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Advanced monitoring of pharmaceuticals and estrogens in the Llobregat River basin (Spain) by liquid chromatography–triple quadrupole-tandem mass spectrometry in combination with ultra performance liquid chromatography–time of flight-mass spectrometry

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ABSTRACT

The occurrence of 28 pharmaceuticals and 10 estrogens has been investigated in waters from the lower part of the Llobregat River basin, where the main intakes for production of drinking water for Barcelona (Spain) are located. Sampling was programmed to monitor the same mass of water on its way down the river to reflect inputs from discharges, contribution from subsidiaries plus persistence of the compounds in the surface water. Analysis of pharmaceuticals was performed by off-line solid phase extraction (SPE) followed by liquid chromatography-tandem mass spectrometry with a triple quadrupole analyzer (LC–QqQ–MS/MS). Further analysis by ultra performance liquid chromatography–mass spectrometry with a time-of-flight analyzer (UPLC–TOF–MS) has been proposed and applied for confirmation of several of these target compounds. Estrogens have been analysed by on-line SPE–LC–QqQ–MS/MS. Within the class of pharmaceuticals, 23 out of the 28 compounds investigated, were detected in at least one sample. The highest concentrations were observed for the β-blockers metoprolol (8042 ng L^{-1}) and sotalol (788 ng L^{-1}), the antibiotic ofloxacin (1904 ng L^{-1}), and the lipid regulator gemfibrozil (1014 ng L^{-1}). Within the group of estrogens, only estrone and estrone-3-sulfate were positively identified, with concentrations for the former (0.82–5.81 ng L^{-1}) close in some locations to those considered sufficient to induce estrogenic effects in aquatic organisms (1–10 ng L^{-1}). As a general pattern, concentration of target compounds increases along the river flow as expected.

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1. Introduction

The present study is focused on monitoring the presence of pharmaceutically active compounds in natural waters. This family of compounds includes prescription drugs, over-the-counter medications and drugs used in hospitals, plus other natural hormones having endocrine disrupting properties.

Interest about the study of the presence and toxicity of these compounds has been reported by some international organizations (GWRC, 2004; Henderson, 2006). The European parliament, for instance, during the preparation of the recently adopted Directive 2008/105/EC on environmental quality standards in the field of water policy, considered the inclusion of various pharmaceuticals (e.g. carbamazepine and diclofenac) in the list of substances subject to a review for possible identification as "priority substances" or "priority hazardous substances", although they were finally withdrawn from the final version. The Global Water Research Coalition in an effort to develop a common list of pharmaceuticals relevant to the water cycle has included carbamazepine, sulfamethoxazole, diclofenac, ibuprofen, naproxen, bezafibrate, atenolol, erythromycin and gemfibrozil as Class 1: high priority pharmaceuticals (GWRC, 2004).

Main reasons for concern are that large quantities of these compounds can enter the environment after use by individuals. During last years, several studies have shown efficiency of water treatment technologies in removing pharmaceutical and endocrine disrupting compounds. Whereas conventional technologies such as sand filtration or flocculation showed poor elimination

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percentages (Ternes et al., 2002), advanced oxidation processes (Zwiener and Frimmel, 2000; Ternes et al., 2003) or nanofiltration and reverse osmosis techniques (Snyder et al., 2007) have been revealed as the most effective ones. Concerning secondary treatments in wastewater treatment plants (WWTPs), literature reveals activated sludge with nitrogen treatment and membrane bioreactor as the most efficient ones(Miège et al., 2009). Other sources are unused or expired medications that are placed in the trash, residues from pharmaceutical manufacturing and residues from hospitals.[1]

Recent advances in technology have improved the ability to detect and quantify these chemicals in environmental samples. Even though they are found in very low concentrations, there is still a lack of knowledge about long-term risks that the presence of a large variety of drugs may pose for non-target organisms as well as for human health (Gros et al., 2006).

The lower Llobregat River basin (NE of Spain) has been the object of several studies dealing with the presence of these target analytes in surface water. High concentration of industrial and agricultural activities, linked to the fact that is a densely populated area, turns these waters into receiving bodies from urban and industrial WWTPs, accidental spills from industries and diffuse pollution from agriculture. This situation, due to the fact that the Llobregat River is source for drinking water for a few millions inhabitants living in the area, rises the necessity of further investigation on the presence of these pollutants and the risks associated.

Pharmaceuticals and their toxicity have been studied in the upper part of the Llobregat basin (Farré et al., 2001) and a short list of them were included in a study covering a wide range of emerging pollutants in the same area (Kuster et al., 2008). Results obtained in the Llobregat basin area, plus other campaigns in other basins in Spain (Gros et al., 2006; Hernando et al., 2006), show levels of pharmaceuticals at the nanogram-per-liter level (ng L^{-1}) or even at the low microgram-per-liter level (μg L^{-1}). Estrogens and progestrogens have also been monitored in this area (Solé et al., 2000; Petrovic et al., 2002; Rodriguez-Mozaz et al., 2004a,b) showing levels of some of them, specially estrone and estrone-3-sulfate, at the low nanogram-per-liter level (ng L^{-1}).

For the analysis of these compounds in the studies reviewed liquid chromatography–mass spectrometry (LC–MS) was the technique mainly selected in the past (López De Alda and Barceló, 2000; Farré et al., 2001), but at present this technique has been largely substituted by liquid chromatography-tandem mass spectrometry (LC–MS/MS) (Gros et al., 2006; Hernando et al., 2006; Kuster et al., 2008). During last years, the Commission Decision 2002/657/EC that was aimed at regulating the performance of analytical methods in the food industry, has also been applied to environmental analysis. According to this regulation, three identification points (IP) are needed when using LC–MS/MS for correct confirmation of the presence of target compounds. The high sensitivity of LC–MS/MS (with triple quadrupole (QqQ) analyzers) makes it a very suitable, accessible technique for analysis in surface waters. The main problem is that the three IP are not obtained for those analytes not showing two selected reaction monitoring (SRM) transitions, that is, when only one product ion can be obtained from the precursor one. This disadvantage makes this technique not reliable enough for the analysis of compounds such as ibuprofen or gemfibrozil.

An innovative aspect of the present study is the performance of an extra analysis based on the use of time-of-flight (TOF) detection for confirmation of the analytes, an approach that has been previously tested for analysis of pharmaceuticals but in wastewaters (Martínez Bueno et al., 2007). Ultra performance liquid chromatography–quadrupole–time of flight-tandem mass spectrometry (UPLC–QTOF–MS/MS) has been applied for drugs identification in wastewater analysis (Petrovic et al., 2006). However, the use of this technique was tested in the present study and finally discarded for our purpose because of the high detection limits found (results not included in this article). UPLC–TOF–MS was also tried and finally selected because of its higher sensitivity. Detection limits were appropriate to confirm peaks of contamination of target analytes in surface waters. TOF–MS measures the accurate mass of the compounds, adding that extra point of confirmation needed for getting a reliable result.

Another innovative aspect concerns the sampling method. Instead of taking samples from several points at the same time, sampling was programmed to try to sample the same mass of water on its way down the river. Monitoring should reflect this way inputs from discharges, contribution from subsidiaries plus persistence of the compounds in the surface water. This sampling method contributes to eliminate some sources of mistakes when interpreting results obtained from traditional samplings (e.g. a peak of contamination caused by a punctual discharge upstream will no be detected in the analysis downstream and it could lead to a wrong conclusion on the natural removal of the compound).

This work provides also for the first time a view on the occurrence of 38 emerging compounds in the lower part of the Llobregat basin area based on the combination of two techniques (LC–QqQ–MS/MS + UPLC–TOF–MS) for their unequivocal confirmation and quantification.

2. Materials and methods

2.1. Chemicals and standards

All standards used were of high purity grade (>90%). Ibuprofen, naproxen, ketoprofen, diclofenac and gemfibrozil were kindly supplied by Jescuder (Rubí, Spain). Indomethacine, acetaminophen, mefenamic acid, clofibric acid, bezafibrate, mevastatin, azythromycin dihydrate, erythromycin hydrate, carbamazepine, fluoxetine hydrochloride, lansoprazole, loratadine, famotidine, ranitidine hydrochloride, sulfamethoxazole, trimethoprim, ofloxacin, atenolol, metoprolol, propanolol hydrochloride and sotalol hydrochloride were purchased from Sigma–Aldrich (Steinheim, Germany). Propyphenazone, pravastatin and paroxetine hydrochloride were from LGC Promochem (London, UK). Pure standards of the natural and synthetic, both free and conjugated, estrogens estriol-3-sulfate, estriol-16-glucuronide, estradiol-17-glucuronide, estrone-3-glucuronide, estrone-3-sulfate, estriol, estradiol, ethynyl estradiol, estrone and diethylstilbestrol were supplied by Sigma Aldrich (Steinheim, Germany).

Individual stock standard solutions were prepared at 1000 μg mL^{-1} in methanol and stored at −20 °C. A mixture of all pharmaceutical standards and another mixture containing all estrogens were prepared by appropriate dilution of the individual stock solutions. Further dilutions of the pharmaceutical mixture were prepared in methanol–water (25:75, v/v) before each analytical sequence and were used as working standard solutions for external calibration. Working standard mixtures of the estrogens were prepared by dilution in methanol and used as spiking solutions for preparation of the aqueous calibration standards (content of methanol <0.1%).

HPLC-grade acetonitrile and water (Riedel de Haën) were supplied by Sigma Aldrich (Steinheim, Germany). Methanol (J.T. Baker) was supplied by Serviquimia (Constantí, Spain). Hydrochloric acid 37%, ammonium acetate (NH4Ac) and acetic acid (HAc) were from Merck (Darmstadt, Germany). Nitrogen for drying 99.995% of purity was from Air Liquide (Madrid, Spain).

[1] www.epa.gov/ppcp

2.2. Site description and sampling procedure

The Llobregat River is located in the northeast of Spain and flows into the Mediterranean Sea south of the city of Barcelona. This is a densely populated area where agriculture and industrial activities (tannery, textile, pulp, paper and salt mining) are also present. The river receives discharges from urban and industrial WWTPs and runoff from agriculture and salt formation areas. Water from the Llobregat River basin is also used for production of drinking water. Several drinking water plants are located next to the river. This high urbanization of the basin is especially significant in Mediterranean climate basins. River water flows fluctuate heavily along the year and wastewater effluents can account for the majority of the river water flows during the dry season. It constitutes, together with its two main tributaries, Cardener River and Anoia River, a good example of overexploited Mediterranean streams.

In the present study a total of 16 water samples were collected at eight selected sites of the lower reach of the Llobregat River basin (see Fig. 1) in two different sampling campaigns performed in November 2006 and December 2006. The first site is located upstream just before the Terrassa Drinking Water Treatment Plant (DWTP) intake (site 1). The five following samples were taken at sites (2–6) located downstream the Llobregat basin. According to the flow, estimation of the time to take the samples was done with the aim of monitoring the same water mass as it flows to the sea. Site 2 is located in the Llobregat River just before the union with its tributary, Anoia River, which carries the discharge of the Abrera WWTP. The next site is located in the Anoia River itself close to its confluence with the Llobregat River (site 3). The following samples were taken from the Llobregat River at Capdevila Dam (site 4, before the input of the Rubí Creek), downstream of the town Molins de Rei (site 5), and before the intake of the Sant Joan Despí DWTP (site 6), the biggest DWTP supplying water to the city of Barcelona. Additionally, two more sites (A and B) having less influence in the water quality of site 6 were monitored: a channel receiving polluted water from the Anoia River, the Rubí Creek and the Sant Feliu WWTP (site A) that was constructed to avoid these waters being discharged to the Llobregat River before the Sant Joan Despí DWTP intake (site A), and the Rubí Creek itself (site B) which receives wastewater from industries in the area.

Water samples (2 L) were collected in amber glass bottles to avoid photodegradation of the analytes. Upon reception, samples were filtered through 0.45 μm Nylon filters (Whatman, Maidstone, UK) to eliminate particulate matter and other suspended solid matter and then stored at 4 °C in the dark until analysis which was always carried out within 48 h of collection to keep microbial degradation to a minimum.

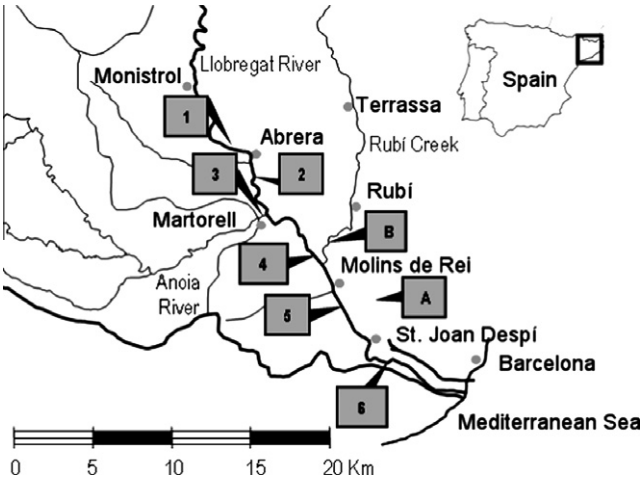

Fig. 1. Map of the low Llobregat basin where the eight sampling sites are indicated (1–6, A and B).

2.3. Analytical methods

Pharmaceuticals were initially analysed by off-line solid phase extraction (SPE) followed by LC–QqQ–MS/MS. Additional confirmation was performed by UPLC–TOF–MS. Estrogens were monitored using an on-line SPE–LC–QqQ–MS/MS method.

2.3.1. Determination of pharmaceuticals by off-line SPE followed by LC–QqQ–MS/MS

Pharmaceuticals were initially analysed by off-line SPE followed by LC–QqQ–MS/MS according with a method described in the literature (Gros et al., 2006).

MS/MS detection was performed in the SRM mode acquiring two SRM transitions per compound. Only 1 SRM transition was acquired in the case of ibuprofen, gemfibrozil, and pravastatin due to poor fragmentation. A second transition was acquired for ofloxacin and ketoprofen according to the method followed, but it could be not detected in the experiment. In order to increase the sensitivity, the SRM transitions were classified into different elution time windows. The first transition, the most abundant one, is used for quantification, and the second transition, less abundant, was used for confirmation purposes.

Identification of the target analytes was accomplished by comparing the retention time and the LC–MS/MS signals of the target compounds in the samples with those of standards analysed under the same conditions. For positive identification the following criteria had to be met: (1) LC chromatographic retention time agreement within 2%; (2) relative abundance of the two selected precursor ion-product ion transitions within a margin of 20% (93/256/EEC).

2.3.2. Determination of estrogens by on-line SPE–LC–QqQ–MS/MS

Fully automated on-line SPE–LC–QqQ–MS/MS analysis of estrogens was performed with a SPE sample processor Prospekt-2 (Spark Holland, Emmen, The Netherlands) coupled on-line to the LC–MS/MS system. The method was previously developed for the analysis of free estrogens (Rodriguez-Mozaz et al., 2004a) but it has been modified to include three conjugated compounds (estrone-3-glucuronide, estriol-3-sulfate and estriol-16-glucuronide).

MS/MS detection was performed in the SRM mode with an electrospray interface operated in the negative ion (NI) mode. Two SRMs transitions were monitored per compound. For quantification of the analytes, the external standard method was used, based on the peak areas obtained in the first SRM transitions.

Positive confirmation of the target analytes in the samples was based on the same criteria (retention time and relative abundance of the two SRM transitions signals) described in Section 2.3.1.

2.3.3. Confirmation of pharmaceuticals by UPLC–TOF–MS

Confirmation of pharmaceuticals in the water sample extracts previously analysed by LC–QqQ–MS/MS was performed by UPLC–TOF–MS. The method is a modification of an UPLC–QTOF–MS/MS method previously developed (Petrovic et al., 2006).

Positive identification of the target compounds was based on: (a) accurate mass measurement of the analyte base peak with an error <5 ppm; and (b) LC retention time of the analyte compared to that of a standard within ±2%.

In all cases (the only exception was pravastatine, which formed the sodium adduct) the base peak corresponded to the protonated $[M+H]^+$ or deprotonated $[M-H]^-$ molecular ion of the analyte, depending on whether the analysis was performed in the positive

ion (PI) or NI mode. The errors obtained in mass measurements (between 0.0 and 4.7 ppm (0.0–1.2 mDa)) were within the widely accepted accuracy threshold of 5 ppm.

For low contaminated waters (river, ground and drinking water) the instrumental detection limits (IDLs) might be not sufficient to detect low concentrations occurring in these samples, and the UPLC–TOF–MS method should be complemented with a sensitive quantitative analysis using a QqQ in SRM mode (Petrovic et al., 2006). That is the reason why only analytes showing one transition or achieving the highest levels in the QqQ analysis were selected for confirmation, as QqQ analyses have been proved to be more sensitive and accurate for quantification. Thus, confirmation analysis via UPLC–TOF–MS has been done only for diclofenac, ibuprofen, gemfibrozil, atenolol, sotalol, metoprolol and ofloxacin in the most polluted samples (sites 3, 5, A and B).

3. Results and discussion

3.1. Levels of pharmaceuticals

Table 1 lists the method detection and quantification limits calculated for the quantification and confirmation SRM transitions monitored for the various target pharmaceuticals in the off-line SPE–LC–QqQ–MS/MS method, together with the percentage of positive samples and the minimum, maximum and average concentrations quantified with this method in the samples investigated.

For all pharmaceuticals but ibuprofen, gemfibrozil, pravastatin, ketoprofen and ofloxacin, two SRM transitions were detected per compound thus achieving four IPs (2002/657/EC), which is in compliance with the minimum confirmation requirements established in the Council Directive 96/23/EC. However, in the case of ibuprofen, gemfibrozil, pravastatin, ketoprofen and ofloxacin, for which only one SRM transition was detected or monitored due to poor fragmentation in the MS/MS system, only 2.5 IPs were earned, which are not enough to comply with the aforementioned Directive.

In order to gain sufficient confirmation in the analysis of these five compounds, water sample extracts previously analysed by LC–QqQ–MS/MS were subjected to a second analysis by means of UPLC–TOF–MS, which earns two additional IPs per ion monitored (2002/657/EC). As it is shown in Table 2, ibuprofen and gemfibrozil could be positively confirmed in the samples through the second analysis (LODs 150 and 50 ng L^{-1}, respectively). Pravastatin and ofloxacin levels, in spite of reaching 78 and 1904 ng L^{-1}, respectively, in the samples, were too low for confirmation with this second technique (LODs 350 and 500 ng L^{-1}, respectively). Ketoprofen had not been found by means of LC–QqQ–MS/MS so no confirmation was performed by UPLC–TOF–MS (LOD 150 ng L^{-1}).

This approach was also used to confirm the presence of compounds detected at very high concentrations in heavily polluted samples. Since the sensitivity provided by LC–QqQ–MS/MS working in the SRM mode is higher than that achieved by UPLC–TOF–MS in the scan mode not all positive results obtained with the for-

Table 1
Method detection and quantification limits for the various target pharmaceuticals in the off-line SPE–LC–QqQ–MS/MS method, together with the percentage of positive samples and the minimum, maximum and average concentrations quantified with this method in the samples investigated.

Therapeutic group	Compound	LOD (ng L^{-1})	LOQ (ng L^{-1})	LOD (ng L^{-1})	% Rec.	% Positive samples	Max	Min	Average	Max[a]	Average[a]
		SRM 1	SRM 1	SRM 2							
Analgesics and antiinflammatories	Ketoprofen	0.6	1.3	–	54–115	0	–	–	–	144	10.8
	Naproxen	0.3	0.7	0.5		100	105.5	14.4	42.6	4500	14.8
	Ibuprofen	0.5	1.4	–		100	490.4	29.4	152.9	6400	101.1
	Indomethacin	0.6	1.6	3.0		50	46.7	10.1	23.8	220	3.5
	Diclofenac	0.4	0.9	3.1		100	358.1	17.6	87.7	1800	19.6
	Mefenamic acid	0.1	0.2	0.7		14	2.4	2.4	2.4	242	16.0
	Acetaminophen	0.1	0.4	0.9		100	96.5	14.1	34.8	3600	30.9
	Propyphenazon	0.1	0.4	0.4		100	18.8	1.5	7.3	880	25.0
Lipid regulators and cholesterol lowering statin drugs	Clofibric acid	0.1	0.1	0.6	101–116	21	13.3	2.1	7.0	630	16.9
	Gemfibrozil	0.1	0.3	–		100	1014.1	26.1	242.8	58 000	15.9
	Bezafibrate	0.1	0.2	0.5		100	305.2	3.4	48.3	780	12.5
	Pravastatin[b]	4.2	11.1	–		21	77.7	52.6	65.1	0	0.0
	Mevastatin	0.1	0.1	0.1		0	–	–	–	0	0.0
Antiulcer agent Histamine H1 and H2 receptor antagonists	Lansoprazole	0.6	1.5	2.5	111	14	76.7	42.0	59.3	0	0.0
	Loratadine	0.2	0.4	0.4	38–70	57	201.6	0.9	45.5	–	–
	Famotidine	0.3	0.7	0.7		21	8.6	3.5	6.4	–	–
	Ranitidine	0.4	0.9	0.3		100	69.6	2.3	16.5	142	10.0
Antibiotics	Erythromycin	0.9	2.3	1.6	52–113	93	111.9	6.9	32.9	1 209 000	52.3
	Sulfamethoxazole	0.3	0.9	0.4		100	119.3	4.1	24.0	1900	35.6
	Trimethoprim	0.2	0.5	0.3		100	252.0	2.4	38.5	800	18.2
	Ofloxacin[b]	0.9	2.3	–		100	1903.6	8.0	285.3	306	40.6
β-Blockers	Atenolol	0.1	0.4	0.2	58–102	100	199.7	5.8	41.5	465	106.3
	Sotalol	0.1	0.3	0.2		100	787.6	1.9	66.8	950	49.3
	Metoprolol	0.3	0.7	0.4		79	8041.1	1.2	738.0	2200	47.4
	Propranolol	1.1	3.0	0.5		64	17.3	1.6	6.7	590	8.0
Psychiatric drugs	Carbamazepine	0.1	0.1	0.2	83–95	100	178.7	8.3	64.0	2500	68.9
	Fluoxetine	1.4	3.8	0.4		7	4.2	4.2	4.2	34	10.5
	Paroxetine	0.4	1.0	1.3		0	–	–	–	0	0.0

[a] Concentration in surface water according to <http://www.knappe-eu.org/fichiers/44-D1.2.> environmental indicator final version.pdf.
[b] Not confirmed by UPLC–TOF–MS.

Table 2
Examples of accurate mass measurement of selected pharmaceuticals in real samples at selected sites.

Compound	Theoretical mass (m/Z)	Site B November 2006			Site A November 2006			Site 3 November 2006			Site B December 2006		
		Experimental mass (m/Z)	Error (mDa)	Error (ppm)	Experimental mass (m/Z)	Error (mDa)	Error (ppm)	Experimental mass (m/Z)	Error (mDa)	Error (ppm)	Experimental mass (m/Z)	Error (mDa)	Error (ppm)
Diclofenac	294.0089	294.0083	−0.2	−0.6	294.0890	0	0				294.0076	−1.3	−4.4
Ibuprofen	205.1229	205.1229	0.0	0.0	205.1238	0.9	4.4	205.123	0.1	0.5			
Gemfibrozil	249.1491	249.1489	−0.2	−0.8	249.1483	−0.8	−3.2				249.1487	−0.4	−1.6
Atenolol	267.1708	267.1704	−0.5	−1.9									
Sotalol	273.1273				273.1263	−1.0	−3.7						
Metoprolol	268.1912				268.1921	0.8	0.3						
Ofloxacin	362.1516	–	–	–	–	–	–				–	–	–

mer technique can be confirmed with the latter. However, for those cases where the concentrations found are remarkably high an additional font of confirmation is possible and valuable. Table 2 shows as an example the results obtained in the UPLC–TOF–MS analysis of diclofenac, atenolol, sotalol and metoprolol in samples collected from sites 3, A and B. In this case, all results were positively confirmed.

With respect to the results obtained, 14 out of the 28 pharmaceuticals investigated were detected in all samples (see Table 1). Only three compounds, namely, ketoprofen, mevastatin, and paroxetine were not detected in any sample. Fig. 2 shows the range of concentrations measured for the various compounds positively identified in the samples. The highest concentrations, above 500 ng L^{-1}, were found for the lipid regulator gemfibrozil (up to 1014 ng L^{-1}), the antibiotic ofloxacin (up to 1904 ng L^{-1}), and the β-blockers sotalol (up to 788 ng L^{-1}) and metoprolol (up to 8042 ng L^{-1}). Average concentrations higher than 100 ng L^{-1} were calculated for the analgesic antiinflammatory ibuprofen (153 ng L^{-1}), and also for gemfibrozil (243 ng L^{-1}), ofloxacin (285 ng L^{-1}) and metoprolol (738 ng L^{-1}) (Table 1). Carbamazepine was among the most ubiquitous compounds (detected in all samples). Concentrations ranged from 8 to 179 ng L^{-1}. Although its consumption is not very high, carbamazepine is not or very poorly removed in conventional treatment processes operating in WWTPs (Zhang et al., 2008).

Recently, an extensive data compilation on the environmental occurrence of pharmaceuticals products in surface water and WWTP influents and effluents has been performed in the frame of the project KNAPPE – Knowledge and Need Assessment on Pharmaceutical Products in Environmental Waters funded by the European Commission within the 6th Framework Programme (Sadezky et al., 2008). Comparing the average concentration calculated for each compound within the present study with the average of those compiled in the frame of the above project, all detected pharmaceuticals but mefenamic acid, propyphenazon, clofibric acid, erythromycin, sulfamethoxazole, atenolol, propanolol, carbamazepine, and fluoxetine (i.e. 16 compounds in total), showed higher values. Remarkably higher values were those corresponding to gemfibrozil (243 ng L^{-1} vs. 16 ng L^{-1}), ofloxacin (285 ng L^{-1} vs. 41 ng L^{-1}), and metoprolol (738 ng L^{-1} vs. 47 ng L^{-1}). However, the maximum values reported were only exceeded by ofloxacin (1904 ng L^{-1} vs. 306 ng L^{-1} found in Italy) and metoprolol (8041 ng L^{-1} vs. 2200 ng L^{-1} found in Germany), in addition to those never detected in the reviewed literature, namely, pravastatin (maximum concentration in the present work 78 ng L^{-1}), lansoprazole (77 ng L^{-1}), loratadine (202 ng L^{-1}), and famotidine (9 ng L^{-1}).

Classified by therapeutic groups, the highest average concentrations were detected for the β-blockers (average of positive results = 191 ng L^{-1}), followed by lipid regulators and cholesterol lowering statin drugs (118 ng L^{-1}), antibiotics (91 ng L^{-1}), analgesics antiinflammatories and antiulcer agents (both 59 ng L^{-1}), psiquiatric drugs (58 ng L^{-1}), and histamine H1 and H2 receptors antagonists (23 ng L^{-1}). For results discussion it is important to mention that samples from site 2 and site A taken in the second sampling period were lost during sample preparation.

Fig. 3 shows the total charge of pharmaceuticals, grouped by therapeutic class, detected along the course of the river basin, at both sampling campaigns. Sites showing the highest concentra-

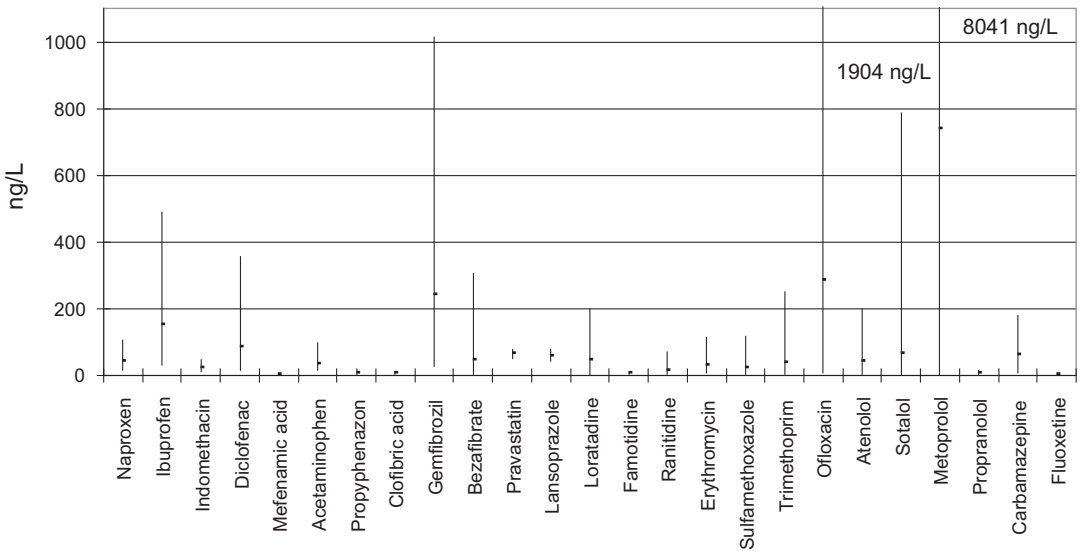

Fig. 2. Range of concentrations (minimum, maximum and average) measured for the various compounds positively identified in the samples (ng L^{-1}).

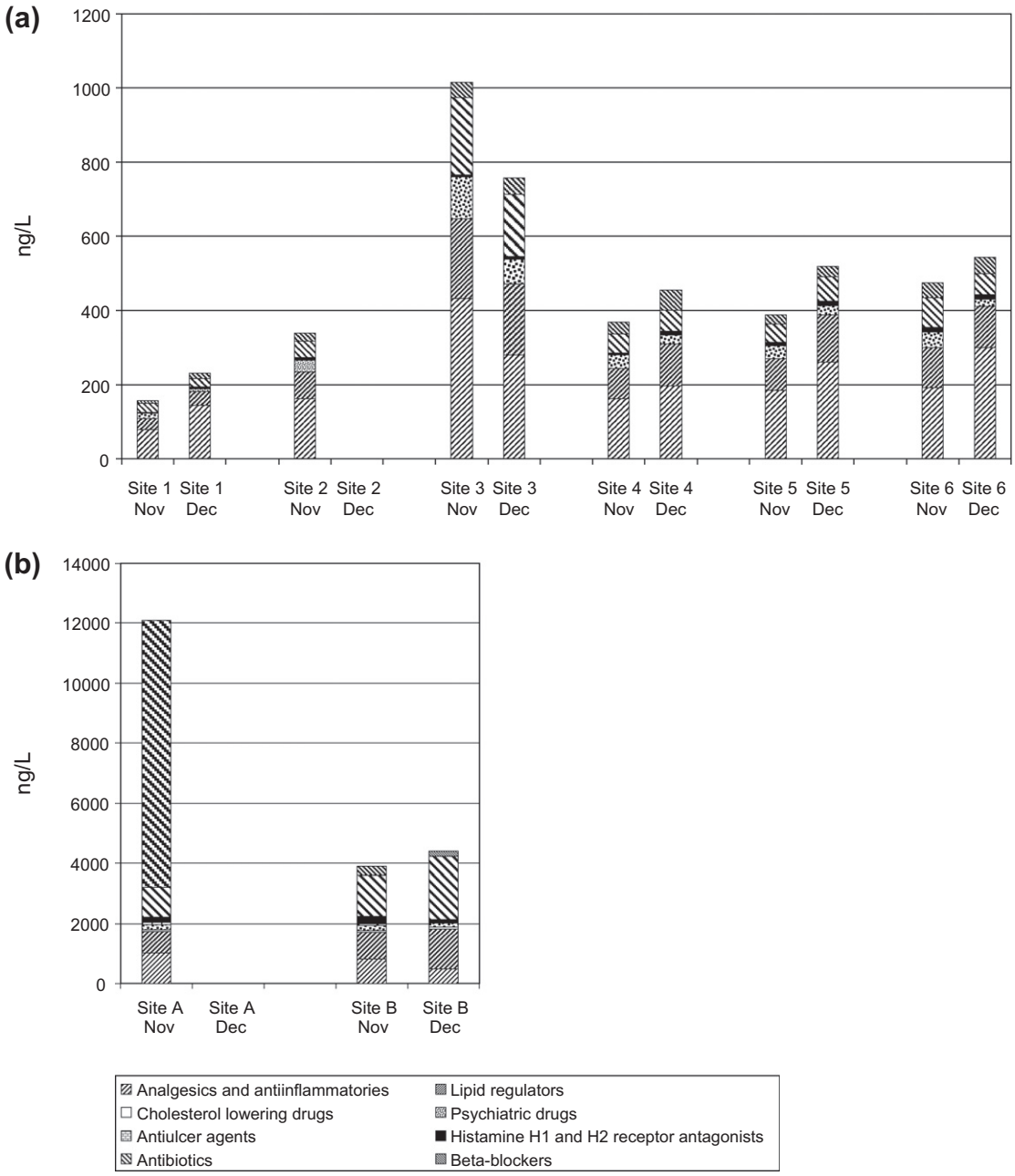

Fig. 3. Cumulative levels of pharmaceuticals, grouped by therapeutic class, detected at sites 1–6 (a) and sites A and B (b), at both sampling campaigns.

tions of the target compounds were site A (channel receiving waters from the Anoia River, Rubí Creek and Sant Feliu WWTP) and site B (Rubí Creek). A decision was taken in the past to avoid these waters discharging to the Llobregat River upstream of the Sant Joan Despí DWTP.

Monitoring of surface water along the river flow showed, in general, an increase of the total pharmaceuticals concentrations from sites 1 to 6. The exception was site 3, which is not located in the Llobregat River itself but in the tributary Anoia River before its union to the previous one. Levels of target compounds were usually higher in the Anoia River (site 3). The low flow of the Llobregat River, linked to the fact of its seasonal fluctuations, makes this river very sensitive to the levels of target analytes present in its tributaries and the water coming directly from WWTPs. Comparing both campaigns, slightly higher values were found in the samples collected in December. Taking into account proximity of sampling periods, results indicate a fairly constant input of contaminants in the river basin.

Information on acute and, especially chronic, toxicity of pharmaceutical aquatic residues is scarce. Pharmaceutical concentrations measured in surface waters are generally well below concentrations that are known to cause acute toxicity to aquatic organisms (Cooper et al., 2008). However, pharmaceuticals enter the aquatic environment continuously leading to fairly constant environmental water concentrations. Chronic exposure to pharmaceuticals has the potential for numerous subtle effects, such as metabolic or reproductive changes on non-target organisms (Cooper et al., 2008). The UK Environment Agency (Boucard, 2006) has recently compiled a database with data on chronic aquatic ecotoxicity of human pharmaceuticals towards various aquatic organisms belonging to different taxonomic groups. The maximum concentrations measured in the Llobregat basin were

Table 3
Method detection and quantification limits of the various target estrogens in the on-line SPE–LC–QqQ–MS/MS method.

Compound	LOD (ng L^{-1})	LOQ (ng L^{-1})	LOD (ng L^{-1})
	SRM 1	SRM 1	SRM 2
Estriol-3-sulfate	0.05	0.12	0.23
Estriol-16-glucuronide	0.16	0.41	0.22
Estradiol-17-glucuronide	0.23	0.62	0.67
Estrone-3-glucuronide	0.14	0.38	0.35
Estrone-3-sulfate	0.02	0.06	0.10
Estriol	0.49	1.32	0.62
Estradiol	0.53	1.42	0.65
Ethynyl estradiol	2.20	5.86	2.90
Estrone	0.12	0.32	0.35
Diethylstilbestrol (DES)[a]	0.30	0.79	0.94

[a] For DES, SRM 1 really refers to the first peak and SRM 2 to the second peak of the first transition.

always below the reported chronic toxicity values. Maximum concentrations measured were on average more than 5, 3, 6, and 5 orders of magnitude lower than the lowest toxicity values reported for algae, invertebrates, fish and plants, respectively, which indicates no ecological risk. However, the potential for synergistic or additive toxicity to aquatic organisms and/or other toxicity effects, not yet studied, cannot be ruled out. According with the inventory of chemicals purported to be endocrine disrupters compiled by the Institute for Environment and Health (IEH, 2005), which includes a total of 966 compounds, carbamazepine (detected in all samples at concentrations up to 179 ng L^{-1}) affects circulating thyroid hormones and sulfamethoxazole (detected in all samples at concentrations up to 119 ng L^{-1}) alters thyroid function.

3.2. Levels of estrogens

Among the group of target estrogens, estrone and estrone-3-sulfate were the only analytes found at the Llobregat basin surface waters and at very low concentrations (in the low ng L^{-1} range). Table 3 lists the LODs achieved for the various compounds monitored. Estrone concentrations, measured in all but one samples, ranged from 0.82 to 5.81 ng L^{-1}. The highest levels were detected at site A (2.38 and 5.81 ng L^{-1} in the first and second sampling campaign, respectively) and site B (2.80 ng L^{-1} in the first sampling campaign). These levels are within the range of those (1–10 ng L^{-1}) from which estrogenic effects can be expected (1–10 ng L^{-1} depending on the estrogenic assay used) (Petrovic et al., 2004). Conversely, the most potent estrogenic compounds (estradiol, ethynyl estradiol, and diethylstilbestrol) were not found in any of the samples analysed.

Estrone-3-sulfate was found in 80% of the samples and reached values between 0.25 and 1.46 ng L^{-1}. Additionally, estriol-3-sulfate was detected at very low levels in some samples but its presence could not be confirmed because the concentration measured with the most abundant SRM transition was lower than the method limit of detection achieved with the second transition. UPLC–TOF–MS could not be used in this case for confirmation due to insufficient sensitivity. Estrogenic activity for conjugated estrogens is lower than for the free estrogens; levels found seem to have no risk for the environment.

4. Conclusions

The combination of two LC–MS techniques has been used to unequivocally detect and quantify levels of 38 compounds in surface waters of the Llobregat River basin. LC–QqQ–MS/MS has been used for detection and quantification because of its high sensitivity and possibility of confirmation when two transitions of the parent ion to product ions are recorded. When a second transition could not be selected, accurate measurement of the mass of the base ion was performed using UPLC–TOF–MS for confirmation. This approach was used to confirm the presence of ibuprofen and gemfibrozil in all samples and of diclofenac, atenolol, sotalol and metoprolol in samples showing high levels of these compounds. Confirmation of pravastatin and ofloxacin by UPLC–TOF–MS was not possible due to insufficient sensitivity. The main disadvantage of this solution is the extra cost related to the performance of two analyses. This problem can be partially solved by analysing exclusively the samples containing those analytes for which extra confirmation is needed.

Results from the monitoring performed confirmed the presence of drugs of high consumption as expected in a densely populated Mediterranean basin. Significant levels, higher in general than those previously reported in the literature, were found for the β-blockers metoprolol and sotalol, the antibiotic ofloxacin and the lipid regulator gemfibrozil. Within the group of estrogens, only estrone and its conjugated derivative estrone-3-sulfate were confirmed to be present. Estrone levels were in some sites close to those considered sufficient to cause estrogenic effects in aquatic organisms.

Two sites, out the eight monitored, showed distinctly high concentrations of both classes of compounds; however, their waters are diverted to reach the river at locations close to the mouth and downstream of the inlet of the Sant Joan Despí DWTP, which supplies water to a great part of the Barcelona metropolitan area, in order to protect the quality of the source water. Along the river, the contamination load was observed to increase from upstream to downstream.

This study confirms the presence of some of the target compounds in concentrations that could lead to a potential risk to the environment and human health. Further studies on the risk of these compounds should be undertaken.

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