



Gracia-Lor, E. et al. (2017) Estimation of caffeine intake from analysis of caffeine metabolites in wastewater. *Science of the Total Environment*, 609, pp. 1582-1588.

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**ESTIMATION OF CAFFEINE INTAKE FROM ANALYSIS OF CAFFEINE  
METABOLITES IN WASTEWATER**

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46

47 **ABSTRACT**

48 Caffeine metabolites in wastewater were investigated as potential biomarkers for  
49 assessing caffeine intake in a population. The main human urinary metabolites of caffeine  
50 were measured in the urban wastewater of ten European cities and the metabolic profiles in  
51 wastewater were compared with the human urinary excretion profile. A good match was  
52 found for 1,7-dimethyluric acid, an exclusive caffeine metabolite, suggesting that might be a  
53 suitable biomarker in wastewater for assessing population-level caffeine consumption. A  
54 correction factor was developed considering the percentage of excretion of this metabolite in  
55 humans, according to published pharmacokinetic studies. Daily caffeine intake estimated  
56 from wastewater analysis was compared with the average daily intake calculated from the  
57 average amount of coffee consumed by country per capita. Good agreement was found in  
58 some cities but further information is needed to standardize this approach. Wastewater  
59 analysis proved useful to providing additional local information on caffeine use.

60

61 **Key words:** Caffeine; 1,7-dimethyluric acid; back-calculation; correction factor; wastewater-  
62 based epidemiology; urinary biomarkers

63

64

65 **1. INTRODUCTION**

66 History suggests that caffeine has been used, in one form or another, since ancient  
67 times. In 2737 BC a Chinese Emperor used the leaves from a nearby bush to prepare a tea  
68 (Arab and Blumberg, 2008; Heckman et al., 2010). An old legend dates the use of coffee to  
69 the 9th century in the southern tip of the Arabian Peninsula when a shepherd noted euphoria  
70 and stimulating effects on his goats caused by eating wild coffee berries. He then decided to  
71 try them himself. Coffee later crossed to Africa and in the 1600s reached Europe becoming,  
72 over the centuries, the most commonly consumed beverage worldwide after water (Butt and  
73 Tauseef, 2011).

74 Caffeine is a naturally occurring alkaloid found in beans, leaves and fruits of more  
75 than 60 plant species. The world's main sources are coffee beans (*Coffea arabica* and *Coffea*  
76 *robusta*) and tea leaves (*Camellia siniensis*). It is also naturally found in kola nuts (*Cola*  
77 *acuminata*), cocoa beans (*Theobroma cacao*), yerba mate (*Ilex paraguariensis*) and guarana  
78 berries (*Paullinia cupana*). Most caffeine is consumed with beverages such as coffee, tea and  
79 soft drinks (including “energy drinks”), while products containing cocoa or chocolate, and  
80 medications such as some analgesic formulations and dietary supplements contribute small  
81 amounts to the diet (Heckman et al., 2010). Total daily intakes vary throughout the world  
82 although coffee usually contributes significantly more than other drinks to overall caffeine  
83 consumption (coffee 71%, soft drinks 16% and tea 12%), particularly among adults  
84 (Heckman et al., 2010; Mitchell et al., 2014). Carbonated Soft drinks are the main source of  
85 caffeine for children (Mitchell et al., 2014).

86 Chocolate contains on average around 1.3% of theobromine, 0.75% of caffeine and  
87 theophylline in small amounts; cola nut between 2 and 3.5% of caffeine, theobromine  
88 (between 1 and 3.5%) and small amounts of theophylline, and tea leaves around 3% of

89 caffeine (theophylline and theobromine in small amounts). This results in around 40-80 mg of  
90 caffeine per cup of tea (150 mL) while caffeine content in cocoa commercial products ranges  
91 from 2 to 7 mg (Barone and Roberts, 1996) and 5-20 mg/100 g in chocolate candy products.  
92 In soft drinks, variable levels of caffeine have been reported depending on the brand but the  
93 typical content is around 40 mg/360 mL (Chou and Bell, 2007). All these products contain  
94 relatively little caffeine compared to the average content of a coffee cup (60-150 mg/150 mL).

95 Caffeine is extensively metabolized by the human liver to form three major  
96 metabolites by demethylation: 3,7-dimethylxanthine (known as theobromine), 1,7-  
97 dimethylxanthine (paraxanthine) and 1,3-dimethylxanthine (theophylline). These are then  
98 broken down further in the liver by additional demethylation and oxidation and are excreted  
99 mostly in the urine (Heckman et al., 2010).

100 While there is no specific recommendation for human caffeine intake, it is considered  
101 that average consumption of approximately 300 mg/day is not associated with adverse health  
102 effects (Fitt et al., 2013; Higdon and Frei, 2006). However, data about caffeine intake in the  
103 population are scarce. Caffeine consumption is usually assessed by dietary surveys, but  
104 getting accurate information in this way presents many limitations. For instance, subjects may  
105 under-report their caffeine intake when food diaries are completed or information is missing  
106 about the strength, brand or amount of caffeine product they have consumed, which may  
107 greatly affect the intake. Another limitation is that in caffeine dietary surveys the subjects are  
108 usually asked about the consumption of certain beverages (mainly coffee and tea) but other  
109 products containing caffeine are not considered: for example, analgesics can contain as much  
110 as 200 mg caffeine per tablet (Derbyshire and Abdula, 2008). Another limitation for  
111 estimating the total caffeine intake is that the caffeine content of various drinks, food and  
112 dietary supplements is only known in some countries such as the USA (Fitt et al., 2013).

113 A complementary method would be to estimate consumption in the general population  
114 by using the levels of caffeine and its metabolites measured in urban wastewater as  
115 biomarkers of intake. This approach, called *wastewater-based epidemiology* (WBE), has been  
116 mainly applied in the last decade for estimating illicit drug consumption (Baker et al., 2014;  
117 Ort et al., 2014; Thomas et al., 2012; Zuccato et al., 2008) and more recently has also been  
118 proposed for the quantitative measurement of lifestyle habits such as tobacco and alcohol use,  
119 exposure to environmental and food contaminants or factors related to health and illness in a  
120 community (Lopes et al., 2014; Reid et al., 2011; Rodríguez-Álvarez et al., 2015; Rousis et  
121 al., 2017; Thomas and Reid, 2011; Yang et al., 2015). The main advantage of WBE is that it  
122 provides objective, up-to-date information about the use of these substances in a population  
123 and can therefore complement current epidemiological methods.

124 In this study, the presence of caffeine and some selected metabolites was assessed in  
125 untreated wastewater in ten European cities. Levels in wastewater were compared with those  
126 measured in urine and with the human excretion profiles of caffeine reported in the literature  
127 in order to correlate the results from the different sources. 1,7-dimethyluric acid, an exclusive  
128 caffeine metabolite, was selected for estimating collective caffeine consumption. The  
129 reliability of this compound for caffeine back-calculation was evaluated by comparing the  
130 amounts measured by wastewater analysis with the average amount of coffee consumed in  
131 each country per capita.

132

## 133 **2. MATERIALS AND METHODS**

### 134 **2.1 Chemicals and reagents**

135 Caffeine (1,3,7-trimethylxanthine), paraxanthine and 1-methylxanthine were purchased  
136 from Sigma Aldrich (St. Louis, MO, USA); 1-methyluric acid, 1,7-dimethyluric acid 7-  
137 methylxanthine were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, California,

138 USA). Standard solutions at 1 mg/mL were prepared in methanol, except for 1-  
139 methylxanthine, 7-methylxanthine, paraxanthine and 1,7-dimethyluric acid which were  
140 prepared in methanol-water (50/50) at pH 8.5-10 (adjusted with 25% ammonia to enhance  
141 solubility). A mix of all compounds at 10 ng/ $\mu$ L was prepared in methanol and then diluted to  
142 1.0, 0.1 and 0.01 ng/ $\mu$ L. Isotopically labeled compounds were caffeine- $^{13}\text{C}_3$  purchased from  
143 Sigma Aldrich and 1,7-dimethyluric acid- $\text{d}_3$  from Santa Cruz Biotechnology. Labeled internal  
144 solutions were prepared separately. Internal standard mixtures with 1 ng/ $\mu$ L of caffeine- $^{13}\text{C}_3$   
145 and 10 ng/ $\mu$ L of 1,7-dimethyluric acid- $\text{d}_3$  were used as surrogates.

146 All solvents were of reagent grade or higher. Methanol for pesticide analysis and  
147 ammonium acetate were from Carlo Erba Reagents (Italy). Ammonium hydroxide solution  
148 (25%) was acquired from Fluka (Buchs, Switzerland). LC-MS grade acetonitrile and  
149 hydrochloric acid (37%) were supplied by Riedel de Haen (Seelze, Germany). Water was  
150 purified using Milli-RO Plus 90 apparatus (Millipore, Molsheim, France). Solid-phase  
151 cartridges (3 mL Oasis HLB, 60 mg) and HPLC XTerra C18 column (3.5  $\mu\text{m}$ , 1 mm  $\times$  100  
152 mm) were obtained from Waters Corp., Milford, MA, USA.

153

## 154 **2.2 Wastewater samples**

155 24-hour composite influent wastewater samples were collected from ten wastewater  
156 treatment plants (WWTP) in different European cities: Bristol (UK), Brussels (Belgium),  
157 Castellón (Spain), Copenhagen (Denmark), Lugano (Switzerland), Milan (Italy), Oslo  
158 (Norway), Porto (Portugal), Utrecht (Netherlands) and Zurich (Switzerland) (**Table S2**).  
159 Samples were collected daily for seven consecutive days in March 2015 and April 2015  
160 (Porto), frozen immediately after collection to prevent degradation of the compounds and sent  
161 to Milan within 24 hours in cooler boxes with dry ice or ice packs to keep them frozen.

162 Samples were stored at -20°C until analysis. For each sample the flow rate of the sewage  
163 stream (L/day) was recorded.

164

### 165 **2.3 Extraction and analysis**

166 Before solid phase extraction, samples were thawed in a warm bath, then filtered to  
167 remove suspended particulate matter through 1.6 µm GF/A glass microfiber filters and 0.45  
168 µm mixed cellulose membrane filters from Whatman (Kent, UK). Then 3 mL of filtered  
169 wastewater were spiked with labeled internal standards (20 ng of caffeine-<sup>13</sup>C<sub>3</sub> and 200 ng  
170 1,7-dimethyluric acid-d<sub>3</sub>) and, if necessary, the pH was adjusted to 6.0-7.5 with 12% ~~HCl~~  
171 (v/v). Samples were loaded on Oasis HLB cartridges (3 mL, 60 mg), previously conditioned  
172 with 6 mL of MeOH and 3 mL of water. Cartridges were vacuum-dried for 10 minutes,  
173 wrapped in aluminum foil and immediately stored at -20 °C. For analysis, cartridges were  
174 eluted with 2 mL of methanol and the extract was evaporated to dryness under a nitrogen  
175 stream. Dry residues were redissolved in 100 µL MeOH-ultrapure water (20:80, v/v),  
176 centrifuged and transferred into glass vials for instrumental analysis. One µL of the final  
177 extract was injected into the liquid chromatography coupled to tandem mass spectrometry  
178 system (LC-MS/MS). The analyses were done by high-performance liquid chromatography  
179 (1200 Series pumps system, Agilent Technologies, CA) coupled to a triple quadrupole mass  
180 spectrometer (AB SCIEX QqQ 5500, Ontario, Canada). Samples were analysed using the  
181 positive electrospray ionization mode. Experimental conditions and detailed analytical  
182 conditions are described in **Table S3** and **S4** and in more detail in Senta et al., 2015.

183

### 184 **2.4. Daily mass loads and back-calculation of consumption**

185           The daily mass loads (g/day) of the selected analytes were calculated multiplying the  
186 measured concentrations of caffeine and metabolites (ng/L) by the daily flow rate of  
187 wastewater (L/day) at the entry of each WWTP.

188           Caffeine consumption was back-calculated using the approach proposed for illicit  
189 drugs by Zuccato et al., 2008. Specific correction factors were developed taking into account  
190 the percentage of urinary excretion of each metabolite and the molar mass ratio of the parent  
191 compound to the metabolite. All the pharmacokinetic studies accessible in the literature which  
192 reported data on the human urinary excretion of caffeine after oral administration (eight in all,  
193 see **Supplemental Information**) were reviewed to develop a specific correction factor for  
194 back-calculating caffeine intake by the population. The mean percentage of excretion of  
195 caffeine and its metabolites was calculated by weighting the number of subjects in each study.  
196 The total uncertainty related to the back-calculation procedure was evaluated as the standard  
197 deviation (SD) of the mean percentage of excretion (**Table 1**). This method had been  
198 previously proposed for refining the correction factors of the most used illicit drugs  
199 (Castiglioni et al., 2013; Gracia-Lor et al., 2016).

200 **Table 1.** Metabolic profiles of caffeine and its main metabolites in human urine (from pharmacokinetic studies and spot urine analysis) and from  
 201 the levels measured in wastewater.

Compound	Mean excretion (%) from pharmacokinetic studies (SD)	Geometric mean from spot urine analysis (95%CI) (2466 subjects) <sup>a</sup>	Mean excretion (%) from wastewater analysis (SD) (70 samples)
caffeine (1,3,7-trimethylxanthine)	1.7 (1.0)	1.81 (1.57-2.08)	20.9 (6.0)
paraxanthine (1,7-dimethylxanthine)	4.6 (1.4)	7.47 (6.73-8.29)	22.1 (4.0)
1-methylxanthine	10.0 (3.4)	17.1 (15.4-19.0)	15.8 (3.5)
7-methylxanthine	3.1 (1.2)	31.4 (28.6-34.3)	24.9 (6.4)
1-methyluric acid	16.5 (6.2)	39.4 (35.8-43.4)	4.7 (1.1)
1,7-dimethyluric acid	6.7 (2.3)	12.2 (11.0-13.6)	11.6 (2.0)
theophylline (1,3-dimethylxanthine)	0.6 (0.4)	0.872 (0.796-0.955)	Not analyzed
theobromine (3,7-dimethylxanthine)	1.5 (1.3)	12.4 (11.4-13.5)	Not analyzed
1,3-dimethyluric acid	1.6 (0.7)	3.51 (3.17-3.89)	Not analyzed
3,7-dimethyluric acid	0.2 (0.4)	0.784 (0.714-0.861)	Not analyzed
3-methylxanthine	2.0 (1.1)	19.2 (17.5-21.0)	Not analyzed

202

203 <sup>a</sup>Data taken from Rybak et al., 2014

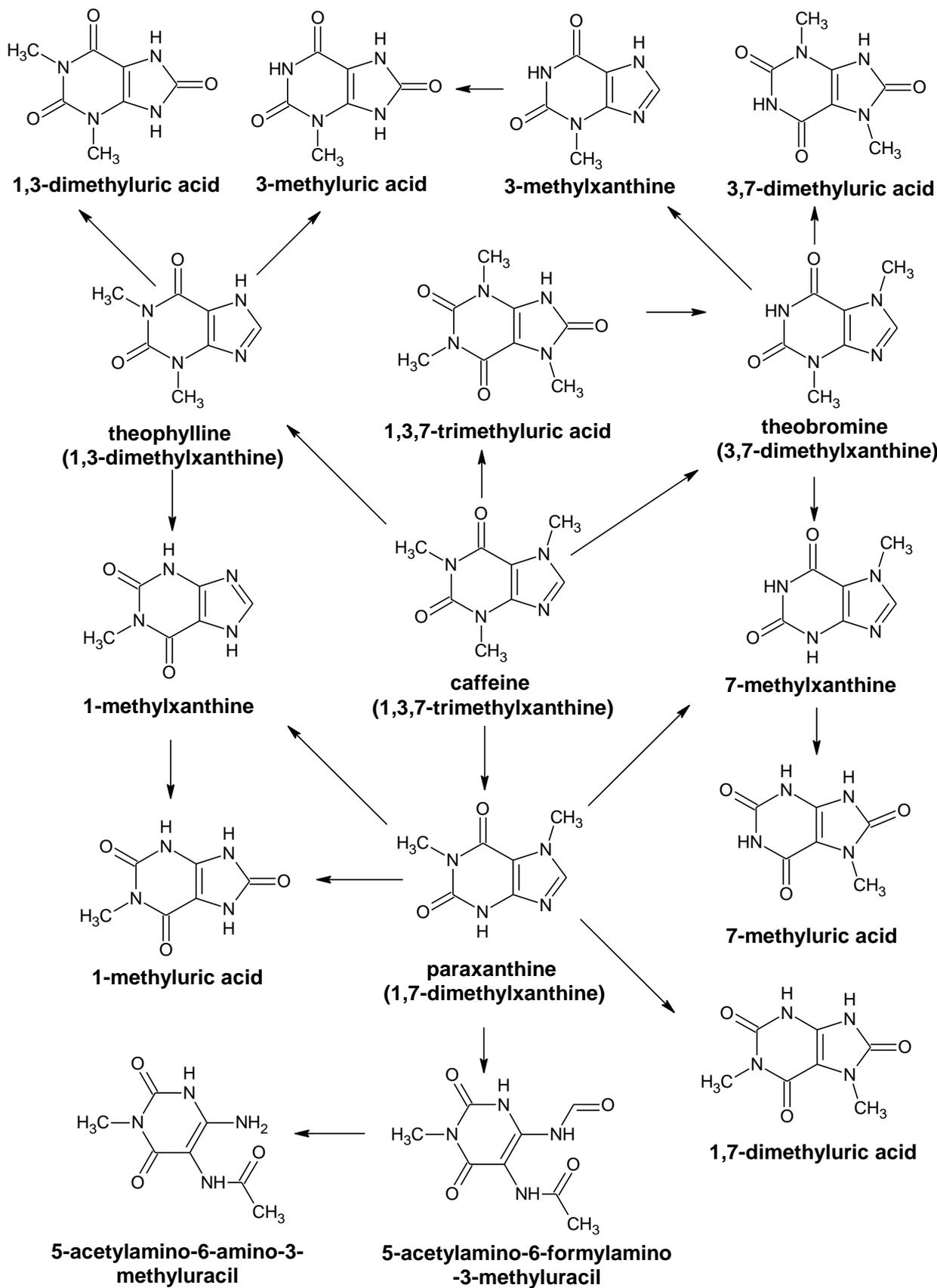
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## 205 3. RESULTS AND DISCUSSION

### 206 3.1 Caffeine biomarkers for back-calculation

207 Selecting a substance as a biomarker is not easy to achieve as it must have specific  
208 characteristics (Gracia-Lor et al., 2016): i) be excreted in measurable quantities in wastewater;  
209 ii) be released to sewers exclusively from human excretion; iii) be unique to human  
210 metabolism to ensure that it comes only from human excretion and not from exogenous  
211 sources; iv) have low adsorption for suspended particulate; v) be stable in wastewater during  
212 in-sewer transport, and during storage and analysis.

213 Each substance for this investigation was tested as a suitable biomarker of caffeine  
214 consumption as described above. Caffeine itself is not a good candidate because it comes not  
215 only from coffee but also from other sources. Caffeine metabolites too may originate from  
216 other naturally occurring alkaloids with similar structures, such as theobromine and  
217 theophylline, which themselves are also caffeine metabolites (**Figure 1**). Theobromine is  
218 present in cocoa beans (and subsequently in chocolate), tea leaves and cola beans.  
219 Theophylline is present in tea leaves in small amounts but is also used medically, for instance  
220 for asthma and other lung diseases (Senchina et al., 2014). Specifically, among five caffeine  
221 metabolites studied, 1-methylxanthine and 1-methyluric acid are also metabolites of  
222 theophylline, while 7-methylxanthine is the major metabolite of theobromine. Paraxanthine  
223 and 1,7-dimethyluric acid however, are exclusively metabolites of caffeine (**Figure 1**). Thus,  
224 they are potentially the most suitable biomarkers to back-calculate the amount of caffeine  
225 consumed, i.e. the consumption of all products containing caffeine (coffee, chocolate, tea,  
226 etc). As they come only from human excretion and not from exogenous sources, their  
227 presence can play an important role in identifying fresh water or ground water contaminated  
228 by sewage.



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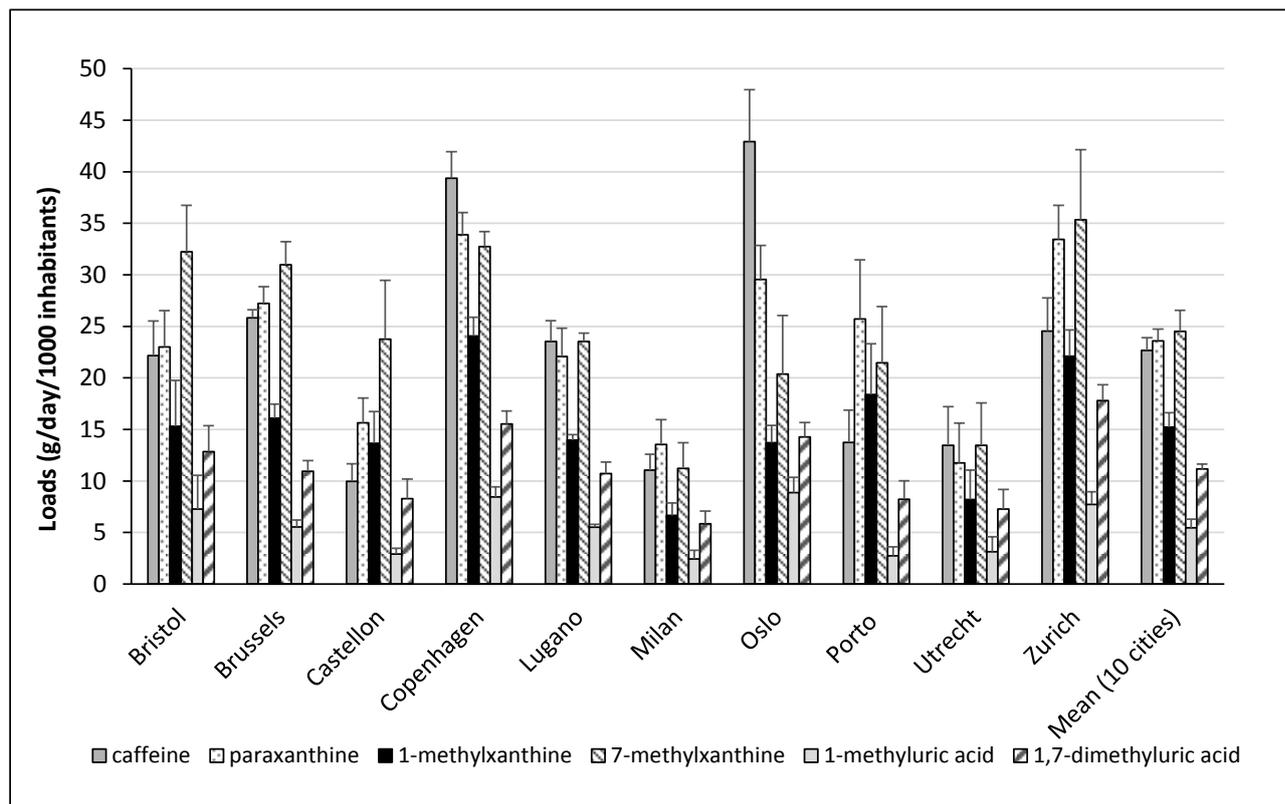
231 **Figure 1.** Metabolic pathway of caffeine in humans

232

### 233 **3.2 Metabolic profiles in wastewater and in human urine**

234 According to the human urinary excretion profile of caffeine, the mass loads of 1-  
235 methyluric acid should be the highest, followed by 1-methylxanthine, 1,7-dimethyluric acid,  
236 paraxanthine, 7-methylxanthine and finally, caffeine (**Table 1**). However, the quantitative  
237 profiles of caffeine and the metabolites calculated from wastewater analysis did not  
238 completely agree with the human excretion profile. The mass loads (mean of the ten cities)  
239 decreased as follows: 7-methylxanthine > paraxanthine > caffeine > 1-methylxanthine > 1,7-  
240 dimethyluric acid > 1-methyluric acid (**Figure 2**). Hence, there are large differences from the  
241 human excretion profile of caffeine. We therefore included supplementary data from spot  
242 urine analysis in our comparison (**Table 1**). These percentages (geometric mean, 95% CI)  
243 were obtained from Rybak et al., 2014, who recently measured caffeine and 14 metabolites in  
244 more than 2000 urine samples. We calculated also the percentages of excretion using the  
245 concentrations measured in wastewater in the ten European cities (**Table 1**). Each metabolite  
246 is reported as a percentage of the sum of the levels of metabolites plus caffeine measured in  
247 wastewater, following the procedure employed by Castiglioni et al., 2011 to calculate the  
248 metabolic profile of cocaine in wastewater and in human urine. The excretion profiles of  
249 caffeine and its metabolites were calculated using median values because of the high  
250 variability of the concentrations.

251



253

254 **Figure 2.** Normalized mass loads (g/day/1000 inhabitants) of caffeine and its metabolites in  
 255 ten European cities in March 2015 and April 2015 (Porto). Means  $\pm$  standard deviation (SD)  
 256 of seven-day samples (only the upper limit of the SD bar is shown).

257 Data from wastewater could be reasonably compared with the profiles in spot urine  
 258 samples, since they indicate respectively the profiles of excretion from an entire community  
 259 and from single individuals. Percentages were comparable for 1-methylxanthine and 7-  
 260 methylxanthine acid in wastewater and spot urine samples, but higher than in  
 261 pharmacokinetic studies (**Table 1**). This can be easily explained by the fact that they are also  
 262 metabolites of theophylline and theobromine respectively. The percentage of caffeine in  
 263 wastewater (21%) was much higher than expected from spot urine analysis and  
 264 pharmacokinetic studies (1.8% and 1.7%). There might therefore be other sources of caffeine  
 265 contributing to the total amount in wastewater (e.g., coffee grounds that are disposed down of  
 266 the sink drain, disposal of coffee that was not drunk or improper disposal of caffeine for  
 267 pharmacological use). In contrast, for 1-methyluric acid the percentage in wastewater was  
 268 lower than in urine and in pharmacokinetic studies. A possible explanation could be  
 269 degradation of this compound in wastewater such as in-sewer, during transport or during  
 270 storage. This should be verified by in-sewer experiments and additional modeling studies.

271 Some differences were observed for paraxanthine (22.1% of the total in wastewater,  
 272 4.6% in pharmacokinetic studies and 7.5% in spot urine samples); however for 1,7-  
 273 dimethyluric acid the results were comparable (approximately 12% of the measured  
 274 concentrations in wastewater and in spot urine samples, and 4.3-12.6% of the administered  
 275 dose in pharmacokinetic studies (see data in SI)). Taking to account of all these  
 276 considerations, 1,7-dimethyluric acid seemed to be the most suitable biomarker for the back-  
 277 calculation of caffeine. The mean percentage of excretion of this metabolite weighted by the  
 278 number of subjects in each study (6.7%) and the 1,7-dimethyluric acid/caffeine molecular  
 279 mass ratio were used to obtain the correction factor (CF), according to the following equation:

$$CF = \frac{Mw_{caffeine} / Mw_{1,7-dimethyluric\ acid}}{Mean\ excretion_{1,7-dimethyluric\ acid}} = \frac{194.08 / 196.06}{0.067} = 14.8$$

281 where  $M_w$  is the molecular weight and the mean excretion is the weighted mean of the  
282 percentage of excretion of the target metabolite.

283

### 284 **3.3 Estimation of caffeine consumption**

285 Using the proposed correction factor, caffeine consumption (in mg/day/person) in  
286 each city was calculated based on the wastewater measurements of 1,7-dimethyluric acid. The  
287 mean daily consumption of caffeine per capita ranged from 263 mg/day/person in Zurich to  
288 87 mg/day/person in Milan (**Table 2**). These data match the mean daily caffeine intake in  
289 Europe of around 300 mg/day/person estimated by the European Food Safety Authority  
290 (means range from 37 to 320 mg/day/person estimated from individual surveys for adults  
291 between 18 and 64 years) (European Food Safety Authority (EFSA), 2015).

292 For a more accurate comparison, we compared our wastewater analysis data to the  
293 amount of coffee consumed per country per capita (per person on average), which reflects the  
294 imports of coffee by each country, according to the International Coffee Organization (ICO)  
295 (International Coffee Organization (ICO), 2015). We converted the per capita consumption  
296 (in kg/person) of coffee to the daily intake of caffeine per person considering that dry coffee  
297 beans contain about 1.1% of caffeine in Arabica and about 2.2% in Robusta coffee. In 2015,  
298 around 60% of the coffee exported was Arabica (“International Coffee Organization,” 2015),  
299 but the proportion can change from country to country. For instance, according to Garattini,  
300 1993, consumer countries can be classified in three levels: (a) where consumption of Arabica  
301 accounts for more than 70% (Switzerland and Northern European countries, i.e. Norway and  
302 Denmark); (b) where consumption of Arabica is around 50% (Italy, the Netherlands, Belgium  
303 and the UK); (c) where consumption of Robusta predominates (Spain and Portugal) (**Table**  
304 **2**). In addition, the amount of caffeine extracted varies with the preparation method, ranging  
305 from 75% in boiled coffee to nearly 100% in filtered coffee. To estimate the amount of

306 caffeine in the coffee we took 1.1% for countries classified in group (a), 1.6% (i.e. mean  
307 caffeine content in Arabica and in Robusta) for countries belonging to group (b) and 2.2% for  
308 countries in group (c). In all cases, we assumed 95% extraction efficiency, as previously  
309 proposed (Fredholm et al., 1999).

310 For four cities (Oslo, Copenhagen, Zurich and Brussels), the difference was 20% or  
311 less. The amounts for Castellón, Utrecht, Milan, Lugano and Porto estimated from wastewater  
312 analysis were lower than indicated by the coffee trade figures, and higher in Bristol. This  
313 might be due to different factors: first of all, we compared data from whole country with data  
314 in a specific city, while population habits might be different. This was the case for Zurich and  
315 Lugano, two Swiss cities: a 20% difference was obtained for Zurich (410,000 inhabitants),  
316 whilst it was around 50% for Lugano (100,000 inhabitants). Secondly, we compared annual  
317 coffee trade figures with caffeine estimated through wastewater analysis in one week. Finally,  
318 data obtained through back-calculation refer to the amount of caffeine consumed in all  
319 products that contain relatively large amounts such as coffee, chocolate, soft drinks and  
320 medications. Thus, larger amounts of caffeine estimated through the wastewater analysis in  
321 Zurich, Copenhagen, and especially in Bristol, might be due to higher consumption of other  
322 products in those countries. Switzerland is in fact the country with the highest per capita  
323 consumption of chocolate, and the UK is also among the countries with the highest  
324 consumption, according to different sources (Statista, 2015; Target Map, 2015)). Another  
325 reason might be the fact that the caffeine content of coffee in the UK is higher than in other  
326 countries (Barone and Roberts, 1996). Furthermore, tea containing around 3% of caffeine is  
327 the most popular drink in the UK today, and contributes to caffeine consumption. In five  
328 cities, the difference was of at least 50%.

329 **Table 2.** Caffeine consumption estimated from wastewater analysis and using coffee trade data for the countries investigated. The difference was  
 330 calculated between the estimates from international statistics and from wastewater analysis.

Cities investigated (country)	Caffeine from wastewater analysis	Caffeine from international statistics*			Difference (%)
	mg caffeine/day/person (SD)	Kg coffee/year/person*	Type of coffee mostly consumed <sup>a</sup>	mg caffeine/day/person	
Bristol (UK)	190 (37)	3.3	50% Arabica-50% Robusta	137	-38
Brussels (Belgium)	162 (15)	4.3	50% Arabica-50% Robusta	179	16
Castellón (Spain)	122 (28)	4.5	Robusta	258	53
Copenhagen (Denmark)	229 (19)	6.9	Arabica	198	-16
Lugano (Switzerland)	97 (16)	7.6	Arabica	218	55
Milan (Italy)	86 (18)	5.6	50% Arabica-50% Robusta	233	63
Oslo (Norway)	211 (21)	8.7	Arabica	249	15
Porto (Portugal)	121 (27)	4.8	Robusta	275	56
Utrecht (The Netherlands)	107 (28)	5.3	50% Arabica-50% Robusta	221	51
Zurich (Switzerland)	263 (23)	7.6	Arabica	218	-20

331

332 \*Source: International Coffee Organization (ICO), 2015 (<http://www.ico.org/coffee-trade-statistics-infographics.asp>)

333 <sup>a</sup>(Garattini, 1993)

334           The aim of the comparison between the amount of caffeine consumed, estimated from  
335 the wastewater analysis, and coffee consumption figures from international trade was mainly  
336 to check whether the proposed metabolite was a suitable biomarker of consumption. The  
337 results indicate that 1,7-dimethyluric acid can be used for this purpose, although additional  
338 studies are needed to validate this approach, including more extensive wastewater sampling  
339 campaigns in different countries.

340           Additional information on the current proportions (percentages) of commercial  
341 varieties of coffee consumed in each country is also needed for more accurate comparisons.  
342 There are some differences between coffee consumption data, in terms of the amount  
343 consumed in each country per capita, published by different sources (for instance, between the  
344 ICO (International Coffee Organization (ICO), 2015) which is based on coffee imports and  
345 exports and Euromonitor International (Caffeine Informer, 2016), which deals with local  
346 business information). This is another factor that may influence the accuracy of a data  
347 comparison.

348           Additionally, only eight studies could be found dealing with the human excretion of  
349 caffeine, so more pharmacological studies are essential to improve the reliability of urinary  
350 excretion profiles and the correction factors used to back-calculate caffeine consumption. At  
351 present, these studies are scarce and most are quite old and based on a small number of  
352 subjects (Gracia-Lor et al., 2016).

353

#### 354 **4. CONCLUSIONS**

355           Profiles of caffeine metabolites in wastewater reasonably matched the profiles in spot  
356 urine samples suggesting that the analysis in wastewater might reflect the collective  
357 consumption of caffeine-containing products.

358           We selected 1,7-dimethyluric acid for caffeine back-calculation because it is an  
359 exclusive human metabolite of caffeine and so it is only produced by consumption of products

360 containing caffeine (i.e. coffee, tea, chocolate, etc.). The percentage of its excretion from  
361 pharmacokinetic studies is similar to the profiles found in urine and in wastewater (estimated  
362 from 70 influent wastewater samples collected in ten European cities). The mean daily  
363 consumption of caffeine per capita, estimated from wastewater analysis using the correction  
364 factor proposed, matched the mean daily caffeine intake (from 37 to 320 mg/day/person  
365 estimated from individual surveys for adults 18-64 years old). In four cities a good correlation  
366 was seen between wastewater analysis and the amount of coffee consumed in the country per  
367 capita. Several factors might explain discrepancies in the other six cities. For instance the  
368 estimation of coffee consumption on the basis of the imports of coffee by each country is  
369 influenced by many uncertainties, so it is hard to estimate the consumption of other  
370 commodities contributing to caffeine intake. Furthermore, the correction factor may be  
371 imprecise due to uncertainties in the metabolism studies in the literature. Thus, new studies  
372 are needed about the metabolism and urinary excretion of caffeine in realistic intake amounts.  
373 Stability tests of biomarkers in sewers are also needed.

374

## 375 **CONTRIBUTIONS**

376 Emma Gracia-Lor, Ettore Zuccato and Sara Castiglioni planned and designed the  
377 study. The collection of the wastewater samples was organized by all authors. Emma Gracia-  
378 Lor analyzed the samples and interpreted the results with the input of Nikolaos I. Rousis and  
379 Sara Castiglioni. Emma Gracia-Lor drafted the manuscript, which was critically revised by all  
380 co-authors. All authors are aware of the content, and accept responsibility, for the manuscript.

381

## 382 **ACKNOWLEDGMENTS**

383 Financial support from the SEWPROF Marie Curie ITN project ‘A new paradigm in  
384 drug use and human health risk assessment: Sewage profiling at the community level’ [grant

385 agreement 317205] supported by the European Union's Seventh Framework Programme for  
386 research, technological development and demonstration and from the COST Action ES1307  
387 “SCORE – Sewage biomarker analysis for community health assessment” is gratefully  
388 acknowledged. Emma Gracia-Lor extends her gratitude to Generalitat Valenciana, Conselleria  
389 d’Educació, Investigació, Cultura i Esport for her postdoctoral contract (APOSTD/2015,  
390 Programa VALi+d). Alexander van Nuijs acknowledges FWO Flanders for financial support.  
391 We are grateful to Christoph Ort for his comments and suggestions and to the personnel from  
392 all WWTPs for their support in wastewater sampling, particularly Francesco Poretti and  
393 Armando Foletti from Consorzio Depurazione Acque Lugano e Dintorni (CDALED) in  
394 Lugano for their kind support.

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