A method for simultaneous determination of four benzodiazepines (bromazepam (BMZ), carbamazepine (CBZ), diazepam (DZP) and nordiazepam (NDZ)) and four barbiturates (barbital (BTL), pentobarbital (PTB), phenobarbital (PNB) and secobarbital (SCB)) in river water and wastewater using solid-phase extraction (SPE) followed by liquid chromatography-(electrospray) tandem mass spectrometry (LC-(ESI)MS/MS) was developed. LC-(ESI)MS/MS analysis was performed in positive and negative modes for benzodiazepines and barbiturates, respectively, and in selected reaction monitoring (SRM). Limits of detection (LODs) were in the range of 0.2–5 ng/L for benzodiazepines and 2.5–50 ng/L for barbiturates. Precision (repeatability and reproducibility between days) expressed as %RSD (n = 5) was lower than 17% for low concentration (depending on the matrix between 50 and 250 ng/L for barbiturates, and between 5 and 25 ng/L for benzodiazepines) and lower than 15% for high concentration (between 200 and 1250 ng/L for barbiturates, and between 20 and 125 ng/L for benzodiazepines). Low matrix effect was observed for all compounds, except for BTL (75%) and PTB (−48%) in wastewater. The method was applied to water samples from two sewage treatment plants (STPs) and the rivers Ebre, Ter and Llobregat, located in Catalonia. CBZ was the target compound found at the highest concentration in river water (2.1–3.3 ng/L). In both influent and effluent wastewater samples, PNB, BMZ, CBZ, DZP and NDZ were determined at concentration levels ranging from 5.0 to 2337.3 ng/L.

**Keywords**: benzodiazepines; barbiturates; liquid chromatography-tandem mass spectrometry; Core-Shell particle; solid phase extraction; aquatic environment

**1. Introduction**

A huge amount of residues generated by the different activities involved in our daily lives enters sewage treatment plants (STPs). These residues may contain diverse groups of organic contaminants, including a broad spectrum of pharmaceuticals, personal care products, illegal drugs, pesticides and veterinary products, among others [1,2]. Despite the attempt by STPs to remove these contaminants from wastewater, their elimination by conventional designs is not always complete. Consequently, effluent discharges introduce the contaminants into the environment [2].

Psychiatric pharmaceuticals are among the most frequently used pharmaceuticals, and these include barbiturates and benzodiazepines. According to data provided by the Catalan Health Department, in 2012 approximately 10 million prescriptions of sedative hypnotics were prescribed in Catalonia [3].
Benzodiazepines are normally halogenated compounds, making them resistant to biodegradation [4]. Degradability studies of barbiturates under aerobic conditions and hydrolysis do not show any evidence of degradation, underlining their high stability in the environment [5]. Once introduced into the environment, psychiatric pharmaceuticals may have unexpected pharmacological and biological activities on non-target organisms, as this group of pharmaceuticals acts on the endocrine and central nervous system, directly affecting the regulation of behaviour and reproduction patterns [6].

Faced with this problem, the need arises to develop sensitive and selective analytical methods to evaluate the incidence of these contaminants in the environment. In Table 1 some of the analytical methods published to determine sedative hypnotic in aqueous environmental samples are compiled.

The most widely reported technique for extracting psychiatric pharmaceutical compounds from aquatic environmental samples is solid-phase extraction (SPE) [7–11]. Even though the majority of the methods reported are based on the simultaneous extraction of pharmaceuticals belonging to different therapeutic groups, only three or four psychiatric pharmaceuticals have been included among the target compounds to be studied. The most widely studied psychiatric compounds are carbamazepine (CBZ), diazepam (DZP) and phenobarbital (PNB). Four types of sorbents have been used to extract them: copolymer hydrophilic–lipophilic balanced Oasis HLB [5,8,12,13], mixed-mode polymeric sorbent Oasis MCX [7,14,15], mixed-anion exchange (MAX) [11] and strong cation-exchange Strata-XC [16]. In general, as can be seen in Table 1, acceptable recoveries were given for each type of sorbent when they were used to extract the compounds from wastewater. Moreover, automated methods based on online SPE coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS) have also been reported for the determination of pharmaceuticals including some sedative hypnotics in environmental waters [17,18]. For instance, López-Serna et al. [18] reported recoveries for four sedative hypnotics spiked in effluent and influent wastewaters between 25% and 78% using a HySphere Resin GP cartridge. Regarding the determination of sedative hypnotic, several methods involving gas chromatography-mass spectrometry (GC-MS) [4,5,7] or LC-MS/MS [8,12,14,16,18] have been published, with successful application in environmental aquatic samples. Despite the fact that many benzodiazepines are fairly polar and non-volatile compounds [19], for some of them the determination has been feasible by GC-MS using derivatisation of the active amide group of the benzodiazepines [4] or even without derivatisation [7]. Likewise, derivatisation of barbiturates is not required to be determined by GC-MS as was shown in a previous study by Peschka et al. [5], in which six barbiturates were determined in river water and wastewater from Germany.

LC-MS/MS methods have been successfully applied to determine benzodiazepines and barbiturates using either electrospray ionisation (ESI) [14,18] or atmospheric pressure chemical ionisation (APCI) [20]. As can be seen in Table 1 to date, methods based on a triple quadrupole (QqQ) mass analyser have been most widely developed for the determination of psychiatric pharmaceuticals [12,14,15,16,17,21], compared with other mass analysers such as ion trap (IT) [11,22] and quadrupole time-of-flight (Q-TOF) [10]. For instance, Hernández et al. [10] used Q-TOF for qualitative screening of 76 illegal drugs, including three sedative hypnotics. The identification of compounds was feasible in complex environmental samples, even without reference standards by means of the accurate mass, isotopic distribution and MS data provided by Q-TOF. Moreover, QqQ enables accurate quantification and high selectivity and sensitivity. Regarding this issue, several multi-residue methods published (see Table 1) have reported low limits of detection (LODs) ranging from 0.1 and 54 ng/L depending on the matrix and the compound. Quadrupole-linear ion trap (Q-LIT) [8,9,13,18] mass spectrometer has also been used to determine psychiatric pharmaceuticals such as PNB, pentobarbital (PTB), DZP and CBZ.
Table 1. Some examples of analytical methods reported in the literature to determine sedative hypnotic in environmental aquatic samples.

| Country          | Compounds studied | Compound | Extraction technique | Instrumental technique | LOD ng/L | Conc range ng/L | R % | LOD ng/L | Conc range ng/L | R % | LOD ng/L | Conc range ng/L | R % | Ref |
|------------------|-------------------|----------|----------------------|------------------------|----------|-----------------|-----|----------|-----------------|-----|----------|-----------------|-----|-----|
| Germany          | (6)               | SCB      | SPE with GC-MS       | 62<sup>a</sup>         | 0.10     | 5               | 86<sup>a</sup> | 62<sup>a</sup> | 20              | n.d | 5<sup>a</sup> | 4<sup>a</sup> | 86<sup>a</sup> | 5<sup>a</sup> | [5] |
| France           | (17)              | PTB      | Oasis HLB            | 103<sup>a</sup>        | 1        | <LOD-5.4        | 74<sup>a</sup> | 88<sup>a</sup> | 10              | n.d | 5<sup>a</sup> | 4<sup>a</sup> | 86<sup>a</sup> | 5<sup>a</sup> | [7] |
|                  |                   | PNB      | SPE with GC-MS       | 105<sup>a</sup>        | 1        | 0.3–1.5         | 120<sup>b</sup> | 120<sup>b</sup> | 22.3            | 157–295 | 5<sup>a</sup> | 4<sup>a</sup> | 86<sup>a</sup> | 5<sup>a</sup> | [7] |
| Slovenia         | (3)               | CBZ      | SPE with GC-MS       | 101<sup>b</sup>        | 1.4      | n.d-2.4         | 87<sup>b</sup> | 87<sup>b</sup> | 13.8            | n.d | 5<sup>a</sup> | 4<sup>a</sup> | 86<sup>a</sup> | 5<sup>a</sup> | [4] |
| Spain            | (23)              | NDZ      | Oasis HLB            | 101<sup>b</sup>        | 1.4      | n.d-8.3         | 101<sup>b</sup> | 101<sup>b</sup> | 13.8            | n.d | 5<sup>a</sup> | 4<sup>a</sup> | 86<sup>a</sup> | 5<sup>a</sup> | [14] |
| United Kingdom   | (65)              | BMZ      | SPE with RPLC-       | 101<sup>b</sup>        | <LOD-69  | 1<sup>a</sup>   | 101<sup>b</sup> | 101<sup>b</sup> | 21–18           | 21<sup>a</sup> | 5<sup>a</sup> | 4<sup>a</sup> | 86<sup>a</sup> | 5<sup>a</sup> | [15] |
| Greece           | (68)              | DZP      | SPE with RPLC-       | 101<sup>b</sup>        | <LOD-9  | 1<sup>a</sup>   | 101<sup>b</sup> | 101<sup>b</sup> | 21–18           | 21<sup>a</sup> | 5<sup>a</sup> | 4<sup>a</sup> | 86<sup>a</sup> | 5<sup>a</sup> | [16] |
| Spain            | (74)              | CBZ      | Oasis HLB            | 101<sup>c</sup>        | 0.4      | n.d-35.5        | 101<sup>c</sup> | 101<sup>c</sup> | 2.4             | 303  | 5<sup>a</sup> | 4<sup>a</sup> | 86<sup>a</sup> | 5<sup>a</sup> | [12] |
| Serbia           | (81)              | PTB      | Oasis HLB            | 108<sup>c</sup>        | 0.7      | <LOD-35.5       | 108<sup>c</sup> | 108<sup>c</sup> | 2.4             | 303  | 5<sup>a</sup> | 4<sup>a</sup> | 86<sup>a</sup> | 5<sup>a</sup> | [13] |

(continued)
Table 1. Continued.

| Country | Compounds studied | Compound | Extraction technique | Instrumental technique | Effluent ww | River | Influent ww |
|---------|-------------------|----------|----------------------|------------------------|-------------|-------|------------|
|         |                   |          |                      | R % LOD ng/L Conc range ng/L | LOD ng/L Conc range ng/L | LOD ng/L Conc range ng/L | Ref |
| china (22) | CBZ | On-line SPE | HLB disk | QqLIT RPLC-(ESI)MS/MS | 95<sup>d</sup> 0.3<sup>*</sup> 13–22 | 88<sup>d</sup> 0.5<sup>*</sup> 19–23 | [17] |
| Spain (74) | DZP | On-line SPE | Hysphere | QqLIT RPLC-(ESI)MS/MS | 82<sup>a</sup> 0.7 <LOD-6.5 | 72<sup>a</sup> 0.5 18.9 | 42<sup>a</sup> 3.5 n.r | [18] |
|          | CBZ  | Hysphere | QqLIT       | 59<sup>a</sup> 0.3 31.2–58.4 | 50<sup>a</sup> 38 156.8 | 25<sup>a</sup> 32 n.r | 25<sup>a</sup> 32 n.r |
|          | PNB  | resin | QqLIT       | 83<sup>a</sup> 2.3 n.d | 52<sup>a</sup> 18 n.d | 61<sup>a</sup> 0.3 n.r | 61<sup>a</sup> 0.3 n.r |
|          | PTB  | GP cartridge | QqLIT | 91<sup>a</sup> 2.2 n.d | 78<sup>a</sup> 0.3 n.d | 57<sup>a</sup> 8.3 n.r | 57<sup>a</sup> 8.3 n.r |
| Portugal (23) | BMZ | SPE with | RPLC-(ESI)MS/MS | 63<sup>a</sup> 2 n.d | 68<sup>a</sup> 2 n.d | 68<sup>a</sup> 2 n.d | [11] |
|          | DZP  | Oasis MAX | RPLC-(ESI)MS/MS | 92<sup>a</sup> 1 n.d | 145<sup>a</sup> 1 n.d | 145<sup>a</sup> 1 n.d | 145<sup>a</sup> 1 n.d |

Notes: Conc: Concentration, GC-MS: Gas chromatography-mass spectrometry, RPLC-(ESI)MS/MS: Reversed phase-liquid chromatography (Electrospray) tandem mass spectrometry, QqQ: triple quadrupole, QqLIT: quadrupole-linear ion trap, IT: ion trap. * Value corresponds to LOQ.
Recovery calculation (superscript): " matrix spiked before SPE against pure solvent standard, " ultrapure water spiked before SPE against pure solvent standard.
" matrix spiked before SPE using internal standard calibration, " matrix spiked before SPE against matrix spiked after SPE.
in environmental samples. As Table 1 shows, the different reports using Q-LIT archived LODs between 0.2 and 2.3 ng/L in river waters and between 0.3 and 38 ng/L in wastewater.

The purpose of this research is to develop a method based on SPE/LC-(ESI)MS/MS for the simultaneous determination of four benzodiazepines (DZP, nordiazepam (NDZ), BMZ and CBZ) and four barbiturates (PTB, secobarbital (SCB), barbital (BTL) and PNB) in river water and wastewater, and to evaluate their presence in different environmental samples from Catalonia. Target compounds were selected based on little information about the presence of sedative hypnotics in the aquatic environment in Catalonia and a large increase of consumption in the last few years [23].

2. Experimental

2.1. Reagents and standards

DZP, BMZ, CBZ, BTL, PTB, PNB and SCB were acquired from Sigma-Aldrich (St. Louis, USA). NDZ, DZP-\textsubscript{d5} and PTB-\textsubscript{d5} were acquired from Cerilliant (Round Rock, TX, USA) as a solution of methanol at 1000 mg/L. Purity of all standards was higher than 97%. Stock solutions of individual standards were prepared for each compound in methanol (MeOH) at 100 mg/L and stored at –18°C in the dark. Mixed working solutions were prepared daily by diluting the stock solutions with MeOH:water (50:50 v/v).

Ultrapure water was obtained with a Purelab ultrapurification system (Veolia Water, Sant Cugat del Vallés, Spain). MeOH (HPLC-grade) was supplied by SDS (Peypin, France), formic acid for LC-MS analysis was purchased from Merck (Darmstadt, Germany) and nitrogen was bought from Carburos Metálicos (Tarragona, Spain).

2.2. Collection of water samples

River water samples were collected from three rivers (Llobregat, Ter and Ebro) located in Catalonia (Spain). These rivers are sources of drinking water that supply a population of over four and a half million inhabitants with rather extensive farming and animal activity. The effluent and influent wastewater samples were collected at the same time from two STPs in Tarragona (STP 1) and Reus (STP 2). These STPs receive an average flow of 21,000 m\textsuperscript{3}/day of wastewater from approximately 120,000 inhabitants and receive both urban wastewaters and some industrial discharges. In this case, influent water was collected after mechanical treatment and effluent water was collected after secondary treatment, which involves primary settling, biological treatment and secondary sedimentation. One sample per month of river water and wastewater were taken during three and five months, respectively, between February 2013 and July 2013 by grab sample using pre-cleaned polyethylene bottles and stored at –18°C until analysis. Influent wastewater samples were centrifuged at 9000 rpm for 7 min (Hettich Zentrifugen, Tuttlingen, Germany) and then filtered using a 0.22 μm nylon filter (Whatman, Maidstone, UK). Effluent wastewater and river water samples were directly filtered with a 0.22 nylon filter.

2.3. SPE

The cartridges tested for the SPE procedure were 500 mg Oasis HLB from Waters (Milford, MA, USA) and 200 mg Bond Elut Plexa from Varian (Middelburg, the Netherlands). 500 mg Oasis HLB cartridges were connected to an SPE manifold (Teknokroma, Barcelona, Spain), which was connected in turn to a vacuum pump. Cartridges were conditioned with 5 mL of
MeOH followed by 3 mL of Milli-Q water (pH = 7). Water samples (100 mL of influent wastewater, 250 mL of effluent wastewater and 500 mL of river water) were then passed through SPE cartridges at a flow rate of approximately 10 mL/min. The cartridges were then washed with 5 mL of 5% of MeOH in water (v/v) and completely dried under vacuum. After eluting the analytes with 5 mL of MeOH, the extracts were evaporated to dryness by nitrogen stream and were finally reconstituted with MeOH:water (50:50 v/v) to a volume of 1 mL in the case of river water and 5 mL in the case of wastewater.

2.4. **LC-MS/MS**

Chromatographic separation was performed by reversed-phase liquid chromatography and detection was performed by tandem MS with QqQ and ESI. The LC instrument was an Agilent 1200 series (Waldbronn, Germany) equipped with a binary pump, vacuum degasser, autosampler and a thermostatted column compartment. The chromatographic column was a Kinetex C\textsubscript{18} (4.6 × 100 mm, 2.7 \textmu m) with Core-Shell Technology from Phenomenex (Madrid, Spain) and a pre-column Krudkatcher\textsuperscript{TM} Ultra column in-line filter, 0.5 \textmu m porosity × 0.1 mm ID was also used. The binary mobile phase was Milli-Q water with formic acid (pH 3) and MeOH. The gradient of elution was as follows: 45% of MeOH for 10 min, increased to 100% in 5 min, kept constant for 1 min and finally returned to 45% of MeOH in 2 min. All of the compounds were eluted within 18 min. The flow rate was 0.5 mL/min, the column temperature was kept at 25ºC and the volume injected was 25 \mu L.

The QqQ mass spectrometer (Agilent 6410 Triple Quad MS) operated in positive mode for benzodiazepines and negative mode for barbiturates. Data acquisition was performed in selected reaction monitoring (SRM) and the protonated [M + H]\textsuperscript{+} or deprotonated [M-H]- pseudo-molecular ion of each compound was chosen as the precursor ion. Three SRM transitions were selected for BMZ, DZP and NDZ and only two transitions were possible to select for CBZ and for all barbiturates (see supplementary information in Table S1). The optimised conditions for the ESI were: high-purity nitrogen (>98%) as the desolvation nebuliser; nebuliser pressure of 45 psi; drying gas flow rate of 12 L/min; drying gas temperature of 350ºC and spray potential of 3500 V. Cone voltages and collision energy voltages were established for each compound (summarised in Table S1) and were from 90 to 150 V and from 5 to 35 V, respectively.

3. **Results and discussion**

3.1. **LC-(ESI)MS/MS**

A good chromatographic separation was obtained with the Kinetex C\textsubscript{18} (4.6 × 100 mm, 2.7 \textmu m) with Core-Shell Technology, which allowed excellent resolution, efficiency and short retention times to be obtained for all compounds. An SRM chromatogram of the chromatographic separation in spiked influent sample is depicted in Figure S1 of the supplementary information. In order to reach a compromise between the chromatographic resolution of the analytes and effective ESI performance in negative (barbiturates) and positive (benzodiazepines) ionisation modes, different aqueous mobile phase compositions were tested in combination with MeOH. Aqueous mobile phases tested were: ultrapure water adjusted to pH 3 with formic acid, to pH 4.5 with acetic acid and to pH 8 with the buffer CH\textsubscript{3}COONH\textsubscript{4}/NH\textsubscript{4}OH 10 mM. The signal intensities in ESI-MS for barbiturates were similar using any of the three compositions, although it is known that a basic mobile phase favours the deprotonation of analytes in negative ESI. On this point, a study conducted by Wang et al. [24] demonstrated poor sensitivity for barbiturates when neutral or basic eluent was used, because the analyte’s response in ESI depends on gas-
phase deprotonation rather than on solution-phase ionisation equilibrium. Acid modifier in the aqueous mobile phase considerably enhanced the signal intensities for benzodiazepines. Meanwhile, in terms of chromatographic separation, the maximum resolution of the analytes, short run time and good peak shape were obtained with the aqueous phase at pH 3. Thus, the mobile phase chosen was water at pH 3 and MeOH with the following gradient: from 60% to 100% of MeOH in 6 min, kept constant for 1 min and returned to 60% of MeOH in 2 min. However, the chromatographic separation was smoothed to avoid an isobaric interference observed at the same retention time of BTL when real samples were analysed. Therefore, the elution gradient was changed to the conditions described previously in Section 2.4 (chromatogram Figure S1).

The full-scan mass spectrum showed two protonated molecules for DZP (m/z 285 and 287), NDZ (m/z 271 and 273) and BMZ (m/z 316 and 318). This is due to the natural ratio of the isotopes of the halogen present in the molecules. For instance, both DZP and NDZ molecules contain chlorine and the natural ratio of their isotopes $^{37}\text{Cl}/^{35}\text{Cl}$ is 1:3; thus, the relative intensities of the ions at m/z 285 (DZP) and m/z 271 (NDZ) were three times more abundant than those ions with m/z 287 (DZP) and 273 (NDZ), respectively. Therefore, the $[\text{M}+\text{H}]^+$ at m/z 285 and 271 were selected as the precursor ions of DZP and NDZ, respectively. In the case of BMZ, the relative intensities’ signal of the protonated molecules was equal due to the natural ratio of the isotopes ($^{79}\text{Br}/^{81}\text{Br} = 1:1$), and the $[\text{M}+\text{H}]^+$ at m/z 316 was selected as the precursor ion, in accordance with the literature [22]. Cone voltages were optimised for each precursor ion and these were between 90 and 150 V (see supplementary information Table S1).

As can be seen in Figure 1, the benzodiazepines showed rich fragmentation spectra originated from the protonated molecular ion. The loss of CO and the halogen is a feature

![Figure 1](image_url). Structures and product ion spectrum of protonated benzodiazepines and deprotonated barbiturates. ♦ Precursor ion.
shared by all benzodiazepines to give product ions at m/z 209 for BMZ, 222 for DZP and 208 for NDZ. In the case of BMZ, it showed a fragment at m/z 288 formed by the elimination of CO, resulting in the contraction of the seven-membered ring to a resonance stabilised six-membered ring [25]. In addition to the loss of CO and Br, the molecule of BMZ also presented neutral loss of hydrogen cyanide (HCN) to give the major product ion at m/z 182.

DZP and NDZ showed product ions at m/z 154 and 140, respectively, which would correspond to the loss of benzene and HCN from the cation formed after the loss of CO. DZP gave the major product ion at m/z 193 and was interpreted as being formed by the elimination of CH$_3$NCO and Cl.

The product ion mass spectrum of CBZ showed poor fragmentation and the predominant fragment at m/z 193 corresponds to the loss of CONH$_2$.

Barbiturates shared similar fragmentation patterns with the formation of the most abundant product ion [M-H-43]- corresponding to a neutral loss of isocyanic acid and the ion at m/z 42 [24]. Consequently, only one confirmation transition for each barbiturate and carbamazepine could be selected, while two transitions were selected for the rest of the compounds.

3.2. SPE optimisation

One highly important aspect in SPE is the selection of the sorbent according to the chemical properties of the compounds of interest. Two copolymer sorbents based on polar and non-polar retention mechanisms (500 mg Oasis HLB and 200 mg Bond Elute Plexa) and three different sample pHs (not adjusted, 3 and 10) were tested. The experiments were conducted using 100 mL of ultrapure water spiked at 2.5 μg/L (barbiturates) and 0.25 μg/L (benzodiazepines) and eluting with 5 mL of MeOH. All compounds showed SPE recoveries (Figure 2) between 89% and 105% using Oasis HLB and between 80% and 95% using Bond Elute Plexa at neutral pH and acidic pH. When the sample at pH 10 was passed through cartridges, recoveries decreased by 15% in the case of most of the compounds. Moreover, PNB showed the lowest recovery (37%) at basic pH when Oasis HLB was used and it was not recovered with Bond Elute Plexa. BTL was not retained in either of the two sorbents at basic pH. Since basic pH did not aid the retention of compounds, particularly in terms of the barbiturates, which are weak acids, it was discarded. Therefore, the sample pH was not adjusted to avoid additional manipulation of the sample. Between the two copolymer sorbents tested, Oasis HLB was chosen because the recoveries were between 6% and 18% higher than with Bond Elute Plexa.

![Figure 2](image-url)
The breakthrough volume was evaluated in order to select the appropriate loading volume for each matrix. First, the breakthrough volume was examined by loading different volumes of spiked ultrapure water between 100 and 1000 mL onto the cartridge and eluting with 5 mL of MeOH. Different concentrations of analytes were added in order to obtain a concentration of 25 µg/L in the 10 mL of redissolution volume. Results showed (data shown in supplementary information Figure S2) that up to 1000 mL, recoveries of the analytes were not affected. Hence recoveries of each compound (81–103%) remained almost constant for all of the volumes tested. Subsequently, the breakthrough volume was evaluated with river water, and influent and effluent wastewater. It is well known that working with these types of matrices at large sample volumes may lead to breakthrough or severe matrix effect. Therefore, taking into account the results from ultrapure water, matrix complexity and previous experience, the following sample volumes for each matrix were evaluated: 500 and 1000 mL for river water, 250 and 500 mL for effluent wastewater and 100 and 250 mL for influent wastewater. In all cases, a non-spiked sample (blank) was analysed to subtract the compounds present in the sample. Recoveries involving matrix effect and SPE extraction are depicted in Figure S2. Most of the barbiturates showed an increase in the recoveries (compared with ultrapure water results) ranging from 116% to 138% when the highest sample volume of each matrix was loaded into the cartridge, contrary to benzodiazepines and BTL, where recoveries decreased to 22–83%. The lowest recoveries were for BTL (22% in river water and 25% in wastewater), CBZ (59% in river water and 64% in wastewater) and DZP (47% in river water and 39% in wastewater). Thus, the sample volume chosen was 500 mL for river water, 250 mL for effluent and 100 mL for influent, since good results for most of the compounds were obtained, with recoveries between 61% (DZP) and 125% (SCB) except for BTL (39%). In the optimisation of the redissolution volume, blank sample extracts obtained in SPE were evaporated until dryness under nitrogen steam, then spiked at a concentration in the final redissolution volume of 25 µg/L for barbiturates and 2.5 µg/L for benzodiazepines and reconstituted with MeOH:water (50:50 v/v). Three different redissolution volumes were tested: 0.5, 1 and 10 mL for river water and 1, 5 and 10 mL for wastewater and the matrix effect was evaluated in order to choose the appropriate redissolution volume. It was calculated by comparing the peak areas of compounds spiked in the SPE extract with the peaks areas of a pure solvent standard solution at the same concentration. The matrix effect was expressed according to López-Serna et al. [12] and Tran et al. [26] as a percentage of ion suppression (positive values) or ion enhancement (negative values). As can be seen in Table 2, the matrix effect increased considerably for all analytes in the three matrices when extracts were redissolved to the lower volume tested. In the case of extracts eluted from river water, the matrix effect at 10 mL was between −3% (BMZ) and 57% (BTL) and these

| Compound | River water | | Influent wastewater | | Effluent wastewater |
|----------|-------------|----------|---------------------|----------|---------------------|
|          | 10 mL | 1 mL | 0.5 mL | 10 mL | 5 mL | 1 mL | 10 mL | 5 mL | 1 mL |
| BTL      | 57 | 72 | 75 | 73 | 70 | 86 | 64 | 75 | 80 |
| PNB      | 24 | 20 | 36 | 11 | −36 | 35 | 10 | −14 | 28 |
| BMZ      | −3 | −27 | −55 | 5 | 15 | 28 | −2 | 21 | 20 |
| CBZ      | 4 | 20 | 26 | 8 | 13 | 26 | 8 | 19 | 22 |
| PTB      | −10 | −16 | −45 | 4 | −48 | 30 | −12 | −29 | 37 |
| SCB      | 23 | −11 | −30 | −2 | −43 | 29 | −24 | −16 | 15 |
| DZP      | 11 | 25 | 30 | 34 | 26 | 37 | 19 | 23 | 46 |
| NDZ      | −5 | 16 | 47 | 15 | 7 | 44 | 5 | −1 | 26 |
values were between −55% and 75% when the redissolution volume was decreased from 10 to 0.5 mL. With 0.5 mL, BMZ (−55%), PTB (−45%) and SCB (−30%) were affected by ion enhancement. When the redissolution volume was 1 mL, the matrix effect was between −27% and 72%.

With respect to influent and effluent wastewater, BTL was the compound most affected by ion suppression (64–86%) in the case of all of the redissolution volumes tested. In general, all compounds were affected by ion suppression (15–86%) when the redissolution volume was 1 mL. No significant differences were observed between 5 and 10 mL for BTL, CBZ, DZP and NDZ, and NDZ did not show any matrix effect in effluent wastewater. Only BMZ and PTB displayed an increase in ion suppression and ion enhancement, respectively, when the redissolution volume was decreased from 10 to 5 mL. So, according to the results and taking into account the method sensitivity, the most suitable redissolution volumes for each matrix were 1 mL for river water and 5 mL for effluent and influent wastewater.

3.3. Method validation

For the validation of the optimised method, linear range, LOD, limit of quantification (LOQ), reproducibility between days and repeatability (expressed as relative standard deviation (%RSD) and recoveries (including matrix effect and extraction) were evaluated for each matrix. A blank sample was analysed to subtract the compounds present in the sample and PNB, BMZ, CBZ, DZP and NDZ were determined.

The use of two isotope-labelled compounds (PTB-d$_5$ and DZP-d$_5$) for the correction of the error caused by the matrix effect was evaluated. PTB-d$_5$ was tested to quantify barbiturates and DZP-d$_5$ was tested to quantify benzodiazepines. Nevertheless, the matrix effect for some compounds such as BTL (70–75%) and NDZ (−1% to 16%) was different from the isotope-labelled compounds (PTB-d$_5$ −25% and DZP-d$_5$ 30%), which led to an erroneous quantification. Therefore, isotope-labelled compounds were not used and the quantification for all compounds was carried out with matrix-matched calibration to compensate for the matrix effect.

Calibration curves were generated with six samples spiked before SPE with increased amounts of each analyte. The linear range shown in Table 3 was found at two different levels because benzodiazepines were about ten times more sensitive than barbiturates. Determination coefficients ($R^2$) were higher than 0.992 in all calibration curves.

LOQs were selected at the lowest point of the calibration curves (Table 2). BT presented the highest LOQs at 50, 100 and 125 ng/L in river water, effluent wastewater and influent wastewater, respectively.

Similar or higher LOQs for BMZ, DZP, NDZ and CBZ have been reported in previous studies (see comparative Table 1), which involved multi-residue methods based on SPE/LC-MS/MS [11,14,16] and SPE/GC-MS [7,27].

LODs were calculated as the concentrations giving peaks for which the signal-to-noise ratio was 3. For compounds that were present in non-spiked samples, they were estimated from their recoveries and the LC-MS response. As can be seen in Table 3, LODs were lower in river water between 2.5 and 10 ng/L for barbiturates and between 0.2 and 0.5 ng/L for benzodiazepines. LODs of effluent wastewater were between 10 and 25 ng/L for barbiturates and 1 ng/L for all benzodiazepines, except for CBZ (5 ng/L). As expected, in influent wastewater, LODs were higher than the other matrices at 50 and 5 ng/L for all barbiturates and benzodiazepines, respectively.

The precision was expressed as the relative standard deviation (%RSD) obtained when the repeatability (intra-day) and reproducibility (inter-day) were assessed. Five blank samples were spiked at two levels of concentration before SPE as follows: low concentration of barbiturates at
Table 3. Validation data for all different kinds of water.

| Compound | Ratio range permitted tolerances | L. Range (ng/L) | LOD (ng/L) | % R<sup>a</sup> | % R<sup>b</sup> |
|----------|---------------------------------|----------------|-------------|----------------|----------------|
| BTL      | 35–58                           | 50–400         | 10          | 33             | 38             |
|          |                                 |                |             |                |                |
|          |                                 |                |             |                |                |
| PNB      | 32–54                           | 10–400         | 5           | 58             | 64             |
| BMZ      | 55–79                           | 1.0–40        | 0.25        | 107            | 103            |
|          |                                 |                |             |                |                |
| CBZ      | 11–20                           | 1.0–40        | 0.5         | 77             | 79             |
|          |                                 |                |             |                |                |
| PTB      | 48–72                           | 10–400        | 2.5         | 110            | 102            |
| DZP      | 61–92                           | 2.0–40        | 0.5         | 57             | 62             |
| NDZ      | 56–85                           | 1.0–40        | 0.5         | 84             | 88             |

| River water | Influent wastewater | Effluent wastewater |
|-------------|---------------------|---------------------|
| L. Range (ng/L) | LOD (ng/L) | % R<sup>a</sup> | % R<sup>b</sup> | L. Range (ng/L) | LOD (ng/L) | % R<sup>a</sup> | % R<sup>b</sup> | L. Range (ng/L) | LOD (ng/L) | % R<sup>a</sup> | % R<sup>b</sup> |
| 125–2500 | 50 | 47 | 50 | 100–2000 | 25 | 39 | 44 |
| 125–2500 | 50 | 114 | 104 | 100–2000 | 25 | 119 | 108 |
| 10–400 | 5 | 76 | 79 | 5–200 | 1 | 84 | 88 |
| 12.5–500 | 5 | 77 | 81 | 10–2500 | 5 | 71 | 74 |
| 2.5 | 73 | 77 | 12.5–500 | 5 | 71 | 74 |
| 10–400 | 2.5 | 129 | 117 | 50–2000 | 10 | 136 | 122 |
| 12.5–2500 | 50 | 116 | 109 | 50–2000 | 10 | 131 | 119 |
| 12.5–500 | 5 | 73 | 77 | 5–200 | 1 | 64 | 68 |
| 12.5–2500 | 5 | 98 | 101 | 5–200 | 1 | 93 | 96 |

Notes: a Recoveries (%R), river, effluent and influent wastewater at 50, 100 and 250 ng/L, respectively, for barbiturates and 5, 10 and 25 ng/L for benzodiazepines. 

b Recoveries (%R), river, effluent and influent wastewater at 200, 1000 and 1250 ng/L respectively for barbiturates and 20, 100 and 125 ng/L for benzodiazepines.
50, 100 and 250 ng/L for river, effluent and influent samples, respectively, while the concentration of benzodiazepines was 5, 10, and 25 ng/L for river, effluent and influent samples, respectively. High concentration of barbiturates was 200, 1000 and 1250 ng/L for river, effluent and influent samples, respectively, and the concentration of benzodiazepines was 20, 100, and 125 ng/L for river, effluent and influent samples, respectively. The repeatability RSD% \((n = 5)\) values at low level were <13% and the reproducibility values were <17%, whereas repeatability and reproducibility at high level were <10% and <15%, respectively.

The recoveries (including matrix effect and extraction), summarised in Table 3, were similar at the two levels of concentration tested, between 33% and 110% for river water, 47% and 129% for influent water and 39% and 136% for effluent wastewater.

The identification and confirmation criteria of each compound are based on retention time, presence of two transitions and relative ion intensities between the signal of the qualifier ion (the second-most abundant ion) and the quantifier ion. In accordance with the criteria set out in Commission Decision 2002/657/EC [28], the maximum permitted tolerances of relative ion intensities for each compound were ±20 for all analytes except for CBZ (±30), BTL and PNB (±25). Table 2 shows the range of maximum permitted tolerances or relative ion intensities for each compound.

### 3.4. Analysis of river and wastewater samples

The method was applied to analyse wastewater samples from two different treatment plants in Tarragona and Reus, as well as river water samples from the rivers Ebro, Llobregat and Ter, all of which are located in Catalonia. In total, five samples of influent and five samples of effluent wastewater were collected in each STP, and three samples of each river were collected in different months. The ranges of concentrations and relative ion intensities for each compound present in the samples are shown in brackets in Table 4 and these fall within the permitted tolerance levels. Concentrations found in previous studies are summarised in Table 1.

As summarised in Table 4, PNB was determined in influent wastewater samples with concentrations up to 324.2 ng/L. Results obtained from effluent wastewater samples could indicate little or no removal of this compound, since only a decrease in the concentration between 5% and 30% was observed. The occurrence of PNB has only been reported in effluent wastewater from Greece [16] at levels ranging from 70.0 to 127.0 ng/L. Other studies have also monitored PNB and PTB in wastewater from Spain [8,10,18] and Germany [5], but the presence of these compounds was not reported in any sample.

All of the benzodiazepines were found in both influent and effluent wastewater. The highest concentration found was CBZ, with a maximum value of 2337.3 ng/L. These concentrations are comparable in both influent and effluent samples. Several studies from China [17] and Spain [8] have also shown poor or no removal and even an increase of CBZ in effluent wastewater samples. As can be seen in Table 1, the reported concentrations were lower than those reported in this study, but a high level up to 6822.0 ng/ of CBZ has been reported in Greece. The presence of CBZ can be due to the high prescription volume as an anti-epileptic drug (770 mg person-year) in the European Union [29]. It should be noted that CBZ is excreted together with its metabolites. Some conjugated metabolites, such as CBZ glucoronide, are converted to the parent compound by enzymatic processes that take place in the treatment plant [29].

The rest of the benzodiazepines analysed were found at similar concentration levels, between 30.1 and 94.2 ng/L in influent samples. Concentrations of BMZ and NDZ in effluent samples were about 70% lower than in influent samples. Similar values of NDZ were found by Baker et al. [15] in an STP from the United Kingdom and by Racamonde et al. [14] in Spain, with maximum concentrations of 51.5 ng/L for influent wastewater and 14.2 ng/L for effluent
Table 4. Concentrations range (ng/L) found in river water and wastewater samples.

| Compound | River water | Influent wastewater | Effluent wastewater |
|----------|-------------|----------------------|---------------------|
|          | Ebro | Llobregat | Ter | STP 1 | STP 2 | STP 1 | STP 2 |
| BTL      | < LOQ (37–41) | < LOQ (44–49) | 266.5–324.2 (38–46) | 313.8–320.3 (42–49) | 110.0–308.4 (39–48) | 170.3–223.0 (35–42) |
| PNB      | < LOQ (40–45) | 15.6–15.9 (42–44) | 15.6–15.9 (42–44) | 15.6–15.9 (42–44) | 15.6–15.9 (42–44) | 15.6–15.9 (42–44) |
| BMZ      | < LOD | < LOD | 70.7–94.2 (65–71) | 57.0–68.5 (60–71) | 22.1–37.8 (59–70) | 12.2–25.8 (59–68) |
| CBZ      | 2.6–3.3 (13–15) | 2.1–2.5 (13–16) | 1110.3–2238.1 (13–18) | 870.1–2242.2 (12–19) | 1137.6–2337.3 (12–17) | 940.1–2020.5 (13–18) |
| DZP      | < LOQ (74–79) | < LOQ (76–79) | 36.6–59.0 (73–81) | 50.5–69.3 (71–85) | 28.2–63.6 (72–81) | 40.8–60.0 (75–87) |
| NDZ      | 1.0–1.1 (68–73) | < LOQ (70–72) | 31.5–56.2 (66–78) | 30.1–52.0 (67–77) | 5.0–14.2 (63–78) | 7.2–17.7 (70–83) |

Notes: n = 3 RSD (%) < 12.
Relative ion intensities (%) in brackets.
water. On the other hand, levels of BMZ slightly higher than those reported in this study were found by Borova et al. [16] in wastewater from Greece at concentrations ranging between 33.0 and 315.0 ng/L.

Some studies have shown that DZP is resistant to biodegradability during wastewater treatment and remains in the aquatic environment for relatively long periods of time [30]. As a result, the detected concentrations of DZP were similar in both influent and effluent samples, ranging between 28.2 and 69.3 ng/L. These findings were higher than those concentrations reported in other studies in Spain [14,18], Slovenia [4] and Greece [11]. Moreover, Gros et al. [8] and Sousa et al. [11] did not detect DZP when wastewater samples from Spain and Portugal, respectively, were analysed. In Figure 3, an SRM chromatogram is depicted, showing the presence of PNB, BMZ, CBZ, DZP and NDZ, with their respective concentrations in an effluent wastewater sample, with CBZ being the most abundant compound.

Since wastewaters are discharged into the receiving environment, sedative hypnotics have also frequently been detected in surface waters due to low elimination by STPs. Samples from three different rivers located in Catalonia were analysed: the Ebro, Llobregat and Ter. Results showed the
presence of CBZ in all three rivers at concentrations between 2.1 and 3.3 ng/L. PNB was found below LOQ in all samples, except for the Llobregat River (15.6 ng/L). BMZ and DZP were detected below LOQ. The most commonly studied benzodiazepines have been CBZ and DZP and their occurrence in river water has been reported to similar concentrations levels. These compounds have been reported in surface water from Spain [9], Slovenia [4], the United Kingdom [15] and France [7] at concentrations from below LOQ to 69 ng/L, as reported in Table 1.

Only a few studies have reported the presence of barbiturates in river water. For instance, Peschka et al. [5] reported concentrations in Germany of 5.4 μg/L for PTB, 1.3 μg/L for PNB and 0.1 μg/L for SCB.

4. Conclusions
A method based on SPE and LC-MS/MS for the simultaneous determination of two groups of sedative hypnotics (four barbiturates and four benzodiazepines) was developed and validated in both river water and wastewater. The method enabled the compounds in the study to be determined at low ng/L levels, as SPE with Oasis HLB allowed large-scale pre-concentration of samples and LC-MS/MS offered high sensitivity on detection.

To ensure the accurate identification of the compound in the samples, three SRM transitions were monitored for BMZ, DZP and NDZ and only two transitions were possible for CBZ and barbiturates. The matrix effect was corrected with the use of matrix-matched calibration. Acceptable values of validation parameters were obtained to ensure an accurate, sensitive and selective method. Recoveries for barbitals and benzodiazepines were between 43% and 130% in all matrices. A slight matrix effect was observed for most of the compounds, with BTL being the compound most affected by ion suppression (70–75%).

The method was successfully applied to determine these sedative hypnotics in three rivers and two STPs in Catalonia. The occurrence of benzodiazepines in environment samples and poor removal in STPs were more significant than in the case of barbiturates, especially with respect to CBZ.

Disclosure statement
No potential conflict of interest was reported by the authors.

Funding
The authors gratefully acknowledge the financial support by Ministry of Economy and Competitiveness [CTM2011-28765-C02-01] and the Department of Innovation, Universities and Enterprise [project 2014 SGR 934]. P. Arbeláez would also like to thank the Ministry of Economy and Competitiveness for a grant [BES-2009-028157].

Supplemental data
Supplemental data for this article can be accessed at http://dx.doi.org/10.1080/03067319.2015.1055474.

References
[1] D.J. Lapworth, N. Baran, M.E. Stuart and R.S. Ward, Environ. Pollut. 163, 287 (2012). doi:10.1016/j.envpol.2011.12.034.
[2] P. Vazquez-Roig, C. Blasco and Y. Picó, TrAC-Trend Anal. Chem. 50, 65 (2013). doi:10.1016/j.trac.2013.04.008.
