The illicit drugs consumption is increasing worldwide, mainly in America and Europe, which leads to significant health and social behavior issues that affect 27 million users (1-2). Consumption of heroin, cocaine and other drugs causes around 0.2 million deaths per year (183.000 drug-related deaths were reported in 2012 (3)).

Although cocaine, one of the most potent and addictive illicit drugs, has been consumed for over a century, its consumption seems to be increasing (4-6). Cocaine is now the most commonly consumed illicit stimulant drug in Europe (4, 6). More than 14.5 million Europeans have consumed cocaine at least once, and it has been estimated that 3.5 have consumed cocaine so in the present year. According to previous studies, United Kingdom is the country with the highest number of cocaine-related emergency episodes, followed by Spain (7). Both countries share a high consumption rate among young adults (aged 15–34) (5, 8, 9).

Cocaine consumption in Spain is a critical issue. According to different surveys (10), the number of adult population that have consumed cocaine at least once in their life has constantly increased since 1995 (11). Cocaine is currently the drug that generates more visits (45.1%) to the Emergency Department (ED) (5). Moreover, although mortality after psychoactive substances use and abuse has...
decreased in Spain, the proportion of deaths where cocaine or its metabolites are involved continues increasing (11).

For many years, surveys have been the only affordable analytic method to measure drug consumption. This system has different inconveniences, including underestimation of consumption (12). The treatment of the sample let to obtain acceptable results for some drugs of abuse, but in contrast, these surveys do not permit to obtain trustful results for illicit drugs with less consumption, such as cocaine. One reason is statistical, another important reason is the fear of some minors to answer this kind of surveys, although are anonymous (12). Another analytic method, wastewater-based drug epidemiology (WBDE), has been increasingly employed as an alternative approach to socio-epidemiological surveys. This method consists analyzing drugs and their metabolites or drug biomarkers in wastewater samples from a target community (13).

Different methods have been developed in order to diagnose substances consumption by WBDE. Solid phase and headspace solid-phase methods allow extracting residues and metabolites of illicit drugs from hair and biological fluids like urine, sweat, blood or saliva. However, previous analyses of biological fluids have allowed to measuring drug consumption by individuals, not by a community (1, 14-15). Residues of illicit drugs that are excreted by humans flow into and through wastewater treatment plants. These residues can produce a serious potential effect on humans, being persistent (such as cocaine and its metabolite benzoylecgonine) in the environment (16). The metabolites maintain the same action of the original compound, thus generating toxicological effects on non-target microorganisms (17).

The proposed analysis of illicit drugs in wastewater is a reliable source of data to estimate substances consumption in the population (18-19). This idea was proposed by Daughton in 2001 and applied by him and Zuccato (4, 20-22). Daughton hypothesized that in a similar manner to medicinal compounds, residues of illicit drugs may also be detected in the aquatic environment as a result of human use. As a consequence, it is possible to determine the amount of drug used by a population by measuring the concentration of drugs residues in wastewater (1, 23). There are different procedures used to perform the determination of organic compounds in aquatic environmental matrices, i.e. solid-phase extraction (SPE), solid-phase microextraction (SPME), stir bar sorptive extraction (SBSE) or liquid-liquid extraction (LLE). Besides, they are remarkable methods of separation and determination using liquid (LC) or gas chromatography (GC), coupled with mass spectrometry (LC-MS or GC-MS). Gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) are the techniques of choice for quantitative analysis. To have a tandem mass offers better results than single-quadrupole instruments, in terms of sensitivity and selectivity. In contrast, LC-MS/MS has been the technique most referenced for polar and semi-polar organic compounds analysis until currently (24).

The selection of appropriate DTRs is also essential to obtain reliable estimates of drug consumption. For this reason, the new good practices protocol for WBE studies specifies that the substances should fulfill the following requirements: 1) being excreted in consistent amounts in urine; 2) being detectable in urban wastewater; 3) being stable in wastewater and 4) have human excretion as their unique source (25).

Since the proposal of this method, several studies have been carried out in different countries (26-27). However, in Spain it has only been developed in cities of other regions (Santiago, Barcelona, Castellón, Valencia) and one town from Murcia (Molina de Segura) but it has not become the preferred method to measure drug consumption, although as said above, cocaine is a predominant drug of abuse very difficult to measure. Surveys suggest that the southeast of Spain is one of the regions where drugs consumption is higher. For these reasons, the aim of this study is to determine the concentrations of cocaine and its metabolites in samples of wastewater from a water treatment plant in the city of Murcia, in the Region of Murcia, Spain, in order to back-calculate cocaine consumption.

**EXPERIMENTAL**

**Materials and chemicals**

Cocaethylene-d₄ and benzoylecgonine-d₃ were purchased from Sigma-Aldrich (Steinheim, Germany) as 0.1 mg/mL solutions in acetonitrile or methanol. Diluted standard solutions were prepared in methanol HPLC (Sigma-Aldrich) until 10 µg/mL as required. Ultrapure water was obtained from a Milli-Q Gradient A-10 system (Millipore, Bedford, Massachusetts, USA). Ammonium acetate, acetic acid, formic acid and tetrabutylammonium bromide (TBA, 99% purity) were purchased also at Sigma-Aldrich. Cartridges (6-mL disposable Oasis MCX) for solid-phase extraction (SPE) were acquired from Waters (Milford, Massachusetts, USA).
Wastewater sampling

Raw influent wastewater was collected at the inlet (before the primary clarifier) of the main wastewater treatment plant located in the province of Murcia. The flow rate of this plant is around 108,000 m$^3$/day and it covers 445,000 inhabitants. We decided to collect wastewater samples from the central area of the city, which consists of a population of approximately 170,000 inhabitants and it comprises a 70% of total flow rate according to data from the wastewater treatment plant (Table 1).

Samples of 250 mL were collected by automatic sampling devices every hour during 6 consecutive days (24 samples/day). Water samples were stored in dark bottles at -20°C until analysis.

Sample preparation and analysis

Following the collection, water samples were vacuum-filtered on a glass microfiber filter GF/A 1.6 µm (Whatman, UK) and then through 0.45 µm nitrocellulose filters (Millipore, Bedford, Massachusetts, USA) before extraction. Solid-phase extraction was performed as described below using 6-mL Oasis MCX cartridges. They were conditioned by washing with 6 mL of methanol, 3 mL of Milli-Q water, and 3 mL of acidified water (pH 2). Then 60 mL-aliquots from wastewater samples were adjusted to pH 2.0 with formic acid. Samples were loaded onto the cartridges by gravity, washed with 5 mL of ammonia in water, and then dried for 5 min. Target substances were eluted using 8 mL of a 2% ammonia solution in methanol. The obtained solution was evaporated to dryness at 35°C under a stream of nitrogen.

Immediately, dried samples were dissolved in 1 mL of 10% methanol in water. Analyses were performed by injecting 25 µL of the final extract into the UPLC-qTOF-MS/MS system. A Waters Acquity UPLC BEH C18 1.7 µm particle size analytical column 50 mm x 2.1 mm (Waters) at a flow rate of 0.3 mL/min. Ther acquisition was performed in Selected Reaction Monitoring (SRM) mode. The mobile phases used were A = H$_2$O with 0.01% HCOOH and B = MeOH. The percentage of organic modifier (B) was changed linearly as follows: 0 min, 10%; 14 min, 90%; 7 min, 90%; 9.0 min, 90%; 9.01 min, 10%. Nitrogen (from a nitrogen generator) was used as the drying gas and nebulizing gas. The gas flow was set at 320 L/h. TOF-MS resolution was approximately 45,000 at full width half maximum (FWHM) in V-mode and 17.500 FWHM in W mode, at m/z 556.2771. MS data were acquired over an m/z range of 50–1200. The microchannel plate detector potential was set to 1750 V. A capillary voltage of 4.5 kV and a cone voltage of 25 V were used. The interface temperature was set to 350°C and the source temperature to 120°C. The column temperature was set to 40°C. Calibrations were conducted from m/z 50 to 1200 with a 1 : 1 mixture of 0.01 M NaOH : 5% HCOOH diluted (1 : 5) with isopropanol: water (1 : 1), at a flow rate of 180 µL/h. MS data were acquired in centroid mode and were processed by the Data Analysis application manager (within Compass 1.7; Bruker Corporation). LC retention time was compared to the specified in the standards, considering a deviation of 2.5%, in order to confirm the metabolites detected by mass spectrometry.

In order to assure the quality of analytical methods and to ensure the reliability of the results (28-29), our parameters were defined. The limit of detection (LOD), which is the lowest concentration that the method can differentiate from background results, was calculated for a signal-to-noise (S/N) of three from the chromatograms spiked at the lowest analyte concentration tested. The limit of quantification (LOQ) was calculated as the lowest concentration level for which this method was completely val-

<table>
<thead>
<tr>
<th>Day</th>
<th>Flow rate (Q) (m$^3$/day)</th>
<th>Flow rate target (70%Q) (m$^3$/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Friday</td>
<td>113.552</td>
<td>79.486</td>
</tr>
<tr>
<td>Saturday</td>
<td>104.206</td>
<td>72.944</td>
</tr>
<tr>
<td>Sunday</td>
<td>100.947</td>
<td>70.663</td>
</tr>
<tr>
<td>Monday</td>
<td>102.506</td>
<td>71.754</td>
</tr>
<tr>
<td>Tuesday</td>
<td>108.461</td>
<td>75.922</td>
</tr>
<tr>
<td>Wednesday</td>
<td>114.250</td>
<td>79.975</td>
</tr>
</tbody>
</table>
idated by using spiked samples with an adequate recovery (between 70 and 120%). Confirmation of these data was made through highly selective Multiple Reaction Monitoring (MRM). Instrumental detection and quantification limits for Cocaethylene were 2-5 ng/L and ~14 ng/L respectively and about Benzoylecgonine 1-3 ng/L and ~12 ng/L. The linearity of the method was analyzed by studying standard solutions in triplicate at six different concentrations, ranging from 0.5 µg/L to 25 µg/L for cocaine and its metabolites. Satisfactory linearity was set when the correlation coefficient \((R)\) was greater than 0.99, according to analyte/internal standard peak areas size. Other different validation parameters were measured as accuracy (which was calculated by means of recovery experiments) and of course, precision (quantified as repeatability in terms of Relative Standard Deviation (RSD)) = \(20\%\). Both were analyzed by injecting different water samples spiked at different concentration levels (LOQ and 10 LOQ). Every recovery experiment was performed five times for each water sample tested.

**Estimation of consumption rates from biomarker concentrations**

Rates of consumption were estimated from biomarker levels in sewage samples (10, 14, 20) which consisted in, firstly, to multiply the concentration (ng/L) of each quantified metabolite in wastewater by the influent wastewater flow rate (m³/day) coming from the study area (70% of total flow) in order to calculate the amount of each metabolite that daily reach the sewage plant from the target area (g/day). The number of inhabitants served by the flow under study was calculated dividing these mass loads by the population served and using BOD parameter (Biochemical Oxygen Demand). This parameter measures organic component of mainly domestic wastewater (kg/m³). Previous studies have suggested that each inhabitant provides approximately 60 g of BOD per day (30). Results are expressed in mg/day per 1000 inhabitants.

The excretion rates of cocaine metabolites were used to calculate the amount of the drug consumed by the population under study. A correction factor used in previous studies (22) to back-calculate from mass loads into amount of used illicit drug was applied. This correction factor considers the fraction of consumed parent drug normally excreted as a metabolite in urine, and the parent drug/excretion product molar mass ratio (4, 22). These calculations are only valid for drugs which produce the main excretion product and therefore a reliable direct indicator of consumption (22). Regarding cocaine, its main excretion product is benzoylecgonine, and importantly, 45% of consumed cocaine is excreted by this manner. The ratio molar mass cocaine/benzoylecgonine is 1.05 and the correction factor is 2.33.

**Cocaine and ethanol co-consumption**

We used the ratio cocaethylene/benzoylecgonine to calculate directly the amount of cocaine consumed together with alcohol.

**Statistical analysis**

Data are expressed as mean ± SEM. Statistical analysis was determined by a two-way analysis of variance (ANOVA). The Newman-Keuls was used as a post hoc test whenever a significant result was revealed by an analysis of variance (ANOVA). Differences with a \(p < 0.05\) were considered significant.

**RESULTS**

Cocaine consumption in the southeast of Spain was estimated once the metabolites were identified according to the equation proposed by Zuccato et al. (2008) (22). The quantification of benzoylecgonine (cocaine metabolite) and cocaethylene (alcohol and cocaine concomitant consumption metabolite) by UPLC–qTOF-MS/MS (Fig. 1) showed the highest values at the weekend (Fig. 2). Sunday was the day with the highest mass load value for benzoylecgonine (5.405 µg/L) followed by Friday (3.399 µg/L) and Saturday (2.444 µg/L). Results were statistically significant (\(p < 0.001\)) for Sunday. Monday was the lowest value for benzoylecgonine (0.084 µg/L). As to cocaethylene, Saturday was the day of the week with the highest mass load value (0.083 µg/L), followed by Sunday (0.049 µg/L) (Table 2). No significant interactions were recorded.

The total cocaine per day in the city of Murcia was calculated taking into account flow rate target and benzoylecgonine concentration for each day (Table 1), and applying a correction factor for cocaine (Fig. 3). Total cocaine consumption in the target area during the study (6 days) was 2463.74 milligrams per 1000 inhabitants, with a mean of 410.6 milligrams per 1000 inhabitants and day. The highest cocaine intake was on Sunday, with a result of 889.97 milligrams of cocaine per day and 1000 inhabitants, which is statistically significant (\(p < 0.001\)) when compared with the rest of the studied week. Friday is the day of the week with the second highest consumption, with 629.4 milligrams per 1000 inhabitants.
Cocaine consumption in the city of Murcia (southeast of Spain) estimated by...

Figure 1. Identification of benzoylecgonine and cocaethylene by UPLC-qTOF-MS/MS

Figure 2. Amount of Benzoylecgonine and cocaethylene quantified for each analyzed day

Table 2. Benzoylecgonine and cocaethylene average concentrations of each analyzed day.

<table>
<thead>
<tr>
<th></th>
<th>(Benzoylecgonine) (µg/L)</th>
<th>(Cocaethylene) (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Friday</td>
<td>3.399</td>
<td>n.q.</td>
</tr>
<tr>
<td>Saturday</td>
<td>2.444</td>
<td>0.083</td>
</tr>
<tr>
<td>Sunday</td>
<td>5.405</td>
<td>0.049</td>
</tr>
<tr>
<td>Monday</td>
<td>0.084</td>
<td>0.023</td>
</tr>
<tr>
<td>Tuesday</td>
<td>2.302</td>
<td>0.014</td>
</tr>
<tr>
<td>Wednesday</td>
<td>0.582</td>
<td>0.031</td>
</tr>
<tr>
<td>Average</td>
<td>2.368</td>
<td>0.034</td>
</tr>
</tbody>
</table>
Once the weekdays mean and the weekend mean were compared, an important difference arose. In Murcia, the weekend mean was 644.7 milligrams/1000 people, whereas the weekdays mean was 176.55 milligrams/1000 people, which means that consumption during the weekend was 365.17% higher than during working-week days. Co-consumption was evaluated using the cocaethylene/benzoylecgonine ratio. The ratio was 0.014 and cocaethylene levels were higher at the weekend.

A BOD parameter which measures organic component wastewater (kg/m³) was employed to estimate the number of inhabitants that were at the city during the period analyzed. Under the assumption that each inhabitant provides approximately 60 g of BOD per day, the number of inhabitants served by the flow under study would be in an interval between 194.316 and 253.250 (Table 3). The number of inhabitants obtained is similar to that obtained from the census, around 250,000 inhabitants (in this region is common to leave the City Centre in Summer).

Consequently, it is possible to calculate the amount of cocaine doses consumed per day and 1000 inhabitants. For that, it has to be assumed that 100 mg is an average dose (the equivalent of four 25 mg “lines” of cocaine) (31).

**DISCUSSION AND CONCLUSION**

As cocaine consumption is increasing worldwide, new accurate information about trends in consumption is essential to develop strategies to tackle the problem of drug abuse and to fight drug trafficking (15, 32). Information about the type, scale, and demographics of cocaine consumption is needed in order to fully understand drug use patterns and to develop better methods and actions to reduce the problem (33-34).

The use of cocaine, one of the most potent and addictive illicit drugs, appears to be increasing in some countries (14). Spain, together with the United Kingdom, Italy or Ireland, is one of the countries with the highest cocaine consumption according to the surveys (5).

![Figure 3. Cocaine consumption in each analyzed day, in grams per day and 1000 inhabitants](image)

### Table 3. Inhabitants served by the wastewater flow target of each day.

<table>
<thead>
<tr>
<th>Day</th>
<th>Flow rate target (70%Qe) (m³/day)</th>
<th>BOD₅ (mg/L)</th>
<th>Inhabitants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Friday</td>
<td>79.486</td>
<td>186</td>
<td>246.400</td>
</tr>
<tr>
<td>Saturday</td>
<td>72.944</td>
<td>171</td>
<td>207.883</td>
</tr>
<tr>
<td>Sunday</td>
<td>70.663</td>
<td>165</td>
<td>194.316</td>
</tr>
<tr>
<td>Monday</td>
<td>71.754</td>
<td>180</td>
<td>215.250</td>
</tr>
<tr>
<td>Tuesday</td>
<td>75.922</td>
<td>165</td>
<td>208.783</td>
</tr>
<tr>
<td>Wednesday</td>
<td>79.975</td>
<td>190</td>
<td>253.250</td>
</tr>
</tbody>
</table>
According to a study that compared illicit drug use in 19 European cities using the wastewater analysis method, another city from the east of Spain (Valencia) was the third city with the highest cocaine consumption rate (34). If we compare our results with previous studies, cocaine consumption in Murcia would be in higher than in Helsinki, Zagreb, Oslo or Stockholm, lower than in London, Amsterdam, Zurich or Antwerp, and in Paris, Milan, Dortmund, Lugano and Santiago, also than Spain. However, if we take into account sizes, political importance and population, Murcia would fall behind, i.e. Stockholm has more than 2 million inhabitants, Oslo is around 1 million, and they are both European capitals. However, Murcia is only the seventh largest city in Spain. Much more remarkable is the comparison with Paris, which have nearly 16 million inhabitants and a similar cocaine amount is consumed (22, 34, 35). If we consider the tendencies reported in the 2017 European survey (5) we could find an explanation for these results, which is that Spain is one of the top countries in cocaine consumption together with the United Kingdom, and above Italy and France.

If we compare similar studies in other cities in Spain (36-37), Murcia would be the third city in terms of cocaine consumption after Barcelona and Valencia, with similar consumption levels to Santiago and Castellon, with a similar population. It should be noted that these results are similar to those obtained from Molina de Segura in 2016 (562.2 milligrams per 1000 inhabitants and day), a town close to Murcia (5).

Previous studies have shown a lower pattern of co-consumption of cocaine and alcohol. It has been suggested that this pattern is much more common in Spain, which is in concordance with our results. Co-consumption was evaluated using the cocaethylene/benzoylecgonine ratio. Our results were 0.014 for the whole week which is similar to previous results for Spanish cities of similar size to Santiago (35). Cocaethylene levels were higher during the weekend, which confirms a pattern of co-abused substances.

Cocaine has been widely described as a recreational drug and this pattern has been confirmed by recent surveys (5). According to this, cocaine consumption should be higher during the weekends. In our study, sampling wastewater was analyzed on working days, one weekend, and one-day regional festivity. In our case, and like other studies (38), cocaine consumption was higher on weekend days, with an average of 644.73 milligrams of cocaine per day and 1000 inhabitants. Cocaine consumption on weekend days (176.55 milligrams of cocaine per day and 1000 inhabitants) was 365.17% higher than on working weekdays. This pattern is similar in Nordic countries, which reflect a 300% increase over the weekend (39) but far from the results for Barcelona or Santiago, around 60-80% and Milan (23%) (35, 39). These results do not match with the epidemiological surveys in the entire country and suggest a different pattern in Spain according to the city’s demography. Our data showed that cocaine concentration at Sunday was twice the consumption of the following day. Regarding the weekdays, Tuesday was the date when the regional festivity happened. These results showed that cocaine consumption on Tuesday and Wednesday increased again after Monday reduction. Since the urinary elimination half-life of benzoylecgonine is nearly 4 hours, the weekend increase would show the greater use of cocaine as a recreational drug.

It is important to clarify that cocaine consumption is usually higher at nights and during weekends. Therefore, cocaine concentrations of a specific day would match with consumed cocaine the night before. As well, cocaine quantified on Friday, Monday and Tuesday would correspond with consumed cocaine on Thursday, Sunday and Monday night respectively. Thus, the highest cocaine consumption would occur on Thursday and Saturday night. Thursdays in Murcia are the date on which university students usually go out. About the regional festivity, it took place on a Monday night and a Tuesday in the morning. As a consequence, cocaine concentrations on Tuesday and Wednesday increased again.

Illicit drug use is a socially stigmatized and often a hidden activity. As a consequence, traditional survey methods such as general population survey and interviews can be inaccurate and prone to conjecture (34). It is also known that this kind of surveys underestimates results for illicit drugs with less consumption, such as cocaine, and moreover, minors usually experience some mistrust to the interlocutor resulting in doubtful consumption answers (12). The method used in this study is useful to estimate drugs of abuse consumption in a population, since it allows to measure simultaneously illicit drugs and their metabolites in wastewater from a particular area, obtaining data of the most used drugs and consumption trends almost in real time, that is, within days or weeks, compared to surveys that may take years before publication (1, 34, 40).

UPLC-QTOF MS is a powerful technique that allows the detection and identification of several widely consumed illicit drugs (41). It is a powerful
method that in our case has detected and quantified cocaine and its metabolites every day of the study except for cocaethylene on Friday, offering a wide range of quantification, starting from 0.014 µg/L for cocaethylene and 0.012 µg/L for benzoylecgonine. In this study, we have obtained worthy results in terms of method validation what can be consulted through parameters as linearity, precision, accuracy, or the above commented LOQ and LOD. UPLC-qTOF-MS/MS system has shown to be capable of detecting and quantifying cocaine metabolites in very hard conditions what gives support to the idea of using this method in the future to quantify illicit drugs consumption of a community, but also, different pharmaceuticals or pollutants present in water.

This method is non-intrusive, objective and does not require approval from individuals. When used for monitoring purposes in large populations, it does not raise major ethical concerns because individuals are not identified and the prospects of harming residents of catchment areas are remote (8, 42). In addition, this method is more cost effective and does not have the problems of surveys that may suffer from a limited number of subjects and lack of veracity in the responses. This is clearly a huge advantage because it is very common that people do not trust their interlocutor altering the value of the responses. Besides, wastewater analysis provides an estimate of all the population contributing to the sewage over the sampling period (9).

However, this technique has certain limitations, such as the stability of target residues in the sewage system during the collection and storage of the sample. Besides, although correction factors or parameters as BOD are used, the reliability of population estimates depends on factors that cannot easily be controlled, i.e., the composition of sewage, the accuracy of census data, the quality of the measured flow data and the method itself used to calculate population equivalents. Another disadvantage of this method is the impossibility to know what percentage of people is really consuming cocaine, thus, we only can obtain the amount of drug abused, and of course, it is important to underline that the tolerance to this drug produces a high variability of consumption among individuals.

There is certainly a need to better understand the amount of cocaine consumed in Spain and, in particular, in its southeast. In this sense, sewage analysis can be applied as a supply indicator to assess the size of Spanish consumers. According to our data, total cocaine grams consumed in target area along analyzed days were 2463.74 milligrams. It is only an estimation based on a certain zone of Region of Murcia over a limited period, so further refinement of the methodology is needed.

Cocaine consumption levels obtained in the Region of Murcia or Spain are higher than levels obtained in similar studies carried out in the rest of Europe and the method used is useful for estimating drugs consumption in a population. At the same time, results are consistent with the results obtained in other Spanish cities of similar size. Wastewater analysis provides more objective results and avoids several uncertainties related to surveys being possible to study short-term consumption. Public administration and citizens should be aware of the problem that drugs of abuse represent. It is not only a health public issue but also an economic problem due to ED visits, hospitalization costs or detoxification costs. Moreover, we think that, in order to reduce consumption, public administration should carry out more campaigns against drugs of abuse, warning citizens about their consequences.

Conflict of interests

There are no conflicts of interest to disclose. All the authors contributed to and have approved the final manuscript.

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