

Analysis of cocaine and nicotine metabolites in wastewater by liquid chromatography-tandem mass spectrometry. Cross abuse index patterns on a major community.

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HIGHLIGHTS

- Cross abuse index patterns on licit and illicit drugs by means of sewage assessment
- Data about cocaine and nicotine consumption from a Portuguese population
- Co-evaluation of cocaine abuse estimates based on local drug purity levels

ABSTRACT

A method based on sample preparation by solid phase extraction and analysis by liquid chromatography and mass spectrometry was validated and used for simultaneous analysis of cocaine, benzoylecgonine and cotinine in samples collected at the major wastewater treatment plant in the city of Lisbon. The aim was to estimate the consumption of both cocaine and nicotine in this community and establish an index involving both drugs supported by the relevance of nicotine as a significant anthropogenic marker. The study was made on two different week days during a month in order to evaluate patterns of consumption outside weekends. Cocaine and nicotine ingestion levels were back-calculated and expressed as mass of pure drugs consumed per day and per 1000 inhabitants (mean: 0.604 g and 5.860 g respectively). Cocaine was also expressed on the basis of local drug purity levels (33.7%) with a corresponding increase on dose assessments, and community drug abuse profiles. The authors sustain that this approach should always be included in drug studies of this kind allowing a better drug abuse assessment. No significant different patterns of consumption were obtained during the working days studied with the exception of one case coincident with a national holiday that showed an increased typical profile found on other non-working day studies, namely weekends. A fairly significant relationship was found between nicotine and cocaine consumption that should be further evaluated in future studies. Pharmacokinetic considerations were made and proposed for cocaine assessment based on the impact on back calculations after common simultaneous consumption of cocaine and ethanol.

Keywords: *Wastewater, Cocaine, Benzoylecgonine, Cotinine, Nicotine*

1. Introduction

The analysis of sewage drug biomarkers (SDBs) has recently been established as a complementary approach for monitoring patterns and trends of illicit drug use in communities (Banta-Green et al., 2009; Berset et al., 2010; Castiglioni et al., 2011; Irvine et al., 2011; Karolak et al., 2010; Metcalfe et al., 2010; Reid et al., 2011; Terzic et al., 2010; van Nuijs, et al., 2011a; van Nuijs et al., 2009a; Zuccato et al., 2008). In 2012 it has been published the first harmonized study involving drug wastewater analysis in 19 European cities (Thomas 2012). Moreover, in 2013, using data from this study, a critical review was made to integrally

address the uncertainties associated with the estimations of community drug use through sewage analysis, proposing a set of best practices requirements (Castiglioni et al., 2013).

Cocaine (COC) remains the second most commonly used illicit drug in Europe, although prevalence levels and trends differ considerably between countries (EMCDDA, 2012). In Portugal results from the II National Population Survey on Psychoactive Substances in the Portuguese Population (15-64 years of age), the latest national survey available dated from 2007, indicate that alcohol and tobacco are the licit substances preferably used by the Portuguese population, and cannabis, cocaine and ecstasy, the illicit substances with lifetime prevalence respectively of 11,7%, 1,9% and 1,3%(IDT, 2011). The prevalence of cocaine use in the last 30 days/12 months has increased from 0.1/0.3 (year 2001) to 0.3/0.6 (year 2007), which was a strong incentive for selecting cocaine as the drug of choice for this sewage study in the capital of Portugal, analyzing the influents of the main wastewater treatment plant (WWTP) in the city of Lisbon, Alcântara (Fig.1).

The aims of this work were (1) to assess the consumption of both cocaine and nicotine through the quantification of cocaine (COC), benzoylecgonine (BE) and cotinine (CT) in the influents of a major urban wastewater treatment plant, by the means of an implemented and validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) analytical method; (2) to establish a relationship index between tobacco and cocaine consumption and (3) to propose a complementary data evaluation for better qualification of epidemiological studies by converting the number of cocaine doses adjusted to the average local purity levels of cocaine. This approach will produce a more realistic view on the extent of drug consumption and trafficking and is proposed as another index to be included on every sewage drug studies

2. Material and methods

2.1 Reagents and materials

LC-MS grade acetonitrile (ACN) and methanol (MeOH) were obtained from Scharlau® (Scharlau S.L., Sentmanat, Spain). Analytical grade 37% hydrochloric acid (HCl), ammonium hydroxide (NH₄OH) and ammonium acetate (CH₃COONH₄) were purchased from Merck (Darmstadt, Germany) and VWR (Lisbon, Portugal). Ultra-pure water (18.2 MΩ.cm) was obtained from a Millipore-Direct Q3 UV system (Millipore®, Bedford, MA, USA). Individual methanolic or acetonitrile solutions (1 mg/mL) of cocaine (COC), benzoylecgonine (BE), cotinine (CT) and their deuterated analogues (COC-d₃, BE-d₃, CT-d₃), were of analytical grade (purity > 98%) and were purchased from Cerilliant (Round Rock, TX, USA).

Oasis HLB (500 mg, 6 mL) SPE cartridges were acquired from Waters (Barcelona, Spain) and a Waters Sep-Pak solid-phase extraction (SPE) Vacuum Manifold with 12 ports and dry vacuum system was also used in the SPE procedure.

2.2 Sample site and sample collection

Influent samples were collected from the main wastewater treatment plant (Alcântara-ETAR) in the region of Lisbon, Portugal. Samples were collected twice a week (Tuesday and Thursday) during four weeks, between the months of October and November 2011. Wastewater treatment plant (WWTP) samples were 24-h composite samples starting at midnight, collected on a time proportional sampling mode (120 minutes) and kept refrigerated during that process. The Alcântara WWTP serves the west part (A) and the center (A1) of the city of Lisbon (Fig.1) and two major suburban areas - Amadora and Oeiras. The WWTP is dimensioned to an estimate design capacity of 756.000 inhabitants with a flow processing capability of $6.6 \text{ m}^3/\text{s}$. This system has started functioning in 1989 with a primary treatment and chlorine disinfection. Since October 2009 several major benefits have been introduced including biological treatment and ultraviolet irradiation allowing possible reuse of outflows both internal and external. During the study the total volume treated by the WWTP reached on average $123\,000 \text{ m}^3/\text{day}$.

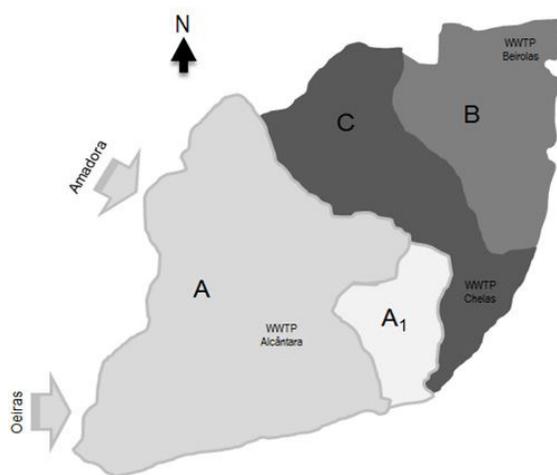


Fig.1- Lisbon WWTP's covering areas: **A,A1**-Alcântara (study); **B**-Beirolas; **C**-Chelas

Sample preparation

All 24h WWTP samples were transferred into 1 L polyethylene bottles, adjusted to pH=2 with 37% HCl and stored at $-20 \text{ }^\circ\text{C}$ until analysis. These conditions have been described as granting negligible degradation of the cocaine and metabolites (Gheorghe et al., 2008) and cotinine (Chen et al., 2012). Concentrations of COD, BOD, phosphorus and azote were measured by the Alcântara WWTP laboratory.

Samples were prepared according to previous published work (Gheorghe et al., 2008). Briefly 500 mL aliquots were defrosted and adjusted to pH=6 with diluted ammonia followed by centrifugation (4 °C, 3800 rpm, 10 min) using a refrigerated centrifuge Hermle Z-360K (Hermle Labortechnik, Wehingen, Germany) and filtering through Millipore 0.45 µm glass microfiber filter. A volume of 100 mL of each sample was then spiked with deuterated internal standards (IS) (20 ng of COC-d₃, 50 ng of BE-d₃ and 50 ng of CT-d₃). Oasis HLB (6 mL, 500 mg) solid-phase extraction (SPE) columns were used to clean-up and concentrate the samples. The SPE columns were previously conditioned with 3 mL of methanol followed by 3 mL of Milli-Q water. After sample elution at 3 mL/min constant flow, SPE columns were washed with 3 mL of a 5% MeOH solution, dried under vacuum for 15 min and finally eluted with 2×4 mL of MeOH. Eluted solutions were then dried under N₂ atmosphere and re-dissolved with 1000 µL of ACN. Samples were extracted in duplicate.

2.3 Standard solutions and calibration

Individual working standard solutions (10 µg/mL) were prepared by dilution in acetonitrile from initial stock standard solutions. These standards were stored at -20 °C in amber glass vial and brought to room temperature before use.

Calibration standard solutions were prepared in acetonitrile from working standard solutions of 10 µg/mL. The concentration range of the solutions was from 0.1 to 100 ng/mL. To all calibration solutions (1 mL) the same amount of internal standards (IS) was added: 20 ng of COC-d₃, 50 ng of BE-d₃ and 50 ng of CT-d₃.

2.4 LC-MS/MS conditions

LC analyses were performed using a Waters Alliance 2695 (Waters®, Ireland) system equipped with a quaternary pump, automatic injector and a thermostated column compartment. The system was coupled to a Photodiode Array Detector Waters 996 PDA (Waters®, Ireland) and a triple quadrupole mass spectrometer MicroMass QuattroMicro® API (Waters®, Ireland) equipped with an electrospray ionization source (ESI). For analysis, a volume of 5 µL was injected. Chromatographic separations were performed using a Phenomenex® Luna 5 µm HILIC 200A (150×3.00 mm) column operating at 35 °C and the autosampler was at 10 °C. A mobile phase consisting of ammonium acetate 5 mM (eluent A) and acetonitrile (eluent B) was used at a flow rate of 0.30 mL/min. The gradient elution profile was as follows: 0-0.5 min: 97% of eluent B; 0.5-4.0 min: 97.0-55.0% B; 4.0-6.5 min: 55-50% B; 6.5-7.5 min: 50% B; 7.5-8.0 min: 50-97% B; 8.0-14.0 min: 97% B in order to equilibrate the column.

Conditions of analysis by MS/MS were optimised by infusion of individual working standard solutions of each compound in the ion source of the mass spectrometer, using a syringe pump at a flow rate of 20 µl/min. The ESI source was used in positive

ionization mode. The source temperature was at 120 °C; desolvation temperature 350 °C, the cone gas flow was 50 L/h and desolvation gas flow 750 L/h. The data acquisition was performed using Selected Reaction Monitoring mode (SRM) with a dwell time of 0.2 s in order to increase the sensitivity and selectivity of the analytical method. The operating conditions for the cone voltage (range from 10-40 V) and collision energy (range from 5-40 eV) were optimised in order to obtain the precursor ion of each target analyte and two product ions. One product ion was used for quantification (SRM1) and the other for qualification (SRM2) purposes. The final conditions used are presented in Table S1 and are according to conditions previously described (van Nuijs et al., 2009b; Srinivasan et al., 2000). MassLynx software (version 4.1) and QuanLynx (version 4.1) were used for controlling the equipment and also for data acquisition and processing.

2.5 Method validation

The validation was performed in accordance with the International Conference on Harmonisation (ICH) guidelines (ICH, 2005) with minor modifications. Briefly, the specificity, stability, linearity, concentration range, precision (intra-day and inter-day precision), accuracy, instrumental limit of detection (ILOD), instrumental limit of quantification (ILOQ) and method limit of quantification (MLOQ) (ISO, 1990; RELACRE, 2000; EC, 2002) were determined (Tables S2 and S3). Criteria requirements were based on Commission Decision 2002/657/EC (EC, 2002). To confirm linearity of the method, the Mandel Test was used according to ISO 8466-1:1990, residuals were calculated and the Rikilt test was performed (Van Trijp and Roos, 1991). Detailed method validation information is presented in Supporting Information.

2.6 Sewage back calculations

2.6.1 Cocaine

Refined cocaine, in the form of the base or hydrochloride salt, is self-administered mainly by snorting, smoking, genital application, and by injection. Cocaine is rapidly absorbed from mucous membrane and pulmonary vasculature depending on the blood rate appearance by the route of administration. After being absorbed, the substance distributes rapidly, mainly into the brain, spleen, kidney and lung where higher concentrations are reached (Bortolotti et al., 2012; Jeffcoat *et al.*, 1989) on their mass balance study with an intravenous administration of radiolabeled cocaine, have determined that less than 1% of the dose is excreted in the unchanged form ($f_{e\text{COC}}$), while a mean of 2.8% (range 2-3%) of the dose is metabolized into ecgonine, 19.3% (range 18-22%) into ecgonine methyl ester and 36.0% (range 26-39%) ($f_{\text{mCOC-BE}}$) into benzoylecgonine (BE) (Jeffcoat et al., 1989). The authors also determined a fraction excreted of 10.8% of unidentified metabolites in urine, to which the later described metabolites can account for, namely p- and m-hydroxycocaine, p- and m-hydroxybenzoylecgonine, norcocaine, N-

hydroxynorcocaine, norcocaine nitroxide and norcocaine nitrosonium ion (Kolbrich et al., 2006). 5.5% of COC dose was also determined to be excreted in feces. The value of 36.0% for BE formation is similar to the average value of 38% suggested by Thomas et al. (2012), after revising pharmacokinetic studies available in the literature. The average elimination half-life of COC is reported to be 244 minutes after intravenous administration, 272 minutes after smoked administration and 299 minutes after intranasal administration (Bortolotti et al., 2012).

Similar fractions values were also obtained in another work (Cone et al., 1998) after an administration of an intravenous dose of cocaine, with means of 1.0% (range 0.15 to 2.97%) and 39.2% (range 28.4 to 50.7%), respectively for $f_{e\text{COC}}$ and $f_{m\text{COC-BE}}$.

However, coadministration of cocaine with alcoholic beverages alters the biotransformation pattern of COC, leading by transesterification in the liver to the formation of an equipotent metabolite cocaethylene (17% of COC dose) and decreasing BE formation due to the inhibition of the ethylesterase hydrolysis by ethanol (Harris et al., 2003). Based on these authors work, it is possible to calculate a metabolized fraction of COC into BE of approximately 24% of COC dose, after intravenous administration of 0.3, 0.6 or 1.2 mg/kg of radiolabeled cocaine together with ethanol (1 g/kg), and considering the mean body weight of studied population (71 kg). Moreover, in the same study, the fraction excreted of unchanged COC is of approximately 5% for all administered doses, representing an increase of this fraction value when compared to COC administration without alcohol (less than 1%). However both values are in the range of the described 1-9% (Baselt, 2008).

More recently in a critical review article (Castiglioni et al., 2013), the authors propose for $f_{m\text{COC-BE}}$ a general value of 29% (RSD 26%), calculated based on human urinary excretion of BE collected from 20 published studies (total of 106 subjects), performed with cocaine administered by different routes. In the calculations the authors weighted not only the number of subjects by study, but also the frequency of COC use by the different routes of administration, based on data from a multicenter European study (Prinzleve et al., 2004).

Coadministration of alcohol and cocaine is common, with 50-90% of cocaine users reporting the ingestion of ethanol simultaneously in order to prolong the "high" and to minimize the dysphoric feelings associated with the use of cocaine (Goldstein et al., 2009).

To estimate cocaine consumptions, back calculations were centered on previous published works (Zuccato et al., 2005; van Nuijs et al., 2011b) and were based on both the concentrations of cocaine and its major metabolite benzoylecgonine, which is present in wastewater at relatively high concentrations and is quite stable in water (Gheorghe et al., 2008).

From the concentrations of benzoylecgonine (ng/L) and with the flow rate (L/day) of the wastewater stream, BE loads (g/day) can be calculated (Table 1). In order to compare the COC consumption results obtained in this study with other 19 European cities, these loads were then further back converted into consumed COC amount (COCEq) (g/day) (Table 2) using the molecular mass ratio of cocaine to benzoylecgonine (1.05) and assuming an average metabolized fraction of COC into BE ($f_{m\text{ COC-BE}}$) of 38% (back-correction factor of 2.76) as suggested by Thomas et al., 2012. However, it is believed that this average value does not take into account the simultaneous administration of cocaine with alcohol, an event likely to happen (EMCDDA, 2009), which reduces BE formation.

Furthermore, in this work unmodified COC was also quantified (ng/L) in the samples (COCww), from which COCww load (g/day) was calculated (Table 1) and then back converted into consumed COC amount (COCww eq) (g/day) (Table 2), using as fraction excreted of unmodified COC ($f_{e\text{ COC}}$) the initial value of 1%, described for COC consumption without alcohol.

Recently (van Nuijs et al., 2012) has assessed sewage degradation of BE and COCww in influent wastewater during 26 h, at relevant conditions (20 °C and pH 7.5) concluding a modest formation of BE (0.2% per hour) on a linear pattern and 40% degradation of COCww after 12 h (quadratic decrease). Having in mind the impact that the use of the degradation factors might have on the final calculations, initial COCww concentration was also tested for correction by the use of a degradation factor of 43% obtained through equation (1) described by van Nuijs et al. (2012) and using 24 h as time delay between excretion and sample collection. For BE, the impact on the use of a formation correction factor was considered negligible.

Equation 1

$$Y = 0.1X^2 - 4.6X + 97.2 \quad (R^2 = 0.917)$$

Analysis of COCww and BE concentration results (Table 1) showed a parallel pattern between them, so that, additionally with an observed closeness of COCww concentration values among all samples analyzed, it suggests non deliberate unused drug waste disposal. According to this assumption, for each sample the same value should be obtained for consumed COC (COCww eq and COCEq), back-calculated from COCww and BE concentrations by the use of wastewater flow and, respectively, the pharmacokinetic parameters $f_{e\text{ COC}}$ and $f_{m\text{ COC-BE}}$. Consequently, best estimates for these parameters were obtained through a Microsoft Excel solver algorithm (Table 3) for each sample, forcing the ratio COCww eq / COCEq to unity and constraining $f_{e\text{ COC}}$

to be less than or equal to 9.00% and $f_{m\text{COC-BE}}$ to be higher than or equal to 24.0%. The ratio $\text{COC}_{ww\text{eq}} / \text{CO}_{eq}$ was computed according to equation (2).

Equation 2

$$\frac{\text{COC}_{ww\text{eq}}}{\text{CO}_{eq}} = \frac{\text{COC}_{ww} \cdot f_{m\text{COC-BE}}}{\text{BE} \cdot 1.05 \cdot f_{e\text{COC}}}$$

For uncorrected stability/degradation COC_{ww} concentration values, the values of 8.36% and 24.2% were obtained as means of the best estimates respectively for $f_{e\text{COC}}$ and $f_{m\text{COC-BE}}$, with a mean ratio $\text{COC}_{ww\text{eq}} / \text{CO}_{eq}$ of 1.08. For corrected stability/degradation COC_{ww} concentration values (equation 1), best estimates were the limits of constraints, with a mean ratio $\text{COC}_{ww\text{eq}} / \text{CO}_{eq}$ of 2.32 that plainly deviates from the initial assumption and therefore COC_{ww} correction was discarded.

Thus, the best estimates obtained support the assumption of cocaine coadministration with alcohol and for this reason it was decided also to use in our calculations 9% as $f_{e\text{COC}}$ value and 24% as $f_{m\text{COC-BE}}$ value, the last one in agreement with the back-correction factor of 3.0 that has been proposed by another author (van Nuijs, et al., 2011b).

The parameters BOD and COD were determined in all samples and used for population assessment. Moreover concentration levels for phosphorus and azote have also been determined for some samples. The number of inhabitants (Table 1) is the mean of population determinations based on available parameters data for each sample.

2.6.2 Nicotine

Nicotine derivatives have also been proposed as possible chemical anthropogenic markers for domestic wastewater (Buerge et al., 2008) considering that nicotine, as a constituent of tobacco, is regularly consumed by a large fraction of the world's population. Approximately 5.5-6 trillion cigarettes are produced globally each year by the tobacco industry (WHO, 2002; Eriksen et al., 2012). Considering a typical nicotine content of 0.8 mg/cigarette (Gorrod J, 1993), the annual global consumption of nicotine can be estimated at 5×10^4 tons. Nicotine would thus rank as a high production volume chemical. We will add to this an important constant outflow as it is widely accepted that most tobacco users strictly maintain daily levels of blood concentration of nicotine, adjusting the number of cigarettes or inhaling frequency. (Ashton and Watson, 1970; Murray et al., 1978; Kolonen et al., 1991; Nakazawa et al., 2004).

Nicotine is distilled from burning tobacco and is carried proximally on inhaled tar droplets, reaching alveolar tissue from which about 80-90% is rapidly absorbed. Consumed nicotine is then extensively metabolized by oxidative enzymatic transformations mainly into primary metabolites cotinine (70-80%) and nicotine N'-oxide (4-7%). Cotinine (CT) is afterwards further metabolized into several sub-metabolites, making that only 10-15% of consumed nicotine is excreted as cotinine. Cotinine has been used as an indicator of tobacco exposure (Haufröid et al., 1998) and will be evaluated as such in this study. Nicotine itself was not investigated as this compound may enter into WW influents through cigarette stubs disposal. Typical concentrations of cotinine in a smoker's urine amount have been estimated to be around 1.6 mg/L (Umweltbundesamt, Dessau-Rosslau, 1998). Assuming a mean urine production of 1.5 L/day, the daily cotinine excretion of a smoker can be estimated at about 2.4 mg/person/day (Buerge et al., 2008).

Cotinine concentration (ng/mL) values obtained for each sample were first corrected based on the work of Chang Chen *et al.* (2012) that suggest an increase fraction of CT initial concentration on samples kept at 20 °C according to the linear equation $0.45 \cdot t$, where t represents the time delay between excretion and sample collection, assumed to be 24 h in our case. Initial CT concentration is increased by the breakdown of excreted cotinine-glucuronide conjugates, which represents a fraction of 12-17% of consumed nicotine excreted in this conjugated form (Hukkanen et al., 2005; Benowitz et al., 1994). Results are presented in Table 1.

Table 1
Cocaine and cotinine quantitative data

Date	Population (RSD%)	Flow (m ³ /day)	BE (ng/L) (RSD% ^a)	COC _{ww} (ng/L) (RSD% ^a)	Load BE ^d (g/day)	Load COC _{ww} ^e (g/day)	Initial CT (ng/L) (RSD% ^a)	Corrected CT (ng/L)	Load CT (g/day)	Consumed NICOT (g/day)	Inhaled NICOT (g/day)	Inhaled NICOT (g/day/1000 inh)
11-Oct (Tue)	515613 ^a (15.3)	125120	529 (2.28)	197 (9.83)	66.2	24.6	3493 (2.27)	3153	394	2576	3030	5.8
13-Oct (Thu)	346370 ^c (39.2)	123100	453 (2.30)	178 (3.85)	55.8	21.9	*	--	--	--	--	--
18-Oct (Tue)	405017 ^b (15.0)	126680	340 (0.96)	124 (4.86)	43.1	15.7	2522 (4.55)	2277	288	1883	2215	5.3
20-Oct (Thu)	468257 ^c (5.1)	126770	567 (0.31)	160 (7.29)	71.9	20.3	3504 (6.41)	3163	401	2618	3080	6.6
01-Nov (Tue)	234102 ^b (15.7)	134780	527 (3.35)	180 (2.28)	71.0	24.3	3075 (10.14)	2775	374	2442	2873	12.9
03-Nov (Thu)	358267 ^c (9.2)	222030	203 (1.53)	90 (4.07)	45.1	20.0	1129 (4.71)	1019	226	1477	1737	4.9
10-Nov (Thu)	332271 ^c (11.9)	177090	292 (1.98)	162 (9.31)	51.7	28.7	1833 (1.81)	1654	293	1913	2251	6.8
Mean	379986	147940	416	156	57.8	22.2	2593	2340	329	2151	2531	5.9

Notes: ^a Lost in process; ^bPopulation mean value based on BOD, COD, N and P levels; ^cPopulation mean value based on BOD, COD and N levels; ^dPopulation mean value based on BOD and COD levels BE (benzoylecgonine concentration in wastewater); COC_{ww} (cocaine concentration in wastewater); ^eCalculated as BE (ng/L) x Flow (m³/day) x 10⁴; ^fCalculated as COC_{ww} (ng/L) x Flow (m³/day) x 10⁴ [†] RSD% represents sample's total variability (duplicate extraction and triplicate injections); [‡] RSD% represents sample's total variability (duplicate extraction and duplicate injections)

Table 2
Cocaine quantitative data

Date	COCEq ^a (g/day)	COCEq ^b (g/day)	COCww eq ^c (g/day)	COCww eq ^d (g/day)	COCEq ^e (mg/day/1000 inh)	COCEq ^f (mg/day/1000 inh)	COC _{Doses} ^g Pure doses / day	COC _{Doses} ^h "st" doses / day	COC _{Doses} "st" doses / day / 1000 inh
11-Oct	182.5	289.0	2464.8	273.9	353.9	560.4	2890	8574	17
13-Oct	153.8	243.4	2191.1	243.5	443.9	702.8	2434	7224	21
18-Oct	118.8	188.0	1570.9	174.5	293.2	464.3	1880	5580	14
20-Oct	198.2	313.8	2028.3	225.4	423.2	670.2	3138	9312	20
01-Nov	195.8	310.1	2426.1	269.6	836.6	1324.6	3101	9202	39
03-Nov	124.3	196.8	1998.3	222.0	346.9	549.2	1968	5839	16
08-Nov	--	--	--	--	--	--	--	--	--
10-Nov	142.6	225.7	2868.8	318.8	429.1	679.4	2257	6699	20
Mean	159.4	252.4	2221.2	246.8	381.7 ⁱ	604.4 ⁱ	2524	7490	18 ⁱ

Notes: COCEq calculated considering $f_{m\text{COC-BE}}$ as ^a0.38 and ^b0.24; COCww eq calculated considering $f_{e\text{COC}}$ as ^c0.01 and ^d0.09; ^ebased on COCEq^a; ^fbased on COCEq^b; ^gassuming 100% fraction absorbed and 100 mg per pure dose; ^hadjusted to local "street" purity of 33.7% (2011); ⁱmean value excluding 1st November data

From the corrected concentrations of cotinine (CT) (ng/L) and with the flow rate (L/day) of the wastewater stream, CT loads (g/day) were calculated. These loads were then further back converted into consumed nicotine amount (g/day) using 14.1% as the fraction of consumed nicotine excreted as cotinine form, calculated from original data published by Benowitz (Benowitz et al., 1994) as well as the molecular mass ratio of nicotine to cotinine (0.92). For the calculation of inhaled nicotine, an absorbed fraction value of 85% was taken into account (Hukkanen et al., 2005). Results are presented in Table 1.

Table 3
Microsoft Excel Solver estimates

Date	$f_{m\text{COC-BE}}$	$f_{e\text{COC}}$	COCww eq / COCEq ^a
11-Oct	0.240	0.0853	1.00
13-Oct	0.240	0.0900	1.00
18-Oct	0.240	0.0835	1.00
20-Oct	0.248	0.0667	1.00
01-Nov	0.244	0.0797	1.00
03-Nov	0.240	0.0900	1.13
10-Nov	0.240	0.0900	1.41
Mean	0.242	0.0836	1.08

Notes: ^a Computed according to equation (2) based on uncorrected COCww and on the best estimates of $f_{m\text{COC-BE}}$ and $f_{e\text{COC}}$ for each sample

Table 4
Cocaine quantitative data (Adapted from Mathieu et al., 2011)

Sample	Flow (m ³ /day)	BE (ng/L)	COCww (ng/L)	Load BE (g/day)	Load COCww (g/day)	COCEq ^a (g/day)	COCEq ^b (g/day)	COCww eq ^c (g/day)	COCww eq ^d (g/day)	Ratio COCww eq ^c / COCEq ^a	Ratio COCww eq ^d / COCEq ^b
A	260247	280	114	73	30	201	318	2967	330	14.8	1.04
B	141236	1788	1928	253	272	696	1103	27230	3026	39.1	2.74
E	71885	1040	297	75	21	206	326	2135	237	10.4	0.73
F	120263	350	124	42	15	116	184	1491	166	12.8	0.90
I	57800	954	336	55	19	152	241	1942	216	12.8	0.90
K	114800	694	322	80	37	220	348	3697	411	16.8	1.18
L	77000	853	300	66	23	181	287	2310	257	12.8	0.90
P	95900	774	311	74	30	205	324	2983	331	14.6	1.02
Q	180000	802	406	144	73	398	630	7308	812	18.4	1.29
R	195000	2400	1000	468	195	1290	2043	19500	2167	15.1	1.06
									Mean	16.7	1.18

Notes: COCEq calculated considering $f_{m\text{COC-BE}}$ as ^a0.38 and ^b0.24; COCww eq calculated considering $f_{e\text{COC}}$ as ^c0.01 and ^d0.09

2.6.3 Estimation of Population Size

The assessment of population served by wastewater treatment plants remains one of the major subjects to be improved in order to reduce biases when producing back-calculations for estimating drug use per number of inhabitants. Several approaches have been suggested through the use of anthropogenic markers such as personal care products, pharmaceuticals used in chronic therapies, sweeteners, methadone, caffeine or the concentrations of P (Phosphorus), N (Azote), BOD (Biochemical Oxygen Demand) and COD (Chemical Oxygen Demand). These last parameters have been proposed on drug wastewater studies based on the previous knowledge that one inhabitant is equivalent to 1.7 g/day P, 12.5 g/day N, 59 g/day BOD and 128 g/day COD (Van Nuijs, et al., 2011; Andreottola et al., 1994; Zessner et al., 2005; Garnier et al., 2006).

3. Results and discussion

The sampling process of the influents was made according to the WWTP-Alcântara collection protocol, on a time proportional schedule of 120 minutes. The uncertainty associated with the different models of sampling has been discussed by several authors (Ort, et al., 2010; Ort et al., 2010b; Castiglioni et al., 2013) regarding optimization of the collection step, being the flow-proportional mode the method that should be adopted for best results, as it minimizes pulse drug flows. In our study, sample collection was made from a WWTP retention tank and short-term fluctuations of the studied substances should be largely attenuated by the mixing process in the tank and thus the error attributable to sampling frequency would be less critical as mentioned by some other authors (EMCDDA, 2008).

The validated method was successfully applied to the 8 wastewater samples collected in Alcântara WWTP (Lisbon). After extraction, each sample was analyzed in triplicate for the determination of COC and BE concentrations (ng/L) and in duplicate for CT. The results from one of the collected samples were disregarded and excluded as outliers after Grubbs test evaluation of load COC_{ww} data. One sample was lost in process during CT quantification. For each catchment day, population estimate's uncertainty based on BOD, COD, P and N parameters ranged from 5.1 to 39.2% (RSD) which is in agreement with data from the harmonized study performed in 19 European studies (Thomas et al., 2012), ranging between 7-55% (Castiglioni et al., 2013). For all catchment days, excluding the 1st November corresponding to a national holiday, the population estimates ranged between 380,000 – 400,000. The day to day population estimate's uncertainty is similar with previous work (van Nuijs, Mougél, et al., 2011), where large variations in the amount of served inhabitants were also observed.

For cocaine consumption analysis, in back calculations the values of 9% and 24%, respectively for $f_{e\text{COC}}$ and $f_{m\text{COC-BE}}$ were used. These are likely to be the most realistic estimates for these pharmacokinetic parameters. In order to validate these estimates, COCww and BE concentration data (10 samples) published elsewhere (Mathieu et al., 2011) were used to back calculate COCww eq and COCeq for the same samples, obtained from four different Swiss cities (Table 4). Considering $f_{e\text{COC}}$ and $f_{m\text{COC-BE}}$ respectively as 1% and 38%, a mean value of 16.7 (range 10.4 to 39.1) is obtained for the ratio COCww eq / COC eq, while the use of 9% and 24% for the same pharmacokinetic parameters enables to obtain a more plausible mean value of 1.18 (range 0.73 to 2.74) for the same ratio.

Despite COCeq and COCww eq were calculated, cocaine consumption analysis back calculations based on BE loads are preferred due to a higher waste water stability of this metabolite when compared to COCww. Cocaine consumed amounts (COCeq) were also normalized to population and expressed per thousand inhabitants. Cocaine consumed doses (COC_{Doses}) are expressed as pure drug, assuming an absorbed fraction of 100% and 100 mg per pure dose, and also expressed at “street” (st) purity level (Table 2). In the year of the study (2011) the purity of cocaine at street level in Portugal was 33.7%, according to data provided by IDT (Institute on Drugs and Drug Addiction, Lisbon).

Based on back-calculations assuming cocaine co-administered with alcohol, the mean amount of consumed cocaine in this study was determined as 252 g/day (range 188 to 314 g/day), representing a daily consumption of 2524 “pure” doses (range 1880 to 3101) or 7490 “street” purity level doses (range 5580 to 9312).

When normalized to population, COCeq and COC_{Doses} show a high value for the sample collected on the 1st of November (National holiday), not seen on absolute values, which might be justified by a “concentration” effect due to increase of cocaine consumption. This is a comparable pattern to common weekend consumption profiles found in other studies. Excluding 1st of November data, mean value of consumed cocaine was determined as 604 mg/day/1000 inhabitants, which represents a daily consumption of 18 “street” purity level doses per 1000 inhabitants. Assuming one dose per person, wastewater analysis suggests an estimate of the real abuse “street” cocaine consumption prevalence of 1.8%, which, when compared to the prevalence of cocaine use in Portugal in the last 30 days/12 months (0.3/0.6), according to the last available survey (IDT, 2011), represents a six fold or three fold increase, respectively for the 30 days or 12 months prevalence data.

Based on back-calculations assuming cocaine administered without alcohol and excluding 1st of November data, our work shows a daily mean estimate of Lisbon's consumption of 382 mg per 1000 inhabitants (range 293 to 444 mg/day/1000 inhabitants). When compared with other 19 cities consumption published elsewhere (Thomas et al., 2012), Lisbon's daily consumption is lower than consumption observed in the cities of Milan, Santiago de Compostela, Paris and Brussels (ranging from 511 to 662 mg/day/1000 inhabitants), and higher than consumption observed in the cities of Budweis, Zagreb, Helsinki, Turku, Oslo, Stockholm, and Umeå (ranging from 2 to 146 mg/day/1000 inhabitants). However, it should be emphasized that consumption data on these cities can be 1.58 times higher if it is assumed cocaine intake with alcohol.

Cotinine concentration in wastewater samples ranged from 1.13 µg/L to 3.50 µg/L (mean of 2.59 µg/L), values considered to be similar to the estimates described for Zurich (2.65 µg/L) (Buerge et al., 2008) or Adelaide (Australia) (3.50 µg/L) (Chen et al., 2012). Based on back-calculations of corrected cotinine concentrations, our work enabled to quantify a mean estimate of inhaled nicotine of 2531 g/day (range 1737 to 3080 g/day). When normalized to population, a daily range of 4.85 to 12.9 g/1000 inhabitants is obtained. As for COCeq, population normalized inhaled nicotine showed a high value for the sample collected on the national holiday, not seen on absolute values, which might be justified by a probable increase of nicotine consumption. When excluding 1st of November data, a daily mean estimate of 5.86 g per 1000 inhabitants is obtained. Assuming a mean of 0.8 mg/cigarette (Gorrod J, 1993), this value corresponds to an estimate of 732.5 cigarettes/day/100 inhabitants. In a European survey (EU, 2012) 44% of the Portuguese interviewed claimed to smoke between 11 and 20 cigarettes per day. Therefore nicotine consumption estimate can be calculated in the range of 36.6 to 66.6% of consumers, which is in accordance to the above survey that mentions to Portugal an average of 23% of consumers with major contributors in the ages of 25-39 years (37%) and 15-24 years (31%).

Moreover, in order to assess cocaine consumption based on back calculations from BE concentration data and the recent proposed general value of 29% for $f_{m\text{COC-BE}}$ (Castiglioni et al., 2013), in our work the mean amount of consumed COC can be determined as 209 g/day (range (156 to 260 g/day)), representing a daily consumption of 2089 "pure" doses (range 1556 to 2597) or 6198 "street" purity level doses (range 4618 to 7706). When normalized to population, and excluding 1st November data, the mean value of consumed COC can be determined as 500 mg/day/1000 inhabitants.

A correlation between inhaled nicotine and COCeq was tested by the means of its absolute and population normalized values. Positive relationships were observed between these variables (Fig. 2 and Fig. 3), with R^2 values of 0.854 and 0.980 and linear

equations $\text{COCEq (g/day)} = 7.56 + 0.0973 \times \text{inhaled nicotine (g/day)}$ and $\text{COCEq (g/day/1000 inhab)} = -0.0875 + 0.114 \times \text{inhaled nicotine (g/day/1000 inhab)}$. Analysis of the regression results indicates that the slope parameters are significantly different from zero, with p values of 0.00842 and 0.000156 respectively for these equations. For both equations the 95% confidence interval for the intercept includes zero value. Regardless of the low number of samples used in this study, the linear relationship that seems to exist between cocaine and nicotine consumptions may characterize a certain pattern for the Lisbon population serving Alcântara WWTP but it should be further evaluated.

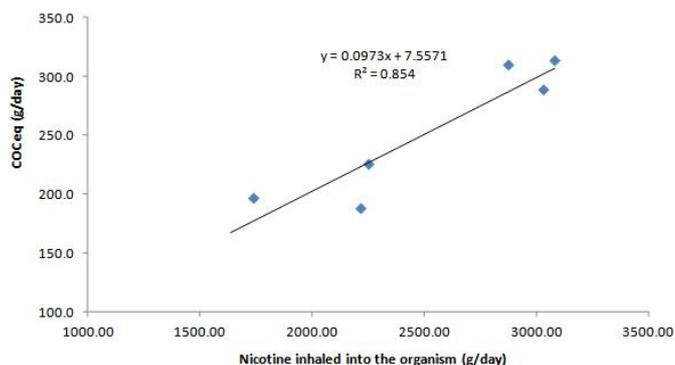


Fig.2 - Plot of consumed COC amount (COCEq) (g/day) vs inhaled nicotine into the organism (g/day).

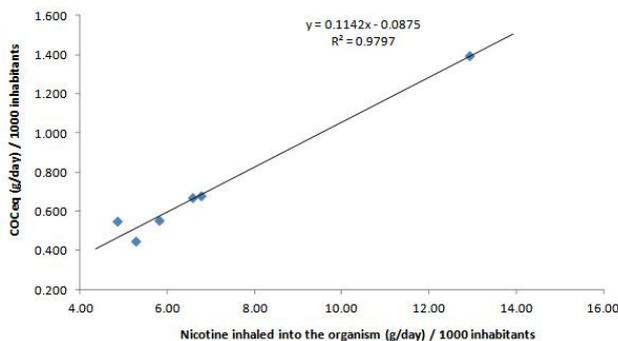


Fig.3 - Plot of consumed COC amount (COCEq) (g/day/1000 inhabitants) vs inhaled nicotine into the organism (g/day/1000 inhabitants)

5. Conclusion

The present project obtained the first sewage forensic estimates (SF/E) in Portugal and confirmed the great potentials of this epidemiological approach in drug abuse assessment. The obtained cocaine and benzoylecgonine concentration results enabled to optimize estimates for the pharmacokinetic parameters to be used on back calculations of cocaine consumption in the population serving the main Lisbon WWTP. Moreover, it is suggested for this community a possible cross abuse index pattern between cocaine and nicotine consumption that should be further evaluated. On a perspective of toxicological and

environmental approach, apart from the levels of cocaine abuse, it is remarkable the toxic nicotine amounts involved on an urban daily base consumption.

Acknowledgements

This work was funded by REDE/1518/REM/2005 for the LC-MS/MS equipment and by funds from former Projecto Vida and Ordem dos Farmacêuticos (Portuguese Pharmaceutical Society).

We wish to thank José Martins and Paulo Coelho from SIMTEJO-Alcântara Wastewater Treatment Plant (Lisbon) for the supply of wastewater samples and analytical control data; and João Goulão and Óscar Duarte from IDT (Institute on Drugs and Drug Addiction, I.P., Lisbon) for the supply of 2011 Portuguese cocaine purity data and Joana Santos from Lisbon Forensic Criminal Laboratory (LPC).

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Lopes A, Silva N, Bronze MR, Ferreira J, Morais J.

Sci Total Environ. 2014 Jul 15;487:673-80. doi: 10.1016/j.scitotenv.2013.10.042. Epub 2013 Nov 5.