7)	13	8	10	0.999

[a]RSD (relative standard deviation).

Table 3. Levels of the main compounds detected during the monitoring study in the influent and effluent of sewage-treatment plants.

	Influent concentration ($\mu g\,L^{-1}$)		Effluent concentration ($\mu g\,L^{-1}$)	
Compound	Range	Mean	Range	Mean
Bisphenol A	0.20–1.70	0.69	0.05–0.5	0.15
Oestrone	0.02–0.26	0.12	0.004–0.08	0.03
Diethylstilboestrol	0.01–0.12	0.05	< LOD–0.03	0.009

need for any additional cleanup. The chromatogram represented in figure 1 shows the presence of very small peaks coming from the matrix, thus clearly indicating the peaks corresponding to the target compounds. On the other hand, the isolation of only one ion for obtaining the product ion spectra provides interference-free spectra, which allow unambiguous identification. This was corroborated by means of studies of variability in the relative abundance of the qualifier ions. Because of the dependence of ion intensity on concentration, the study was performed at a concentration level similar to that present in the samples. An acceptable repeatability was observed in the relative abundance, with RSDs lower than 20% in all cases.

To ensure correct quantification of the analytes in the samples, precision in the chromatographic response was also determined in terms of reproducibility and repeatability. The RSDs obtained were lower than 15% in all cases, indicating a good performance for this method.

The recovery studies were carried out by spiking wastewater samples at a concentration of $50\,ng\,L^{-1}$. Quantitative recoveries (four replicates) were obtained in all cases, varying from 75 to 99% for all compounds with an RSD $\leq 9\%$, except for 4-octylphenol, which yielded a mean recovery of 30%.

Because of the hydrophobicity of these compounds, and since it is generally agreed that suspended particles with a small particle size and high organic carbon content present a high adsorption efficiency [23], a brief study was performed to help estimate the amount of these compounds that could be adsorbed to suspend particulate matter. Particulates retained in the glass fiber filters, after filtration of a spiked wastewater sample ($10\,\mu g\,L^{-1}$), were rinsed with dichloromethane. A percentage of the total amount present in the water samples of around 3% for bisphenol A, diethylstilboestrol and 17 α-ethynyloestradiol, 7% for oestrone and β-oestradiol and 11% for 4-octylphenol was found in the particulates. This behaviour also corroborates the tendency of oestrogenic compounds to be adsorbed to aquatic sediments and STP sludges already described [24].

3.3. *Monitoring studies*

A 1-year monitoring study was performed to evaluate the presence and behaviour of the studied compounds in two WWTPs using primary and secondary treatments, involving an activated sludge process. Measurements were made of WWTP raw influents and treated effluents. The most remarkable result was the presence of bisphenol A in all samples analysed, in both influents and effluents. The mean

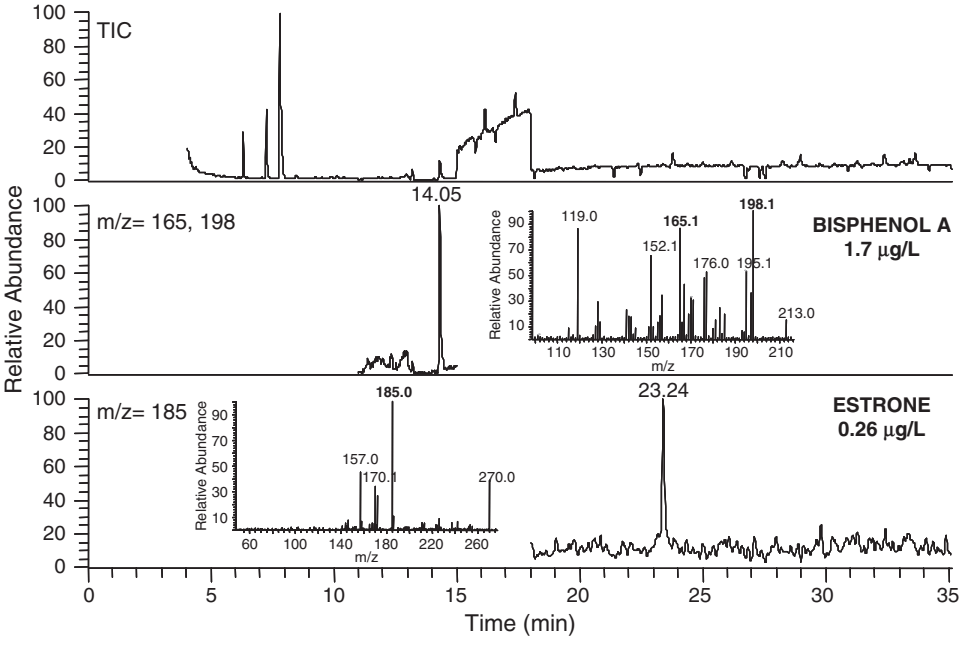

Figure 2. Typical extracted ion current GC-MS-MS chromatogram corresponding to an influent wastewater sample where bisphenol A and oestrone have been identified.

concentration in influent was $0.69\,\mu g\,L^{-1}$, sometimes reaching concentrations up to $1.7\,\mu g\,L^{-1}$. Figure 2 shows an example of an extracted ion GC-MS-MS chromatogram of a real influent wastewater sample in which this compound has been identified. In final effluents, the mean concentration was reduced to $0.15\,\mu g\,L^{-1}$ (range $0.05–0.5\,\mu g\,L^{-1}$). The results suggest that, although a removal efficiency of bisphenol A of about 81% is obtained during the treatment, this compound is continuously discharged into the marine environment. A similar behaviour was observed in the case of oestrone, which was identified in 80% of the samples. Although natural and synthetic oestrogens are mainly excreted as their endocrinologically inactive conjugated forms (sulphated and glucuronated oestrogens), they return to the original form during the wastewater collection and treatment process, presumably by the action of micro-organisms, such as *Escherichia coli*, which exhibit glucuronidase and sulphatase activity [25]. Consequently, free oestrogens are detected in both influents and effluents.

During this study, levels of oestrone of up to $0.5\,\mu g\,L^{-1}$ were detected in influents, with mean values of $0.15\,\mu g\,L^{-1}$. The removal efficiency for oestrone was found to be about 66%, being present in the effluents at mean concentrations of $0.03\,\mu g\,L^{-1}$. The scarce presence of oestradiol (detected only sporadically in the samples) with respect to oestrone can be a consequence of its lower excretion rate for women and the rapid elimination in contact with activated sludge, which gives rise to oestrone via an oxidation process, as evidenced in laboratory experiments [11]. Some authors have referenced levels of oestrone equivalents in the influent and effluent of the plant [14], probably as a consequence of this transformation.

Octylphenol and ethinyloestradiol were not identified during the study, and diethylstilboestrol was detected only sporadically (18% of samples). In general, results obtained during the monitoring are in concordance with previous studies.

4. Conclusions

The method described here has been demonstrated to be a very simple, fast, and viable alternative for routine monitoring in the determination of the group of EDCs selected in wastewater. Traditional derivatization processes during the sample preparation can be omitted, and the combination of the high selectivity of the GC-MS-MS technique with the injection of higher-than-normal sample volumes means that the method performs well. The approach has a satisfactory accuracy, with recovery percentages ranging from 75 to 99% (except for octylphenol: 30%) and relative standard deviations lower than 10%. The method detection limits were in the range of 2–20 ng L^{-1}. The method has been successfully applied to monitoring selected compounds in influent and effluent wastewater samples from WWTPs. Results show the continuous presence of bisphenol A and oestrone in effluents with removal efficiencies during the biological treatments of about 81 and 66%, respectively.

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