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Comparison of APPI, APCI and ESI for the LC-MS/MS analysis of bezafibrate, cyclophosphamide, enalapril, methotrexate and orlistat in municipal wastewater

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The applicability of three different ionization techniques: atmospheric pressure photoionization (APPI), atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) was tested for the liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of five target pharmaceuticals (cyclophosphamide, methotrexate, bezafibrate, enalapril and orlistat) in wastewater samples. Performance was compared both by flow injection analysis (FIA) and on-column analysis in deionized water and wastewater samples. A column switching technique for the on-line extraction and analysis of water samples was used. For both FIA and on-column analysis, signal intensity and signal-to-noise (S/N) ratio of the target analytes in the three sources were studied. Limits of detection and matrix effects during the analysis of wastewater samples were also investigated. ESI generated significantly larger peak areas and higher S/N ratios than APCI and APPI in FIA and in on-column analysis. ESI was proved to be the most suitable ionization method as it enabled the detection of the five target compounds, whereas APCI and APPI ionized only four compounds. Copyright © 2011 John Wiley & Sons, Ltd.

Supporting information may be found in the online version of this article.

Keywords: pharmaceuticals; cytostatic agents; mass spectrometry; LC-MS/MS; APPI; APCI; ESI

INTRODUCTION

In recent years, healthcare spending has outpaced economic growth in developed countries, with pharmaceutical expenditures being a key driver of this trend. According to analysts, the growth in personal use, rather than price, has been the major cause of increased pharmaceutical spending.[1] This growing consumption trend is expected to increase the likelihood that pharmaceuticals may appear in the environment. The issues pertaining to pharmaceuticals in the environment and mainly in the aquatic environment have troubled the scientific community; hence, the current concerns to better understand the fate of anthropogenic substances released in the aquatic environment. This has led to the development of numerous analytical methods predominantly using liquid chromatography-tandem mass spectrometry (LC-MS/MS) to carry out such projects. Nowadays, the most commonly used ionization sources in LC-MS/MS are electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI). These techniques provide a soft ionization process and are highly suitable for the analysis of pharmaceutical drugs in various water matrixes.

So far, only a few studies are available in which the suitability of LC-MS/MS methods coupled to different ionization sources has been compared for the analysis of pharmaceuticals in water.[2,3] In this work, the assessment of three ionization sources and on-line solid phase extraction (SPE) were investigated to choose the optimal methods for the analysis of five pharmaceuticals in wastewater. Given the known matrix effects potentially observed in such complex matrices, a comparison of the ionization sources is critical to optimize methods required to carry out such environmental analyses.

The selected compounds belong to various therapeutic classes: lipid regulators [bezafibrate (BEZ)], anti-cancer [methotrexate (MTX) and cyclophosphamide (CYC)], anti-obesity agents [orlistat (ORL)] and antihypertensives [enalapril (ENA)]. These substances are a good choice for this study because of their variable hydrophobic character and their different physico-chemical properties (Table S1, Supporting information). ORL and CYC are neutral; BEZ and MTX are acids whereas ENA is an ampholyte. Also, these compounds are of special interest because many factors influence the occurrence of these drugs in the environment: total quantities consumed, pharmacokinetics, physico-chemical properties and wastewater treatment processes.

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EXPERIMENTAL

Reagents

BEZ, CYC, ORL and MTX with a certified purity ≥ 99% were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). ENA was kindly supplied as a formulation by a local drugstore. LC-MS grade acetonitrile (ACN), water (H_2O), methanol (MeOH) and toluene (TOL) were purchased from J. T. Baker (Phillipsburg, NJ, USA). Deionized and purified water was obtained from a Milli-Q ultrapure water system (MQ-water). HPLC-grade reagent acetic acid (AA) from Fisher Science (Fair Lawn, NJ, USA) and formic acid (FA) 98% was purchased from Sigma-Aldrich Canada.

Instrumentation

A liquid chromatography-tandem mass spectrometry (LC-MS/MS) and an Equan system made by Thermo Fisher Scientific (Waltham, MA) were used to carry out the preconcentration and analysis. The components and functioning of this system have been described earlier.[4] Shortly it consists of a sample delivery system (an autosampler and a load pump), a switching-column array (six-port switching valve, preconcentration column, LC pump and an analytical column) and an ionization and detection system (source and tandem mass spectrometer). For ESI and APCI the Ion Max API source manufactured by Thermo Scientific was used. For APPI, the PhotoMate orthogonal source manufactured by Syagen (Tustin, CA) was used. This source is composed of a discharge lamp filled with krypton (Kr) and emits photons having energy of 10.0 eV.

Preparation of standards

Stock solutions 500 mg l^{-1} of each compound were prepared by weighting and dissolving the corresponding pure powder in an appropriate solvent (BEZ, CYC, ORL and ENA were dissolved in MeOH; MTX was dissolved with 0.3% FA in H_2O–MeOH 3 : 7). Mixed working solutions containing 50 μg l^{-1} of the compounds were prepared weekly by dilution of the stock solutions in ACN and were kept at 4 °C.

Sample collection and preparation

The Montreal wastewater treatment plant has a capacity of about 7.6 million m^3 day^{-1} and it is the largest primary physico–chemical treatment plant in America. Its treatment process consists in a mechanical removal of large solid matters and grit. To reduce the phosphorus found in water, a coagulant is added to destabilize the colloids, after which an anionic polymer is injected to agglomerate the particles.[4] Water samples were collected in amber glass bottles with Teflon-lined caps and kept at 4 °C in the dark until analysis. They were vacuum-filtered and the sample pH was adjusted to 2.8 with FA (98%). Samples were prepared in 50-ml volumetric glass flasks, adding 2.5% of MeOH to prevent sample loss.

Flow injection analysis conditions

A mass of 1 ng (i.e. 20 μl of a 0.05 ng μl^{-1} solution) of each analyte was infused by flow injection analysis (FIA). Acquisition scan time was 0.2 s and the mobile phase used was 50% of 0.1% AA in MQ-water (eluent A) and 50% of ACN (eluent B). Infusion experiments by FIA using the three sources and individual standard solutions were carried out to obtain the optimal flow rate conditions. For APCI, the corona needle discharge current was also optimized. TOL was tested as APPI dopant in the FIA experiments, because it has an ionization energy (IE = 8.83 eV) lower than the photon energy of the emitted light of the Kr lamp (10.6 eV). Its photoions have a high recombination energy or low proton affinity (PA).[5] This photon-emission energy is higher than the ionization energy (IE) of the target molecules (7–10 eV for many organic molecules) and lower than the IE of the constituents of air and the mobile phase. The optimal flow rate of TOL in our experiments was 20 μl min^{-1}, which produced a sufficient amount of dopant ions in the source.

On-line preconcentration and chromatographic conditions for the analysis on-column

A total mass of 0.5 ng (i.e. 1 ml of 0.5 ng ml^{-1}) of each analyte was preconcentrated and analyzed by on-line SPE. The procedure used for the on-column analysis was based on a previously published method.[4] Briefly, it consisted of the conditioning of the Strata-X preconcentration column (20 × 2 mm, 28 μm, manufactured by Phenomenex, Torrance, CA, USA) with a conditioning solution (0.1% AA in MeOH/MQ-water 1 : 40 v/v) at a flow rate of 1 ml min^{-1}. An aliquot of 1.00 ml of sample was then loaded into the preconcentration column using the same conditioning solution. After the sample was loaded, the preconcentration column was back-flushed and the retained components of the sample were then transferred to the analytical column using the solvent gradient delivered by the analytical pump. ESI experiments were achieved on a Synergi Max RP-C12 column (75 × 2 mm, 4 μm, manufactured by Phenomenex) preceded by a guard cartridge (4 × 2 mm, 4 μm) of the same packing material at a flow rate of 0.2 ml min^{-1}. A Synergi Max RP-C12 analytical column (150 × 4 mm, 4 μm) preceded by a guard cartridge (4 × 2 mm, 4 μm) were used for separation for APCI or APPI applications with a flow rate of 600 μl min^{-1}. The mobile phase consisted of 0.1% AA in MQ-water (eluent A) and ACN (eluent B). Liquid chromatography was carried out at ambient temperature using a mobile phase gradient (Table S2, Supporting Information). At the end of the last step, a new cycle begins for the analysis of the next sample. All the on-line operations are fully automated for routine analysis with a run time of 15 min (combining the sample preconcentration, the chromatographic run and conditioning for the next sample).

Mass spectrometry

The tandem mass spectrometer was operated in positive and negative ion mode. The optimization of the operation parameters for ESI was carried out by the infusion of 1 μg ml^{-1} standards of each compound at a flow rate of 0.2 ml min^{-1}. Sheath gas was set to ten arbitrary units and the auxiliary gas to five arbitrary units. A spray voltage of ±3.5 kV was used for the negative and positive ionization. Ion transfer capillary temperature was set to 350 °C and skimmer offset to 5 V. For the APPI and APCI sources, a concentration of 10 μg ml^{-1} and a flow rate of 0.6 ml min^{-1} were used. Common APPI and APCI parameters and conditions were as follows: vaporizer temperature 500 °C, capillary temperature 270 °C, sheath gas pressure 20 arbitrary units, auxiliary gas 5 arbitrary units and skimmer offset 4 V. For all experiments, the collision gas was argon (Ar) and the sheath and auxiliary gas was nitrogen (N_2). Detection was performed in the selected reaction monitoring (SRM) mode. For both quadrupoles (quadrupole 1 and quadrupole 3), resolution was set at unit resolution (full width at half maximum = 0.7 u). Tube lens and collision energies of

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the SRM transitions are compound-specific and given in Table S3, Supporting information.

Evaluation of detection limits and matrix effects for SPE on-line analysis

Key analytical performance parameters such as limits of detection (LOD) and matrix effects were evaluated. LOD were determined using the standard error of the intercept and the slope of the calibration curve, as proposed by the International Conference on Harmonization (ICH) of Technical Requirements for registration of Pharmaceutical for Human Use.[6] The method of standard additions was used to determine the overall method LOD. Calibration curves contained the analytes at seven different concentrations (0, 50, 100, 200, 500, 700 and 1000 pg/ml) and were injected and prepared in triplicate. The amounts spiked were chosen to represent the average analyte concentration we expected to find in the samples according to previous studies. Standard additions were performed by taking equal volumes of the sample solution; all but one were spiked individually with different amounts of the analyte (0–1000 ng l_1), and all were diluted to the same volume.

Integration of chromatographic peaks and quantitation were performed using the LCquan 2.5 software (Thermo Fisher). Calibration curves were built with the area of the analyte standard as a function of the analyte concentration.

The detection limit and the quantification limit (LOQ) are expressed as:

$$LOD = \frac{3.3\sigma}{S} \text{and}$$
$$LOQ = \frac{10\,\sigma}{S} \quad (1)$$

where σ = the standard error of the intercept and S = the slope of the standard additions calibration curve. Matrix effects were determined with the same extraction processes in wastewater effluent samples according to Salvador *et al*.[7] They were calculated by comparing the peak areas of known amounts of standard spiked in MQ-distilled water (DW) with the peak area of those standards spiked in wastewater (WW) effluent (WW_S) after correcting for the peak area of the analyte in the unspiked matrix (WW_{NS}) according to the following equation:

$$\text{Matrix effects (\%)} = \left(\frac{WW_S - WW_{NS}}{DW}\right) \times 100\% \quad (2)$$

A value of 100% indicates that there is no absolute matrix effect; if the value is above 100%, there is a signal enhancement and there is signal suppression if the value is $< 100\%$. To avoid false positives and increase the scientific certainty, confirmation criteria were set so that the LC retention time remained within 1–2% of the retention time of the standard compound under the same conditions.[8] Three replicate analyses of the samples were compared to the 200 pg ml^{-1} concentration level and used to calculate the repeatability of the retention times, obtaining coefficients of variation under 2%.

RESULTS AND DISCUSSION

Comparison of APPI, APCI and ESI mass spectra obtained by FIA

Full scan and product ion scan of each analyte were performed using the above-described instrumental conditions and parameters. Our experiments showed that the protonated molecule $[M+H]^+$ was the most abundant for four compounds: *m/z* 261 for CYC, *m/z* 377 for ENA, *m/z* 455 for MTX and *m/z* 496 for ORL. The major ion detected for BEZ was $[M-H]^-$ (*m/z* 360), which was more intense and had a higher signal-to-noise (S/N) ratio than the protonated molecule.[9] Observed precursor and fragment ions are given in Table S3, Supporting information. The results show that the three sources generate very clean and identical spectra for the tested compounds. However, all the compounds were better ionized by ESI, producing a higher S/N ratio. APPI and APCI failed to produce the precursor ion of MTX with sufficient intensity for its identification, additionally adducts of MTX were not observed. ESI permitted the ionization of MTX affording a protonated molecule with excellent signal intensity. According to the APCI and APPI theory of ionization, solutes must first be vaporized into the gaseous state before ionization; therefore, it is important that the solutes be nonionic in solution and non labile to thermal degradation. Probably, MTX does not fit this model well: it is ionic in solution or it is not stable at the temperature of the interface (500 °C). Owing to the lack of MTX thermal stability data, the low APCI response was explained by its polarity. MTX has basic amino groups in its structure, capable of retaining a proton during the ionization process.[10] In APCI(+), the mobile phase and the analytes are first vaporized and then ionized by acid–base reactions in the gas phase. In this process, strong gas phase acids produced by the corona discharge transfer a proton to the analyte if the proton affinity (PA) of the former is higher than that of the latter. In addition to proton affinity, signal intensity in APCI is dependent on the volatility of the compounds. Charged species are less volatile than the neutral forms.[11] Therefore, because MTX (pKa = 4.8 and 5.5) is ionic at the pH of the mobile phase (pH = 2.9) it was poorly vaporized in APCI and consequently, not detected.

Similarly to APCI, the solvent in APPI is vaporized with a heated nebulizer, but the ionization process is initiated by using a vacuum ultraviolet (VUV) lamp instead of a corona discharge. The compounds possessing ionization energies (IE) below 10 eV are directly ionized by the emitted photons, producing a molecular ion ($M^{+\bullet}$). In the presence of a protic solvent, the molecular ion of the analyte abstracts a hydrogen atom from the solvent to form a protonated molecule. The analyte is ionized by the proton transfer reaction if its PA is higher than that of the solvent molecule. As CYC, ENA and ORL were detected as $[M+H]^+$ ions, the ionization with APPI occurs via proton transfer reaction under the chosen conditions. For BEZ, the production of $[M-H]^-$ ions could be via electronic capture or charge exchange. The results using APPI were quite similar to those for APCI. Notably, MTX could not be detected by using this ionization source. The reason is probably the same as for APCI, i.e. poor vaporization of MTX in its ionic form.

Optimization of experimental parameters

Flow rate

The effects of the different flow rates on the abundance of the peak areas of the compounds are shown in Fig. S1, Supporting information. For ESI, Fig. S1(a) indicates that the highest signal intensity for each analyte was obtained when the flow rate was low (0.2 ml min^{-1}). In ESI, low flow rate provides the finest droplets during the nebulization process, thereby increasing the surface area of each droplet, which is favorable for ion transfer into the gas phase, resulting in enhanced ionization efficiency.[9] In Fig. S1, this is not reflected for the 0.1 ml/min flow rate, and a possible explanation for the increase observed between 0.1 and 0.2 ml/min

Table 1. Effect of TOL as a dopant on the APPI source studied by FIA[a]

Analyte	Peak area ± SD (×10⁴) APPI + dopant	Peak area ± SD (×10⁴) APPI	Dopant enhancement factor	S/N ratio ± SD (×10⁴) APPI + dopant	S/N ratio ± SD (×10⁴) APPI	Dopant enhancement factor
BEZ	2.0 ± 0.5	29 ± 7	0.1×	0.100 ± 0.003	0.4 ± 0.1	0.3×
CYC	8670 ± 623	2160 ± 138	4×	0.21 ± 0.02	13 ± 2	0.02×
ENA	569 ± 15	751 ± 60	0.8×	4.1 ± 0.2	21 ± 2	0.2×
MTX	[b]	[b]	[b]	[b]	[b]	[b]
ORL	411 ± 4	229 ± 7	2×	0.23 ± 0.03	3.3 ± 0.3	0.1×

[a] Average of triplicate peak areas analysis of FIA were determined for 2 ng injections. Mobile phase = (1 : 1) ACN/H_2O pH 2.9 with AA. Dopant flow rate was 20 µl min^{-1}. TOL IP = 8.8 eV. Kr lamp photon energy = 10.6 ev.
[b] MTX was not ionized with APPI nor APCI.

Table 2. Comparison of APPI, APCI and ESI peak area and S/N by flow injection analysis

Analyte	Peak area ± SD (×10⁴) ESI	Peak area ± SD (×10⁴) APPI	Peak area ± SD (×10⁴) APCI	S/N ± SD (×10⁴) ESI	S/N ± SD (×10⁴) APPI	S/N ± SD (×10⁴) APCI
BEZ	3740 ± 406	29 ± 7	22 ± 1	14.6 ± 0.1	0.4 ± 0.1	0.104 ± 0.009
CYC	1136 ± 267	2160 ± 138	32 ± 3	24 ± 1	13 ± 2	1.3 ± 0.2
ENA	2975 ± 739	751 ± 60	27 ± 5	19 ± 1	21 ± 2	0.215 ± 0.008
MTX	7218 ± 989	[a]	[a]	21.7 ± 0.8	[a]	[a]
ORL	2375 ± 195	229 ± 7	17 ± 2	3.8 ± 0.1	3.1 ± 0.3	0.94 ± 0.02

Average of triplicate peak areas analysis of FIA was determined for 1 ng injections. Mobile phase = (1 : 1) ACN/H_2O pH 2.9 with AA. ESI flow rate was 0.2 ml min^{-1}. APPI and APCI flow rate was 0.6 ml min^{-1}.
For peak area all the results are statistically different according to the *t*-test. No dopants were used for any of the analytes.
[a] MTX was not ionized with APPI nor APCI.

could result from a greater quantity of ions being sampled by the system at 0.2 ml/min. The decrease between 0.2 and 0.4 ml may be the result of a loss of efficiency upon ionization by condensing droplets resulting from the excess effluent liquid flow.

For APPI and APCI, the best signal intensity of the target analytes was obtained at a flow rate of 0.6 ml min^{-1} (Figs S1(b) and (c)). Because the vaporized mobile phase acts as a reagent gas in APCI at higher flow rates more reagent gas molecules are available to react with the target analyte; thus, the ionization efficiency of the APCI process is enhanced.[12] For APPI, some studies have shown that a low flow rate may improve its ionization efficiency resulting from lower photo absorption by the solvent.[13,14] However, our data showed that a lower flow rate does not provide better ionization efficiency in APPI. As solvent molecules are involved in the ionization process, the ions formed by proton transfer are better produced in high flow rate conditions.

Corona needle discharge current

The effects of APCI corona needle discharge electric current on mass spectra and ion intensity of target analytes were investigated by FIA using the parameters and conditions described earlier. Full scan analysis was performed at discharge current of 0–20 µA, and a clear trend was observed as shown in Fig. S2. The magnitude of the discharge current did not significantly affect the overall appearance of the mass spectra over the tested current range, but it affected the absolute intensity of the ions in the spectra. We found that a discharge current in the range of 4–6 µA resulted in maximum ion intensities for the target compounds. Therefore, the discharge current used for all the compounds was 6 µA.

Effect of TOL as a dopant on APPI

Several groups have shown that the addition of a photoionizable substance like TOL to the LC flow can increase the ionization yield of the target compounds.[15,16] As can be observed in Table 1, TOL was found to enhance the APPI signal intensity of only two of the five target compounds: CYC (by a factor of 4.0) and ORL (by a factor of 1.8). However, the dopant was also found to increase the background noise and consequently generated lower S/N ratios than the dopant-free APPI tests. Our findings are in agreement with previous studies showing that the addition of dopants results in an increase of the background noise.[17,18] It has been reported that the type of APPI source in this work is not designed for use with a dopant,[5] nevertheless several applications have made use of it to increase method sensitivity.[15,16] As there was little or no significant improvement using a dopant, it was not introduced into the APPI source in subsequent investigations.

Comparison of signal intensity and S/N ratio

Flow injection analysis

Results obtained by FIA appear in Table 2. All the resulting peak areas obtained using the three sources were statistically different ($p < 0.05$) applying the Student's *t*-test.[19] These results show that ESI peak areas obtained by FIA were always higher than those of APCI and APPI. Also S/N ratio results were better for ESI than for APPI and APCI. MTX presented the best results with the ESI source, while using APPI and APCI the precursor ion of MTX presented insufficient intensity for its identification. MTX is the most polar compound of the five, and it has been demonstrated that ESI works well on species of this kind.[10] We expected better results for ORL

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Table 3. Comparison of APPI, APCI and ESI performance by on-column analysis in MQ-water

Analyte	LOD (pg ml^{-1})			S/N ± SD (×10^4)			Peak area ± SD (×10^4)		
	ESI	APPI	APCI	ESI	APPI	APCI	ESI	APPI	APCI
BEZ	7	15	15	1.5 ± 0.1	0.326 ± 0.004	0.13 ± 0.07	323 ± 5	4.1 ± 0.7	9.1 ± 0.3
CYC	3	14	10	0.84 ± 0.01	0.41 ± 0.01	0.5 ± 0.2	328 ± 5	17 ± 1	23.3 ± 0.3
ENA	13	83	48	0.443 ± 0.001	0.033 ± 0.001	0.12 ± 0.01	65 ± 0.8	2.34 ± 0.06	6.2 ± 0.2
MTX	4	[a]	[a]	4.71 ± 0.01	[a]	[a]	36 ± 1	[a]	[a]
ORL	9	17	17	1.3 ± 0.3	0.242 ± 0.001	0.14 ± 0.01	6 ± 0.04	0.4 ± 0.1	1.3 ± 0.1

On-column LODs were determined using the standard error of the intercept and the slope of the calibration curve. Average peak areas of triplicate analysis were determined for 0.5 ng injections. No dopants were used for any of the analytes.
[a] MTX was not ionized with APPI nor APCI.For peak area all the results are statistically different according to the *t*-test.

Table 4. Comparison of APPI, APCI and ESI performance by on-column analysis in water from the raw sewage collector of the Montreal WWTP

Analyte	LOD (pg ml^{-1})			S/N ± SD (×10^4)			Peak area ± SD (×10^4)			Concentrations measured (pg ml^{-1})		
	ESI	APPI	APCI	ESI	APPI	APCI	ESI	APPI	APCI	ESI	APPI	APCI
BEZ	7	[a]	14	0.72 ± 0.03	0.3 ± 0.1	0.3 ± 0.1	191 ± 8	12 ± 3	30 ± 2	36	[b]	LOD
CYC	5	11	7	0.5 ± 0.1	0.44 ± 0.09	0.64 ± 0.01	317 ± 9	17 ± 1	25 ± 2	12	LOD	LOD
ENA	38	[a]	[a]	1.54 ± 0.03	0.41 ± 0.06	0.23 ± 0.02	116.1 ± 0.3	13 ± 1	28 ± 3	230	[b]	[b]
MTX	11	[c]	[c]	0.31 ± 0.03	[c]	[c]	72.8 ± 0.4	[c]	[c]	LOD	[c]	[c]
ORL	15	9	17	2.41 ± 0.02	0.103 ± 0.002	0.11 ± 0.05	25 ± 2	5.21 ± 0.04	2.0 ± 0.2	LOD	LOD	LOD

For peak area all the results are statistically different according to the *t*-test. No dopants were used for any of the analytes.
[a] A very poor linearity was obtained and no LOD was calculated.
[b] Signal suppression very high that was not possible to calculate the concentration in the sample.
[c] Methotrexate was not ionized with APPI and APCI.

(the least polar compound) with APCI or APPI. However, poor ionization of apolar compounds in APCI can be explained by the formation of protonated solvent clusters. Thus, only compounds with a higher proton affinity (PA) than the solvent clusters are ionized.[20]

On-column analysis

The on-column ESI peak areas presented similar results than those obtained by FIA. Tables 3 and 4 show the results in MQ-water and wastewater, respectively. The ESI on-columns areas for all analytes are higher than those observed when using APCI and APPI. We can also observe that using FIA (e.g. without an HPLC column), ESI areas were also higher than those obtained by on-column analysis. In MQ-water and wastewater on-column analysis, APCI S/N ratio was lower for almost all the compounds among the three ionization sources (Tables 3 and 4). APCI S/N ratio was slightly higher than APPI for CYC in both samples. Only for ENA did it produce a higher response than APPI, but never higher than ESI. Cai *et al*.[17] found identical results in the comparison of APPI and APCI baselines. APCI signals were noisier than those of APPI for the on-column analysis and some possible reasons are the ionization of column bleed components and probably the presence of uneven electron discharge (sparking) at the tip of corona needle in APCI.

Figures 1 and 2 show the separation of the target compounds using the SPE on-line LC-MS/MS, for the injection and preconcentration of 0.2 ng of each compound, respectively. Probably due to the combined effects of ion suppression and elevated APPI or APCI baselines, ENA and ORL produce a poor ion intensity relative to ESI (Figs 1 and 2). The SRM chromatograms of the MQ-water and WWTP effluents show that a spiked standard of BEZ offers a good signal-to-noise ratio by APPI but less than the ESI S/N. From direct S/N comparisons between the two figures, Fig. 2 gives the wrong impression that dirtier samples yield better quality MS data for almost all the experiments, but these results are due to the higher concentration of the analytes already present in the wastewater (BEZ 50, CYC 9, ENA 369, MTX 59 ng/l, respectively).[4]

Evaluation of matrix effects on-column by SPE on-line

A critical aspect in quantitative analysis with LC-MS/MS is the influence of the matrix on the ionization process. Matrix effects can be defined as an unexpected suppression or enhancement of the analyte response due to co-eluting matrix constituents.[21–23] It has been demonstrated that the occurrence of matrix effects may differ between ionization techniques (ESI, APCI or APPI), ionization mode (positive or negative) or between equipment with different source design.[24] Matrix effects in ESI, APCI and APPI sources were investigated using a wastewater treatment plant (WWTP) effluent sample. They were determined using the same on-line extraction processes and by comparing the peak areas of spiked standards in WWTP effluent samples to those of standards spiked in MQ-water. As shown in Table 5, the WWTP effluent matrix led to alterations in the results depending on the source. In all three ionization sources, signal enhancement was observed for ORL (140–173%) and signal suppression for ENA (47–63%) while CYC and was subjected to weak matrix effects (95–118%).

It has been reported that APCI and APPI can be less sensitive to matrix effects than ESI;[21,25–27] however, we observed strong matrix effects using APCI and APPI (Table 5). Similar results were obtained by other groups during the LC-MS/MS analysis of other pharmaceuticals in biological matrices using APCI or APPI

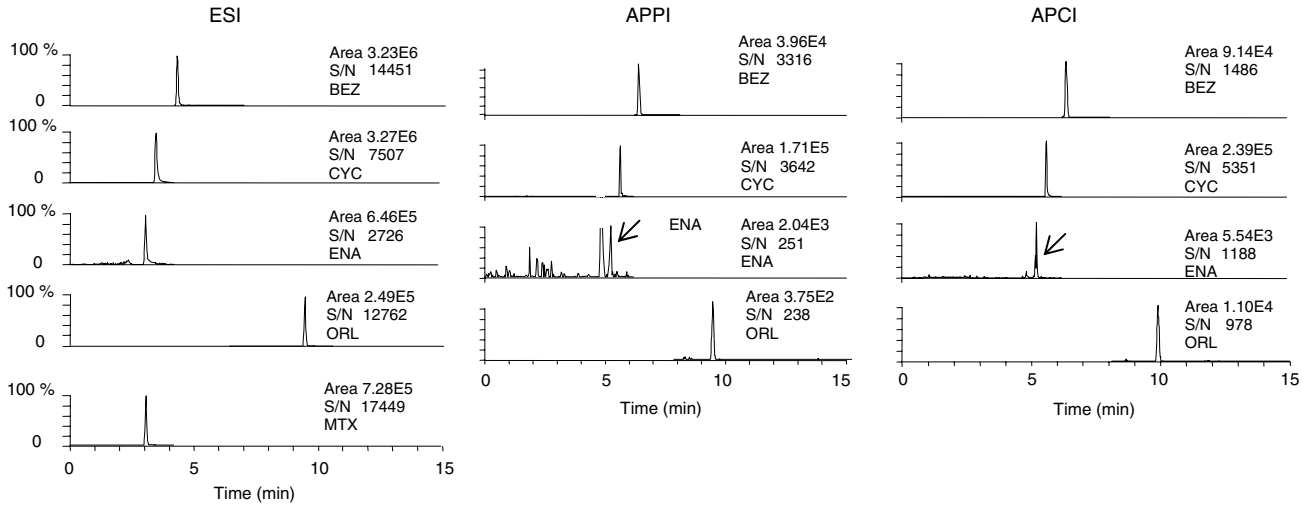

Figure 1. SPE on-line and LC separation of the target compounds. Loading of 1.00 ml of standards (200 pg ml^{-1}) in deionized water.

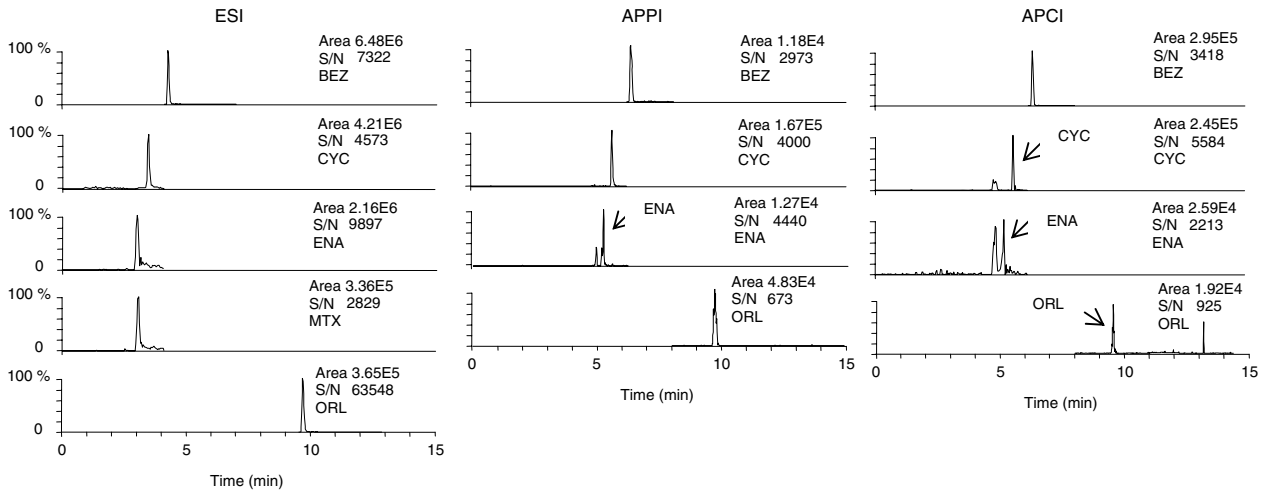

Figure 2. SPE on-line and LC separation of the target compounds. Loading of 1.00 ml of standards (200 pg ml^{-1}) in wastewater.

sources.[28,29] Other studies have also concluded that the extent of matrix effects may be also dependent on the LC-MS interface employed in a given method, because the ionization mechanism is different and may affect the efficiency of formation of the desired ions in the presence of the same co-eluting compounds.[22,30]

The analytes most affected by matrix effects were ENA (47–63%) and ORL (140–173%). The main source of the problem is commonly reported to be the presence of endogenous substances, i.e. organic or inorganic molecules present in the sample that are co-extracted. ENA matrix effects may be explained by its short retention time, close to the solvent front (Fig. 2), where the amount of weakly retained (and possibly interfering) compounds is the highest.[31,32] We did attempt to improve the chromatographic separation by changing the gradients and mobile phases, but none of the combinations we tried provided significant improvements. ORL, an apolar compound, was adequately separated from the other compounds; however, co-eluted components of the sample could interfere with its ionization. A solution to the matrix effects observed might be a more selective analyte extraction.[33,34] The drastic difference between Figs 1 and 2, mainly for ENA, is due to the trace level concentration present in the wastewater matrix (369 ng/l).

Standard additions are useful to compensate for the presence of matrix effects affecting the ionization process usually observed when analyzing environmental samples.[35] We used the standard additions method, because when working with a fully automated on-line preconcentration and detection system, this method is much less time consuming and laborious and becomes the most efficient way to correct for the signal distortion effects caused by matrix components.[35,36]

Comparison of limits of detection

APPI, APCI and ESI on-column LOD were determined by SPE-on line in spiked MQ-water and WWTP effluent samples. The standard additions calibration curve was used to calculate the LOD, expressed as 3.3 times the ratio of the standard error of the intercept and the slope of the curve. For ESI, the lowest LOD was of 2.5 pg ml^{-1} for CYC in MQ-water, the limits achieved for all the compounds were satisfactory for environmental analysis. All the LOD obtained by ESI were significantly lower than those obtained by APPI and APCI. This is explained by a lower background noise with ESI, suggesting that it is a superior ionization source for the analysis of our target substances in such environmental

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Table 5. Matrix effects[a] evaluation and precision[b] values expressed as %CV in MQ-water and wastewater effluent for the three ionization sources

	MEE ± SD			Precision (%) MQ water			Precision (%) WWTP water		
Analyte	ESI	APPI	APCI	ESI	APPI	APCI	ESI	APPI	APCI
BEZ	108 ± 9	30 ± 7	155 ± 21	2.7	17.7	4.1	3.8	[c]	2.8
CYC	118 ± 1	95 ± 7	102 ± 7	3.2	3.6	2.2	6.4	3.1	5.5
ENA	63 ± 8	54 ± 4	47 ± 8	9.2	24.0	14.9	15.8	[c]	[c]
MTX	46	[d]	[d]	4.0	[d]	[d]	6.0	[d]	[d]
ORL	140 ± 31	160 ± 23	173 ± 45	17.0	13.8	6.8	7.3	7.5	11.8

[a] % MEE was calculated by comparing the peak areas of 200 ng l^{-1} standard spiked in DI-H20 and wastewater effluent ($n = 3$).
[b] Precision was calculated on the basis of seven replicates at 200 ng l^{-1} within-run.
[c] Signal suppression very high that was not possible to calculate the %CV.
[d] Methotrexate was not ionized with APPI nor APCI.

samples. Most published methods on the determination of pharmaceuticals in the environment samples with LC-ESI-MS/MS present LOD and limits of quantification in the low pg ml^{-1} range (0.1–9 pg ml^{-1}).[37–40] Castiglioni *et al*. quantified BEZ, ENA, CYC and MTX, in eight Italian effluents and have shown some of the lowest LOQs (0.1, 1.9, 0.71 and 0.83 pg ml^{-1}, respectively).[37] The LOD achieved with APCI in this work are low enough to quantify BEZ, CYC and ORL in contaminated wastewater samples but not in more diluted samples such as tap water. Poor goodness-of-fit ($R^2 < 0.7$) was obtained for the calibration curves of BEZ and ENA, consequently only CYC and ORL could be analyzed in wastewaters by APPI, concentrations of real samples measured with each ionization source are presented in Table 4. As can be observed, standard additions compensates for matrix effects but does not improve the analytical sensitivity, as it was concluded by a study on pharmaceuticals in municipal wastewaters.[41]

CONCLUSIONS

In the above work, the results of the comparison of the three most used atmospheric pressure ionization sources for LC-MS/MS showed that ESI is the best ionization source for the analysis of the target pharmaceutical compounds. ESI detected protonated molecules of the target compounds with higher relative abundance; it was shown to be an ideal ionization technique because of its high sensitivity and high selectivity for the determination of the five selected compounds in municipal wastewater and lower background signal. ESI exhibited superior performance, offering better detection limits, higher peak areas and higher S/N ratio. ESI signal intensity was about 95% higher for all the compounds by FIA, only for CYC was APPI shown to be 90% better than ESI. For on-column analysis in MQ-water, the five studied compounds presented ESI signal intensities 90% higher. In the presence of a complex matrix as wastewater samples, ESI signal was about 80% higher than the APPI and APCI signal, maybe due as a result of interferences from the sample matrix that were lower when using ESI. It was further shown than ESI provides less matrix ionization effects for three of the target compounds, which may suggest than the ESI source is less sensitive to matrix ion effects than either APCI or APPI sources, for the analytes and method developed in this work.

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Supporting information

Supporting information may be found in the online version of this article.

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