

Research Article

Simple and Fast Methods Based on Solid-Phase Extraction Coupled to Liquid Chromatography with UV Detection for the Monitoring of Caffeine in Natural, and Wastewater as Marker of Anthropogenic Impact

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Two concentration methods for fast and routine determination of caffeine (using HPLC-UV detection) in surface, and wastewater are evaluated. Both methods are based on solid-phase extraction (SPE) concentration with octadecyl silica sorbents. A common “offline” SPE procedure shows that quantitative recovery of caffeine is obtained with 2 mL of an elution mixture solvent methanol-water containing at least 60% methanol. The method detection limit is $0.1 \mu\text{g L}^{-1}$ when percolating 1 L samples through the cartridge. The development of an “online” SPE method based on a mini-SPE column, containing 100 mg of the same sorbent, directly connected to the HPLC system allows the method detection limit to be decreased to 10 ng L^{-1} with a sample volume of 100 mL. The “offline” SPE method is applied to the analysis of caffeine in wastewater samples, whereas the “on-line” method is used for analysis in natural waters from streams receiving significant water intakes from local wastewater treatment plants.

1. Introduction

Caffeine is the world most widely-consumed psychoactive stimulant. It is possible to find this alkaloid in foods, beverages and drug preparations, and the daily average load has been estimated at between 16 and $70 \text{ mg person}^{-1} \text{ day}^{-1}$ [1, 2].

The amounts of caffeine reaching wastewaters in urban areas are considerable since (i) large amounts of caffeine go directly down the drains from unconsumed drinks (e.g., coffee, tea, and soft drinks) and the rinsing of pots and cups [3], and (ii) 0.5–3% of human caffeine intake is excreted in a nonmetabolized form by urine [4, 5]. Caffeine has been found to reach values of around $100 \mu\text{g}\cdot\text{L}^{-1}$ [2, 6] in the influents of wastewater treatment plants (WWTPs) and is one of the compounds that most contribute to the total mass loads of pharmaceuticals in these plants [7].

On the Costa Brava (Girona, Spain), a large percentage of the wastewater treated by WWTPs is reused for the irrigation of fields, golf courses, and public gardens as well as to feed

natural streams for the recovery of their natural flow and ecological quality. Given these uses, it is extremely important that microcontaminants be removed in the WWTPs.

In selecting chemical markers of anthropogenic impact, it is necessary to use ones that are able to distinguish wastewater inputs from both treated and non-treated sources [2, 8]. Such a compound would have to be largely eliminated in WWTPs. Caffeine, therefore, may be regarded as a suitable marker given that >99% can be degraded in WWTPs [2, 6, 7, 9, 10]. Henjum et al. [11] used caffeine as an indicator for domestic wastewater discharges in streams located in places where no direct wastewater discharges were expected and so where wastewater inputs could be attributed to combined sewer overflows or septic drainage fields.

The objectives of this study were, firstly, to develop a simple and fast analytical methodology to determine caffeine in environmental and waste waters, and, secondly, to evaluate the efficiency of three selected WWTPs of the Costa Brava area in removing caffeine at different treatment stages, and, finally, to monitor caffeine as a marker of

untreated wastewater in three natural streams receiving significant water intakes from local WWTPs.

2. Experimental

2.1. Chemicals and Reagents. Caffeine (1,3,7-trimethylxanthine, 99%) was obtained from Aldrich (Steinheim, Germany). The solvents used (acetonitrile and methanol) were HPLC grade (Carlo Erba, Milan, Italy). Ultra-pure water was obtained from a MilliQ system (Millipore Iberica S.A., Barcelona, Spain).

Stock solutions of caffeine (ca. 500 mg·L⁻¹) were prepared in methanol. These solutions were stored for up to one week in amber vials at 4°C. Working solutions were prepared daily by diluting and mixing the stock with MilliQ water.

Solid-phase extraction AccuBond II ODS cartridges (500 mg, 3 mL volume, 55 µm particle diameter, and surface area of 546 m²·g⁻¹) were used in the clean-up and concentration steps (Agilent Technologies UK Ltd., West Lothian, UK).

2.2. Study Area. The area under study is located on the Costa Brava, the coastal area of the Girona province, north-eastern Spain. This is a popular tourist area on the Mediterranean and as a result there are significant oscillations in the population between seasons.

Three WWTPs (Empuriabrava, Pals and Palamós) were selected for the evaluation of the efficiency of caffeine removal in the water treatment of urban wastewaters. The treatment process includes grit removal and screening, primary settling and activated sludge biological processing in all the plants. This is followed by UV/chlorination disinfection in Palamós and Pals, whereas Empuriabrava WWTP has a tertiary stage of lagoons and three constructed wetlands that feed the "Aiguamolls de l'Empordà" natural park. In all the WWTPs the study was performed during July 2005, a period when these WWTPs were operating at their greatest capacity due to the significant seasonal increase in the local population, and in October 2005, when the level of tourism was considerably lower.

Surface waters from three streams (Riera de Tossa, Riera d'Aubí and Riu Ridaura) were evaluated. Three local WWTPs that feed these streams were also evaluated to determine whether the source of caffeine detected in the streams was associated to effluent discharges from the plants.

2.3. Sampling and Analysis. Two liters of sample was collected at each sampling point. It was filtered through glass microfiber filters (Whatman, Maidstone, UK) to eliminate suspended particles, and stored in amber glass bottles at 4°C until analysis (maximum 48 hours).

Concentration and clean-up steps were performed by solid phase extraction (SPE). For the "off-line" concentration procedure, SPE cartridges were conditioned with 2 mL methanol and 2 mL milli-Q water. Samples were loaded in the cartridges at 25 mL min⁻¹ and sorbents were then dried for 30 s with a vacuum pump. Desorption of caffeine was achieved with 2 mL methanol:water (60:40). 20 µL of the final eluate was analyzed by HPLC-UV.

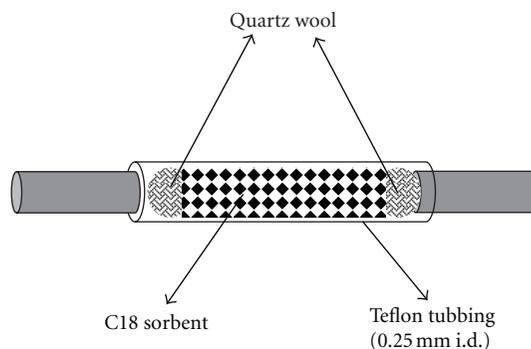


FIGURE 1: The mini-SPE column design.

For the "on-line" concentration procedure, a mini-SPE column (Figure 1) made of Teflon tubing (0.25 mm i.d.) was prepared. A quartz wool plug was inserted in one end and 100 mg of AccuBond II ODS particles were introduced. This was then plugged by a second piece of quartz wool and the excess tubing was cut off. The mini-SPE column was conditioned with 1 mL methanol and 1 mL milli-Q water before a sample was passed through the column. The column was then dried by passing dry air through the column for 60 seconds. Caffeine was desorbed with 0.3 mL methanol and the valve was turned to direct the elution volume to the injection valve of the HPLC instrument for analysis (Figure 2).

HPLC analyses were performed on a Shimadzu chromatograph (Kyoto, Japan) equipped with two pumps (LC-9A) and a UV-Visible spectrophotometric detector (SPD-6AU). Separation was carried out on a 20 × 0.46 cm i.d. column packed with a 5 µm Kromasil 100 C18 silica phase (Teknokroma, Barcelona, Spain) at 25 ± 1°C. An ODS pre-column was used (TR-C-160-1, Teknokroma). The mobile phase consisted of an acetonitrile-water solution (20:80, isocratic) at a flow rate of 1 mL min⁻¹. Samples were injected by means of a Rheodyne 7725i injector (Rohnert Park, CA, USA) with a 20 µL sample loop. UV detection at 272 nm was used.

3. Results and Discussion

3.1. HPLC Analysis of Surface and Waste Water Samples. Determination of caffeine in water samples is usually performed by GC-MS [2, 9, 12] or LC-MS [13–16]. These methodologies allow detection limits in the range of ng L⁻¹ to be reached but require the use of complex and expensive instrumentation. Simpler and cheaper instrumentation is preferred in non-research laboratories where routine analyses are performed. HPLC-UV can also be applied for the analysis of caffeine in water samples. The evaluation of the HPLC-UV method described in the experimental section yielded a limit of detection (LOD) of 1 µg L⁻¹ (3s_{blank} criteria, where s_{blank} was determined as the standard deviation obtained for 5 injections of a standard at 5 µg L⁻¹) and a limit of quantification (LOQ) of 4 µg L⁻¹ (10s_{blank} criteria). Intraday precision (n = 10, standard at 10 µg L⁻¹) was 2.0%

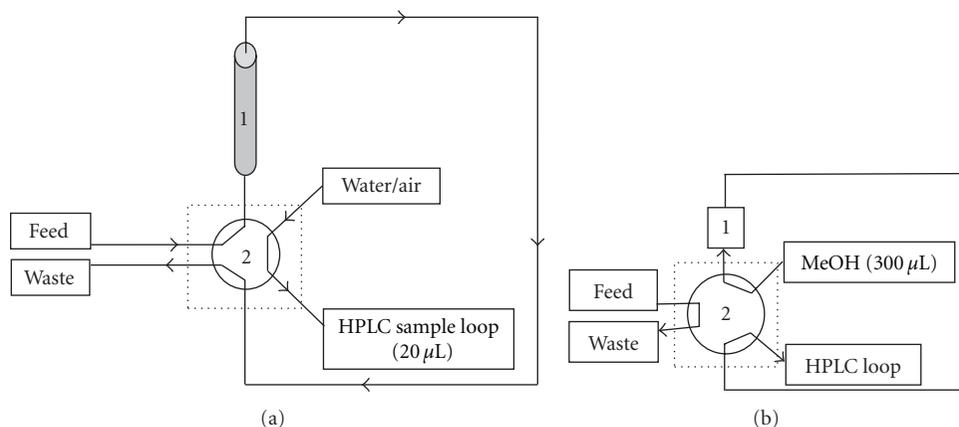


FIGURE 2: Diagram of the “online” mini-SPE column HPLC-UV system developed. 1: mini-SPE column; 2: six-port rotary valve (Model 5020 Low Pressure Valve, Rheodyne). (a) Valve position during feed (concentration) step; (b) valve position during desorption and HPLC analysis.

whilst inter-day precision ($n = 10$ consecutive days, standard at $10 \mu\text{g L}^{-1}$) was 5.6%.

The limits obtained for the HPLC methods are excessive for the appropriate determination of the trace amounts of caffeine likely to be expected in environmental and waste waters (few parts per trillion) and so sample preparation is needed. Moreover, sample treatment is always required when measuring compounds at trace levels in a complex matrix as wastewaters. The analysis of different samples obtained at the influent of a WWTP, after filtration through $0.45 \mu\text{m}$ filters to eliminate particulate matter, showed that the matrix effects (e.g., presence of large amounts of organic matter in these samples) resulted in large peaks at the beginning of the chromatograms with long tailing that made it impossible to analyze caffeine at levels below $20 \mu\text{g L}^{-1}$.

3.2. SPE Clean-Up and Preconcentration. SPE is a standard methodology for the extraction of caffeine for water samples [2, 9, 17, 18]. Here, two different approaches were evaluated and compared for the clean-up and preconcentration of caffeine from water samples: (i) commercial single use SPE cartridges with a C18 stationary phase and (ii) a mini-SPE column filled with the same sorbent.

3.2.1. Commercial SPE Cartridges (“Off-Line” Method). The breakthrough volume of commercial Accubond ODS sorbents was determined to evaluate the efficiency of this sorbent. A breakthrough volume of 26 mg of caffeine (52 mL for a 500 mg L^{-1} solution) was found for the AccuBond cartridges. Bibliographic and preliminary analysis in the laboratory showed that levels of caffeine found in water samples had a maximum expected concentration of ca. $100 \mu\text{g L}^{-1}$ [6], which results in a breakthrough volume of ca. 260 L at this level. As a result, no saturation of the Accubond sorbent is expected in the analysis of up to 1 L samples.

Different elution solvents and mixtures were evaluated. Acetonitrile was not appropriate because it did not yield quantitative recoveries of caffeine from the sorbent. Methanol or methanol-water mixtures yielded quantitative

TABLE 1: Caffeine recoveries obtained with methanol-water mixtures as the elution solvent ($n = 3$, values in brackets correspond to the standard deviation). Sorbent conditioning: 2 mL methanol and 2 mL Milli-Q water; feed solution: 100 mL standard at 0.1 mg L^{-1} ; sampling flow: 1 mL min^{-1} ; desorption by gravity with 2 mL elution mixture.

% methanol	Recovery (%)
100	96.5 (0.7)
80	98 (2)
70	101 (1)
60	92 (1)
40	19.0 (0.7)
20	0 (–)
0	0 (–)

recoveries when a minimum of 60% methanol was present in the elution solution (Table 1). Evaluation of the elution volume showed that 2 mL were enough for the quantitative elution of caffeine in all the conditions tested. A recovery percentage $\geq 96\%$ was obtained at all elution flows tested (1, 2, 5 mL min^{-1} and gravity elution). An elution mixture of 2 mL methanol-water (60:40) by gravity was selected for further analyses.

The low expected concentration of the samples requires large volumes to be concentrated through the SPE sorbent, thus the use of high sampling flows will decrease the time of analysis. The evaluation of sampling different flow rates showed that there were no significant differences between the recoveries obtained in the range from 1 to 25 mL min^{-1} ($P = 0.21$, ANOVA test, 3 replicates at each flow). A sampling flow of 25 mL min^{-1} , resulting in a loading time of 40 minutes, was chosen for the analysis of 1 L samples.

The experimental LOD obtained in the analysis of fortified WWTP samples (1000 mL) with the “off-line” SPE HPLC-UV procedure was $0.1 \mu\text{g L}^{-1}$. This value was sufficiently low as to ensure appropriate determination of caffeine in wastewater samples. However, it was still excessive

for the analysis of surface waters since levels in the order of few ng L^{-1} have been reported [2, 16, 19]. Intraday precision ($n = 10$) was 6%.

3.2.2. Mini-SPE Column (“On-Line” Method). To reduce LODs, different “on-line” SPE/HPLC-UV methodologies for caffeine determination have been proposed with detection limits of 0.1 [18] and 0.05 [17] $\mu\text{g L}^{-1}$, using sample volumes of 100 mL. We evaluated different mini-column designs for “on-line” SPE-HPLC-UV in order to obtain improved efficiency, sensitivity and speed in the determination of caffeine in surface waters.

Recovery was evaluated with minicolumns containing 10, 50, and 100 mg of sorbent. For these assays, the whole elution solution was collected and mixed, and then a 20 μL aliquot was injected into the HPLC system to determine the amount of caffeine recovered. Nonquantitative recovery ($39 \pm 11\%$, $n = 3$) was obtained for the first amount of sorbent. Quantitative recoveries were obtained for both 50 ($95 \pm 5\%$, $n = 3$) and 100 mg ($101 \pm 2\%$, $n = 3$) of sorbent, with no statistical significant differences between both values (t -test, $P = 0.18$). A weight of 100 mg was chosen for subsequent analyses to prevent saturation of the column by matrix components when surface waters were analyzed.

The study of the volume of methanol needed for the quantitative elution with the mini-SPE column showed that volumes below 150 μL did not yield quantitative recoveries. A volume of 300 μL was chosen for further analyses.

For “on-line” analysis, the elution volume was sent directly to the HPLC injection valve. Once the first portion of solution appeared at the end of the loop, the valve was turned to the injection position to send the first 20 μL portion of the elution solution to the chromatographic column.

Reproducibility of the mini-SPE column method was evaluated at different concentration levels (15, 25, 50, and 100 ng L^{-1}) with the conditions selected. Intraday precision ($n = 10$) was $<12\%$. Statistical analysis confirmed that quantitative recoveries ($\geq 95\%$) were obtained at all levels. The evaluation of samples at 10 ng L^{-1} yielded significant smaller recoveries.

LOD of the “on-line” system was determined for a minimum recovery of 50%, and it was experimentally found to be 5 ng L^{-1} . LOQ was fixed at 15 ng L^{-1} . The linearity of the “on-line” method was confirmed in the range between 15 and 200 ng L^{-1} . Note that the LOD obtained with the “on-line” mini-column is one order of magnitude below LOD obtained using MS detection [12, 16].

Figure 3 shows the chromatograms obtained for a standard at 50 ng L^{-1} analyzed with the conventional “off-line” SPE methodology (Figure 3(a)) and the “on-line” mini-SPE column (Figure 3(b)). Detection limit with the “off-line” SPE method (i.e., 100 ng L^{-1}) was above the concentration of the sample and caffeine was not detected with this methodology. The “on-line” method allowed the quantitative determination of the same sample. Besides the reduction in the LOD method, the mini-column methodology allows to work with reduced sample (100 mL) and elution volumes (300 μL), which leads to improved efficiency and speed of analysis.

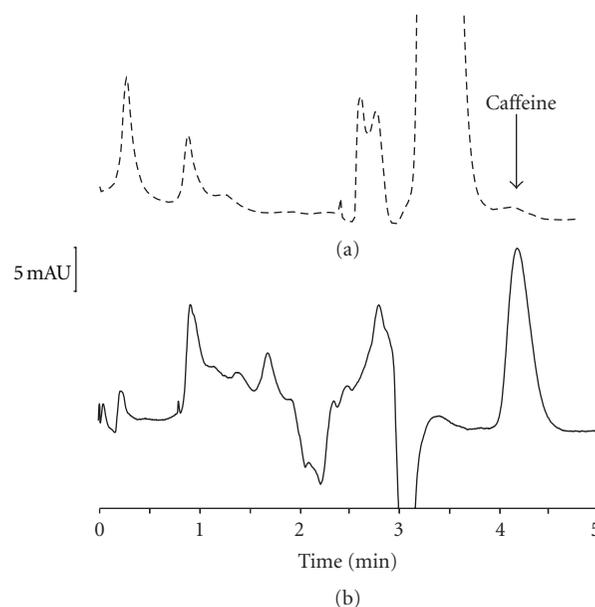


FIGURE 3: Chromatograms obtained for the analysis of a standard at 50 ng L^{-1} . (a) Analysis with the “off-line” SPE method, and (b) analysis of the same standard with the “on-line” mini-SPE/HPLC-UV method.

One of the problems in the analysis of water samples using an “on-line” system is the presence of considerable amounts of organic matter in some samples that can lead to a fast saturation of the mini-SPE columns. It is therefore important to evaluate the number of samples that can be analyzed with a continuous “on-line” system before saturation takes place. To this end, we evaluated two groups of samples: (i) surface waters and (ii) WWTP samples. When relatively “clean” samples were analyzed, as in the case of tap, surface waters and tertiary effluents from WWTPs, levels of organic matter were small and different samples were analyzed with the same mini-SPE column with no significant recovery losses. We were able to analyze ten consecutive fortified surface water samples with the same mini-SPE column with no variations in efficiency (recoveries $\geq 96\%$). WWTP samples, however, present a large amount of organic matter, which cannot be completely removed from the sorbent with the elution solvent. This reduces the efficiency of the sorbent after each analysis. For this type of sample, the “on-line” mini-column was not appropriate for analyzing consecutive samples and it had to be changed after each analysis.

3.3. Water Analysis

3.3.1. Surface Waters. Figure 4 shows the chromatogram obtained in the analysis of a surface water sample with the developed “on-line” method. Caffeine was not detected in the sample (Figure 4(a)). The analysis of the sample fortified at 15 ng L^{-1} gave a 101% recovery (Figure 4(b)). The analysis of all surface waters analyzed during the study showed equivalent results (caffeine levels below detection

TABLE 2: Caffeine levels detected ($\mu\text{g L}^{-1}$) at the three WWTPs evaluated. Sampling dates: (1) 6th of July, (2) 11th of July, (3) 14th of July, (4) 18th of July, (5) 21st of July, (6) 27th of July, and (7) 4th of October.

WWTP	Sampling date						
	1	2	3	4	5	6	7
<i>Empuriabrava</i>							
Influent	104.7	110.7	77.0	82.3	119.7	70.5	85.2
Biological effluent	0.6	1.2	nd	nd	0.5	0.8	nd
Lagoon	nq	nq	nd	nd	nd	nd	nd
Wetlands influent	nd	nd	nd	nd	nd	nd	nd
1st wetland	nd	nd	nd	nd	nd	nd	nd
2nd wetland	nd	nd	nd	nd	nd	nd	nd
3rd wetland	nd	nd	nd	nd	nd	nd	nd
<i>Pals</i>							
Influent	55.3	60.3	59.0	77.6	57.9	49.4	54.7
Biological effluent	nd	nd	nd	nq	nd	nd	nd
Secondary clarifier	nd	nd	nd	nd	nd	nd	nd
<i>Palamós</i>							
Influent	80.7	89.1	75.5	92.4	49.4	82.6	66.6
Primary effluent	58.8	67.8	44.1	64.4	44.4	55.3	53.1
Biological effluent	nq	nd	1.2	nd	0.5	nd	nd
Tertiary	nd	nd	nd	nd	nd	nd	nd

nd: not detected (LOD: $0.1 \mu\text{g}\cdot\text{L}^{-1}$).

nq: detected but below quantification limit (LOQ: $0.5 \mu\text{g}\cdot\text{L}^{-1}$).

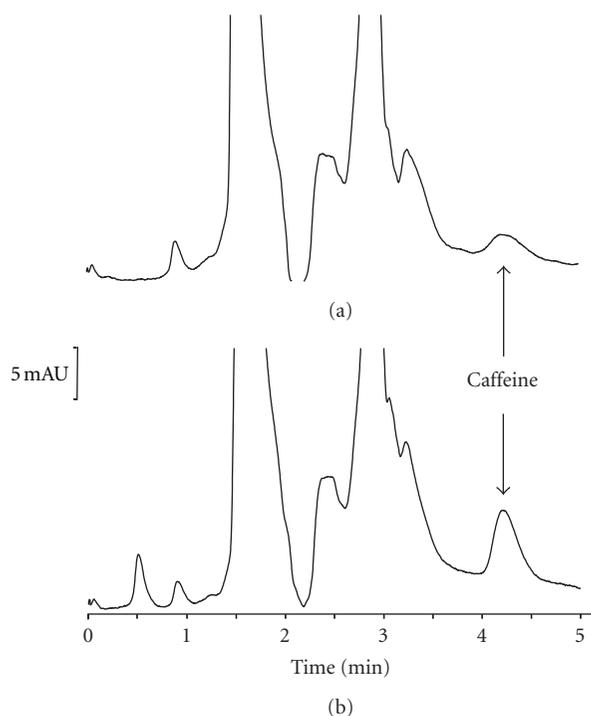


FIGURE 4: Chromatograms obtained for the analysis of a surface natural water with the “on-line” mini-SPE/HPLC-UV method. (a) Direct analysis of the sample and (b) analysis of the same sample fortified at 15 ng L^{-1} .

limit, 5 ng L^{-1}), except for one isolated sample downstream from the Palamós plant where a value of 60 ng L^{-1} was found.

3.3.2. WWTP Samples. As indicated previously, the “on-line” method did not give appropriate clean-up for continuous monitoring of samples within WWTPs. For this reason, the “off-line” method was used as it allows a most effective clean-up of these samples. Table 2 shows the results obtained in the analysis of samples from three WWTPs at different sampling points in each plant.

The aim of this study was to obtain information about the removal of caffeine in the different steps involved in the water treatment of urban wastewaters. Caffeine was quantified in all the influent samples from the three WWTPs at levels in the range $50\text{--}120 \mu\text{g L}^{-1}$, with mean values of $92.9 \mu\text{g L}^{-1}$ in Empuriabrava, $59.2 \mu\text{g L}^{-1}$ in Pals and $76.6 \mu\text{g L}^{-1}$ in Palamós. Calculated amounts of caffeine by person and day were of the same order for each influent plant when concentration values were normalized taking into account the population and the amount of water treated in each WWTP: 23.2 , 14.8 , and $15.2 \text{ mg person}^{-1} \text{ day}^{-1}$, respectively. These results are similar to those obtained in other studies [2, 9, 20].

The effect of a primary treatment to remove solid particles from the water entering the WWTP was evaluated in the Palamós plant. Levels at the effluent of the primary treatment were determined as this treatment was designed

in this plant exclusively to remove solid particles. Previous studies [9, 10] indicated that sorption/sedimentation of caffeine is negligible and attributed this to its low sorption potential ($\log K_{ow} \sim 0$). Thus, this treatment is expected to have a small effect on the removal of such a hydrophilic compound. As can be seen in Table 2, the caffeine reduction was between 10 and 42% (mean = 28%, sd = 11) after this treatment.

The results obtained indicate that the biological treatment had the highest effect on the removal of caffeine with more than 99% of the total caffeine being eliminated after this treatment in all the WWTPs evaluated. These results agree with previous studies [2, 10], where it was found that biodegradation was the dominant elimination process of caffeine and other xenobiotics contaminants in waters and determined residence half-life times between 0.8 and 5.0 hours in WWTPs due to the high microbial activity in activated sludge. Globally, the WWTPs evaluated showed good efficiency in caffeine removal as this compound was not detected in any sample at the effluent of the plants.

4. Conclusions

The developed methodologies based on solid phase extraction followed by HPLC with UV detection have been successfully applied to the determination of caffeine in environmental waters and throughout the wastewater treatment process. Significant removal of caffeine in the four WWTPs examined was demonstrated and the biological stage of the wastewater treatment process was confirmed as being responsible for this removal. The results also indicate the suitability of caffeine as a marker for untreated domestic wastewater.

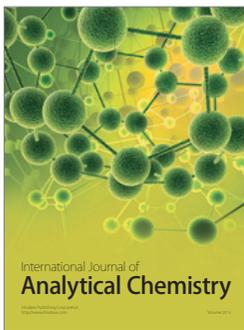
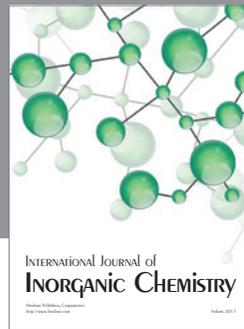
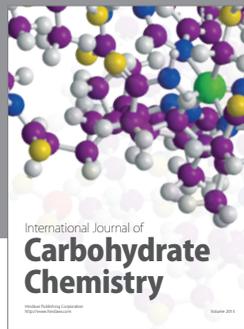
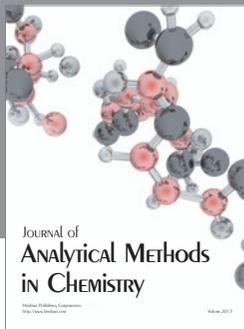
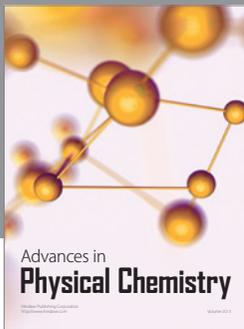
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