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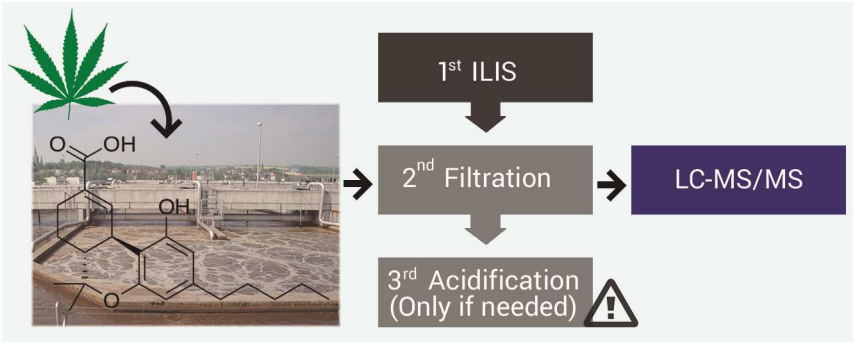

Improving wastewater-based epidemiology to estimate cannabis use: focus on the initial aspects of the analytical procedure

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Abstract

Wastewater-based epidemiology is a promising and complementary tool for estimating drug use by the general population, based on the quantitative analysis of specific human metabolites of illicit drugs in urban wastewater. Cannabis is the most commonly used illicit drug and of high interest for epidemiologists. However, the inclusion of its main human urinary metabolite 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH) in wastewater-based epidemiology has presented several challenges and concentrations seem to depend heavily on environmental factors, sample preparation and analyses, commonly resulting in an underestimation. The aim of the present study is to investigate, identify and diminish the source of bias when analysing THC-COOH in wastewater. Several experiments were performed to individually assess different aspects of THC-COOH determination in wastewater, such as the number of freeze-thaw cycles, filtration, sorption to different container materials and in-sample stability, and the most suitable order of preparatory steps. Results highlighted the filtration step and adjustment of the sample pH as the most critical parameters to take into account when analysing THC-COOH in wastewater. Furthermore, the order of these initial steps of the analytical procedure is crucial. Findings were translated into a recommended best-practice protocol and an inter-laboratory study was organised with eight laboratories that tested the performance of the proposed procedure. Results were found satisfactory with z-scores ≤ 2.

Keywords: drug consumption; carboxy-THC; sewage; sample treatment; wastewater-based epidemiology; proficiency testing

1. Introduction

Drug use has not only a negative impact on health and well-being of individuals and people around them, but also represents a clear threat to the stability and security of entire regions and to economic and social development. Cannabis is the most widely cultivated and trafficked illicit drug, responsible for over 75% of drug seizures in Europe [1]. As the most commonly used illicit drug, it is of great interest from an epidemiological point of view. According to the United Nations Office on Drugs and Crime (UNODC), 3.8% of the global population used cannabis in 2014 [2] and the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) estimated that 13.3% of young adults (15-34) consumed cannabis in the European Union that same year [3]. Although the use of cannabis has remained stable worldwide over the past years, in some regions, particularly North America and Western and Central Europe, its use has recently increased [2]. The development and use of complementary monitoring tools is important to have a more complete understanding of cannabis use and the impact of new cannabis policies.

Estimating community drug use through the chemical analysis of specific human biomarkers in wastewater has demonstrated its potential to become a useful complementary approach to established drug monitoring tools such as epidemiological surveys, treatment demand and law enforcement data. This technique, referred to as wastewater-based epidemiology (WBE), provides near-real-time information on geographically and temporal drug use patterns, particularly relevant against the backdrop of an ever-shifting drug problem. This quantitative approach is well established to estimate the consumption of cocaine, amphetamine, methamphetamine and 3,4-methylenedioxymethamphetamine (MDMA) [3-5]. However, in contrast to these substances, the estimation of cannabis using WBE is problematic [3].

The principal active ingredient of cannabis is Δ^9-tetrahydrocannabinol (THC), but in WBE studies the urinary metabolite of THC, 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH), is used as target biomarker [6]. THC-COOH is specific and, compared to other metabolites, shows high stability over 72 h in wastewater [7, 8]. The metabolism of THC is diverse and extensive, a relatively low percentage of THC is excreted as THC-COOH [3, 6]. One challenge is therefore the need for more research to better understand the excretion percentage of THC-COOH in order to refine back-calculations to estimate THC consumption. This challenge will not be addressed in the present paper. Another challenge is the analytical determination of THC-COOH in wastewater. Some knowledge gaps associated with physical processes were identified, such as its potential to partition on particulate matter [9, 10] and

adsorption onto hydroxyl sites present on the surface of glassware [11]. THC-COOH has different physicochemical properties compared to the other conventional illicit drugs (see Table SI-1). At acidic pH, THC-COOH is present in its non-charged hydrophobic form, which means it may partition to particulate matter, sample containers or filter material, while at neutral pH and the basic pH of natural wastewater the molecule is negatively charged and more hydrophilic. In general, the analytical difficulties and non-instrumental factors have strongly been related to the lower polarity (high lipophilicity) of THC-COOH compared to other illicit drugs when included in multi-residue methods [12-15]. The results of inter-laboratory exercises performed by the Sewage analysis CORe group Europe Network [16] corroborated the difficulties related to the chemical analysis of THC-COOH in wastewater [5]. Although the laboratories involved in those exercises successfully determined THC-COOH in the methanol standards, the recoveries of THC-COOH spiked into wastewater were initially low. This observation suggested that concentrations of THC-COOH in wastewater might be underestimated, probably due to losses during some critical analytical steps.

The present manuscript is a result of studies performed by a working group established within the framework of the pan-European inter-disciplinary network (SCORE), which brings together experts from different disciplines interested in standardizing the WBE approach and in coordinating international studies [17]. The aim of the present work is to investigate and identify the sources of possible bias when analysing THC-COOH in wastewaters and to propose best-practice protocols regarding the initial steps of the analytical procedure. The research is an important step in attempting to provide more accurate estimations of cannabis use through WBE.

2. Materials and methods

This paper describes a study that has been performed by a collaborative group involving 12 institutions, and 10 laboratories. A summary of in-house validated analytical methodologies of each participating laboratory is presented in Table 1 and the full details can be accessed in Table SI-2 (Supplementary Information file). These multi-residue methods were also applied to measure several illicit drugs in wastewater for WBE monitoring studies organized by SCORE [5].

2.1. Reagents and materials

Analytical standards of THC-COOH and its deuterated analogue were prepared starting from certified ampoules, purchased either from Lipomed AG (Arlesheim, Switzerland) or Cerilliant (Round Rock, TX, USA). All laboratories used THC-COOH-d_3 as isotope-labelled internal standard (ILIS), except Lab 9 who used THC-COOH-d_9.

A range of different filter materials with pore sizes ranging from 0.2 to 2.7 µm were tested: glass fibre, regenerated cellulose, mixed cellulose acetate and cellulose nitrate, and polytetrafluoroethylene (PTFE), polyvinylidene fluoride (PVDF) and polyethersulfone (PES) membranes. Filters were supplied by Pall Corporation (Port Washington, NY, USA), Nalgene (Rochester, NY, USA), Phenomenex (Torrance, USA), Whatman (Dassel, Germany), Millipore (Bedford, MA, USA),VWR International (Radnor, PA, USA) and Agilent (California, USA).

The solid-phase extraction (SPE) cartridges used for sample concentration and clean-up were polymer-based: cation exchange mixed mode (Oasis MCX or Strata-XC), or neutral hydrophilic-lipophilic balanced (Oasis HLB). Amino silica-based Strata NH_2 cartridges were used for additional extract clean up by Lab 6. Oasis and Strata cartridges were supplied by Waters (Milford, MA, USA) and Phenomenex (Torrance, USA), respectively (see Table SI-2).

During preliminary tests vials of different materials were tested: glass and polypropylene (PP).

2.2. Analytical methodology

Instrumental analysis was performed with liquid chromatography coupled to mass spectrometry (LC-MS). In all cases, chromatographic separation was performed using reversed-phase LC columns. Eight laboratories used low resolution MS and two used high resolution MS. Electrospray ionization (ESI) was used in all cases, in either positive or negative mode. More

information regarding instrumental parameters can be found in Table SI-2. Statistical analysis of results was performed with GraphPad Prism version 5.01.

2.3. Experimental

Preliminary experiments were set up in order to identify possible sources of bias regarding the sample preservation and treatment. In all experiments, two types of matrices were included: ultrapure water and filtered wastewater (free of solid particles). Samples were spiked at a sufficiently high concentration level (50 ng mL^{-1}) in order to perform analysis without further pre-treatment. The sample pH reduction was recommended as one of the WBE "best practice" requirements [18] to decrease the bacterial degradation and increase the sample stability. However, a study performed by Senta and colleagues [8] indicated enhanced pre-analytical losses of THC-COOH when samples were filtered at pH 2. Therefore, we included pH adjustment as a parameter in our experiments. These preliminary experiments were performed by multiple laboratories in the consortium. Results were evaluated with the recovery, expressed as percentage (%), and defined as the relative response of THC-COOH divided by the deuterated response and compared to t=0. In addition, laboratories were asked to evaluate their instrumental variability (expressed as relative standard deviation, RSD%) by analysing at least 5 replicates over 3 days.

2.3.1. Freeze-thaw cycles

The effect of multiple cycles of freezing and thawing of samples containing THC-COOH was evaluated by spiking 20 mL of matrix at 50 ng mL^{-1} THC-COOH and distributing aliquots of 0.5 mL in 2 mL glass vials. Each vial was exposed to a different number of freeze-thaw cycles: 0, 1, 2, 5, 10 and 20 (n=3 in every case). After all freeze-thaw cycles had been performed, the ILIS was added and the vials were analysed by direct injection into the LC-MS. Three laboratories provided results.

2.3.2. In-sample stability

The in-sample stability of THC-COOH was tested at three temperatures (20 °C, 4 °C and -20 °C) over a period of 7 days, with sampling points at 0, 1, 4, 7 days. The matrix (3 mL) was spiked at 50 ng mL^{-1} of the analyte, homogenized and distributed in 3 vials of 2 mL, and each stored at one of the three temperatures. After the experiments, the ILIS was added to each vial and samples were directly injected into the LC-MS system. Four laboratories provided results.

177 *2.3.3. Filtration*

178 The effect of sample filtration prior to analysis was assessed at natural pH (~7.5) and acidic pH
179 (samples adjusted to pH 2.5). From 20 mL of THC-COOH spiked matrix at 50 ng mL^{-1} level, 1 mL
180 was transferred into a glass vial for direct analysis while the rest was filtered. Different types of
181 filters were used: (1) type GF/A glass microfiber filters + cellulose nitrate and acetate filters, (2)
182 type A/E glass fibre filters + PES membrane filters, (3) type GF/C glass fibre filters, (4)
183 regenerated cellulose filters + PES membrane filters. The filtered aliquots were spiked with ILIS
184 and directly injected into the LC-MS system. The resulting recovery was compared to the non-
185 filtered sample, and the loss during filtration was calculated as follows:

$$1 - ((average\ recovery\ filtered)\ /\ (average\ recovery\ nonfiltered))$$

186 Four laboratories provided results.

187 *2.3.4. Sorption*

188 The potential sorption of THC-COOH to the different container surfaces was investigated by
189 storing 1 mL of matrix spiked with THC-COOH at 50 ng mL^{-1} level in vials of two different
190 materials: glass and polypropylene (PP) (n = 3). The sample pH was considered as a second
191 variable. Therefore, two pHs were investigated: natural pH (7.5) and acidic pH (pH adjusted to
192 2.5). An aliquot was taken after a determined number of days (storage at 4 °C: 0, 1, 4 and 7
193 days), spiked with the ILIS and directly analysed by LC-MS. Three laboratories provided results.

194 *2.3.5. Order of preparatory steps*

195 In addition to the preliminary experiments described above, the order of sample preparation
196 steps, often performed prior to SPE, was evaluated. The steps were: ILIS addition, sample
197 filtration and pH adjustment (acidification). To do so, one wastewater sample spiked at 800 ng
198 L^{-1} was divided into 4 sub-samples. The order of steps for each of the sub-samples was varied.
199 Samples were subsequently extracted and analysed using the validated methodology of the
200 one laboratory (Lab 6) that performed the experiment.

201 *2.3.6. Inter-laboratory study*

202 From the preliminary experiments, a best-practice protocol was derived stating
203 recommendations on the pre-analytical aspects of the analysis of THC-COOH in wastewater
204 (see below). In order to test the performance of this protocol, an inter-laboratory study was
205 organized with eight laboratories.

40 L of wastewater collected at the entrance of the WWTP in Utrecht (The Netherlands) were used as matrix. A stainless steel mixing tank was used to homogenize the bulk by stirring for 30 min at 400 rpm. Homogenized wastewater was distributed in four 5 L glass volumetric flasks. Wastewater test samples were prepared by KWR as followed: Sample 1, non-spiked, at natural pH (7.5); Sample 2, spiked at low level (72 ng L^{-1}), natural pH (7.5); Sample 3, spiked at high level (720 ng L^{-1}), natural pH (7.5); and Sample 4, acidified to pH 2.5 and spiked at high level (720 ng L^{-1}). The low level (72 ng L^{-1}) and high level (720 ng L^{-1}) were prepared by spiking 0.5 mL and 5 mL of a THC-COOH solution of 0.72 mg L^{-1} (in methanol), respectively into the 5 L bottles and filling up with homogenized wastewater. Each of the prepared samples was distributed in 0.5 L PP bottles. Each bottle contained approx. 450 mL of sample. Bottles were stored in a freezer (-25 °C) overnight in order to be shipped frozen the following day to the participants.

218 Table 1. Overview of in-house methods performed by participating laboratories.

Lab #	**Lab 1**	**Lab 2**	**Lab 3**	**Lab 4**	**Lab 5**	**Lab 6**	**Lab 7**	**Lab 8**	**Lab 9** [(1)]	**Lab 10** [(1)]
Sample volume	50 mL	5 mL	100 mL of "sample" (25 mL sample + 75 mL ultrapurewater)	50 mL of supernatant	100 mL	125 mL	100 mL	100 mL	n.a.	n.a.
Particulate removal	Filtration 1.6 µm glass fiber filter	Filtration 0.2 µm RC syringe filter	Dilution	Centrifugation	Filtration (1) Whatman No. 41 filter paper (2) 0.2 µm PTFE syringe filter	Filtration 2.7 µm Whatman, glass fiber filter	Filtration (1) 1.6 µm glass microfiber filter GF/A (2) 0.45 µm mixed cellulose acetate & cellulose nitrate	Filtration (1) 1 µm glass fiber filter A/E (2) 0.2 µm PES membrane filter	Filtration 0.2 µm Whatman PTFE syringe filter Primo 1 mL syringe	Filtration (1) 1.6 µm glass microfiber filter GF/A (2) 0.45 µm mixed cellulose acetate & cellulose nitrate filter
pH at extraction	Natural	Natural	Natural	Natural	Natural	Acid	Acid	Natural	n.a.	n.a.

SPE material	Oasis HLB	Strata-XC	Oasis HLB	Oasis HLB	Oasis HLB	Oasis MCX	Oasis MCX	Oasis HLB	n.a.	n.a.
Analytical instrument [(2)]	LC-QqQ	LC-QqQ	LC-QqQ	LC-QqQ	LC-QqQ	LC-QqQ	LC-QqQ	LC-LTQ-FT-Orbitrap	LC-QqQ	LC-QTOF MS
Ionization mode (ESI)	-	-	+	+	+	-	-	+	+	-
Reference	[19]	Unpublished	[20]	Adaptation from [20]	Unpublished	[10]	Adaptation from [21]	[22]	Adaptation from [23]	Unpublished
Instrumental variability[3] (Intra-day, RSD (%))	6% (n=6)	2% (n=6)	7% (n=6)	3% (n=6)	1% (n=5)	5% (n=6)	4% (n=6)	2% (n=6)	10% (n=5)	8% (n=6)
Instrumental variability[3] (Inter-day, RSD (%))	11% (n=6)	3% (n=6)	7% (n=6)	3% (n=6)	2% (n=5)	7% (n=6)	5% (n=6)	4% (n=3)	6% (n=3)	7% (n=6)

219 [(1)] Labs 9 and 10 did not participate in the interlaboratory study but provided results in preliminary experiments

220 [(2)] QqQ: triple quadrupole; LTQ-FT Orbitrap: linear ion trap-Fourier transform Orbitrap; QTOF: quadrupole-time-of-flight

221 [(3)] Instrumental variability was performed using a standard solution of 50 ng/L in solvent

222 n.a. not applicable

3. Results and discussion

Based on previous inter-laboratory exercises performed by the SCORE consortium [16], the study started from the premise that the instrumental procedures and multi-residue methods of the different laboratories are successful in determining THC-COOH in standard solutions in methanol in the ng mL^{-1} range [17]. Participating laboratories measured THC-COOH in negative- or positive-ESI mode and sample preparation consisted of filtration/dilution/centrifugation and off-line SPE using different types of filters and cartridges (Table 1). Multi-residue methods applied by 3 out of the 8 laboratories consisted in the use of cation exchange mixed mode cartridges for SPE. Although this type of sorbent is most selective towards basic compounds, THC-COOH showed acceptable recovery when interacting with the MCX sorbent through the reversed-phase mechanism [10, 21]. ILIS was used as surrogate in order to ensure the analytical quality of the results. Instrumental variability within the participating labs was < 10% in all cases (Table 1).

3.1. Effect of sample pre-treatment operations

3.1.1. Freeze-thaw cycles

After 20 freeze-thaw cycles, the THC-COOH concentration showed a slight decrease (≤ 10%, RSD = 13%) from the initial concentration (see Figure SI-1.1 for wastewater matrix and SI-1.2 for ultrapure water). However, the variability of the result fell within the level of accepted uncertainty of replicate analyses [18] and, therefore, the decrease was considered not significant.

3.1.2. In-sample stability

The in-sample stability results were calculated relative to day 0 (as the mean recovery of each lab before freezing the sample for the first time) (Figure SI-2.1 for wastewater matrix and SI-2.2 for ultrapure water). THC-COOH remained stable in wastewater up to 7 days at all temperatures tested, with relative recoveries between 80 and 120%.

These results confirm the findings reported by González-Mariño et al. 2012 [21] and Heuett et al. 2015 [24] who reported high stabilities up to 3 and 4 months, respectively when stored at -20 °C. González-Mariño also reported losses of THC-COOH when stored at 4 °C, whereas in our study no significant loss was observed at that temperature. In another study [8] that included pH as a second variable, a lower stability of THC-COOH was observed in the acidified samples

(54% decrease from the original concentration at pH 2) than in the non-acidified samples (10% decrease from the original concentration at pH 7.4) when stored at 4 °C. This result can be explained by the enhanced adsorption of THC-COOH to solid particulate matter observed at pH 2 as compared to natural pH [9].

3.1.3. Filtration

Details on the individual performance of each filter or filter combination at pH 7.5 and pH 2.5 can be accessed in SI (Table SI-3). Results presented in Table SI-3 clearly demonstrate that filtration has a great impact on the THC-COOH recovery, and that it is highly pH dependent. At acidic pH, THC-COOH is not charged and its lipophilicity increases (logD: 5.1 at pH 2.5 vs 2.4 at pH 7; chemicalize.com). In the case of wastewater at natural pH, the small-volume syringe filter of regenerated cellulose (RC) performed the best (no loss during filtration). However, when filtering larger volumes, the loss amounted to 27 – 30% independent of the filter material. In the case of acidified wastewater, results invariably showed losses during filtration > 75%, which is in a good agreement with findings reported by Senta et al. 2014 [8]. As can be seen in Figure 1, the average loss during filtration when sample pH was not adjusted (pH ≈ 7.5) amounted to 20% (RSD = 3%). This impact was even higher when wastewater was acidified to pH 2.5 and the loss amounted to 90% (RSD = 1%). Means differed significantly (paired t-test, p-value = 7e-4).

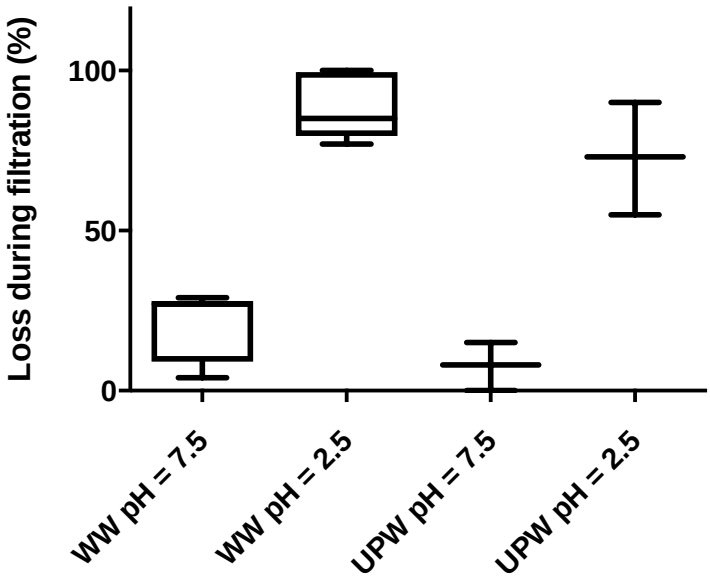


Figure 1. Losses of THC-COOH during filtration and influence of matrix (WW = wastewater, UPW = ultrapure water) and different sample pH. The data are presented as box plots of

277 grouped results (WW = 4 laboratories, 5 different filter types tested, 3 replicates each; UPW =
278 3 laboratories, 3 different filter types tested, 3 replicates each) and expressed as percentage of
279 the average recovery of the filtered versus the non-filtered sample. Boxes represent the mean,
280 25% and 75% percentile values and the whiskers extend to the minimum and maximum values.
281
282 *3.1.4. Sorption*

283 Results from the sorption experiments are shown in Figure SI-3 (.1 for wastewater matrix and
284 .2 for ultrapure water). Sample pH appears to be a more important parameter than the type
285 of sample container (glass or PP) used. Losses due to sorption to container walls occur more
286 rapidly and to a higher extent at pH=2.5, as the compound is in its non-charged hydrophobic
287 form.
288
289 Altogether, the results from filtration, in-sample stability and sorption tests have identified pH
290 as the variable having the most significant impact on the recovery of THC-COOH. This
291 corroborated that, given the specific physico-chemical properties of THC-COOH, its behaviour
292 is highly dependent on wastewater pH.
293
294 *3.1.5. Order of preparatory steps*

295 The order of sample preparation steps was evaluated by comparing the recovery obtained in
296 each case. These preparatory steps are performed prior to SPE and employed to prevent the
297 SPE material from clogging [22] or to prevent and correct for in-sample degradation effects as
298 well as matrix effects (i.e. ILIS addition). They are frequently applied when a multi-residue
299 analysis is foreseen [8]. The results for these experiments were in agreement with those
300 assessed in the previous sections.

301 The conclusion is that sample acidification, if required by the selected enrichment protocols,
302 should be performed only after the sample filtration. Ideally, ILIS should be added before
303 filtration to correct for any potential loss. The results of the preliminary experiments
304 highlighted the influence of pH and the importance of the correct execution order of sample
305 preparation steps before SPE, with sample acidification being critical. When consulting the
306 SCORE inter-laboratory exercise participant laboratories [17], only 5% had performed their
307 analysis using the order of steps identified as the optimal one in this study: 1st ILIS addition 2nd
308 filtration 3rd pH adjustment (only if needed). Therefore, it was decided to perform an inter-

309 laboratory study within the group in order to confirm this hypothesis before making any
310 recommendation.

311 *3.2. Inter-laboratory study*

312 An inter-laboratory study was performed using the optimal approach identified in the
313 preliminary experiments described above. Four samples were prepared as described in section
314 2.3.6 and shipped frozen to each participant. All samples were received within 24 h in frozen
315 conditions. Each laboratory was asked to analyse three independent replicates and report THC-
316 COOH concentrations in ng L^{-1} for each sample. The resulting data was tested for homogeneity,
317 the presence of outliers and normality distribution, and z-scores were calculated in order to
318 measure the performance of each laboratory with regard to the group average.

319 First, the homogeneity of the variances was tested to confirm the correct data comparison
320 (Cochran test). Results showed that the variance for samples 1, 2 and 4 for laboratory 8 was
321 too high (C = 0.738 (sample 1), 0.696 (sample 2), 0.830 (sample 4) > 0.561), therefore those
322 data were removed from the following evaluation. The remaining data set was evaluated for
323 outliers (Grubbs, α=0.05) and the Shapiro-Wilk normality test (α=0.05) was applied to
324 determine if the results derived from a normal distribution. All samples passed with following
325 p-values: sample 1, 0.22 (n=7); sample 2, 0.26 (n=7); sample 3, 0.34 (n=8); sample 4, 0.29
326 (n=6).

327 The group's mean average concentration and relative standard deviation per sample was
328 calculated (see Table 2), following the ISO guidelines [25]. For more details, Table SI-4 shows
329 the mean concentration and standard deviation per laboratory and per sample. Results
330 showed good repeatability (< 10 %) within laboratories, and reproducibility (≈ 30 %, calculated
331 as the RSD for the mean dispersion), except for sample 4. The reproducibility for samples 1 to
332 3 is comparable to other inter-laboratory tests [26]. In contrast, the reproducibility for sample
333 4 was much worse (50%, initially 110 % due to the outlier), due to the issues described in
334 previous sections.

335 Z-scores were calculated to help in the identification of random or systematic errors. To do so,
336 the difference between each individual lab's mean (m) and the group's mean (M) was
337 subtracted, and then divided by the group's standard deviation. This computation provides a
338 value that can be either positive or negative (when the mean is above or below the group's
339 average, respectively), as a measure of the accuracy of each laboratory. The accepted cut-off

value is z-score ≤ |3|, whilst a value between 2 and 3 is considered questionable, in accordance with the IUPAC [27] terminology. Graphical results are presented in Figure 2.

Z-scores were in general consistently positive or negative for each of the laboratories, which might indicate some type of systematic bias, but within the acceptance criteria. Certain laboratories seemed to be grouped systematically in the lower or higher end, however these groupings appear to be independent of extraction and analysis procedures. Laboratory 8 showed high results for all samples, particularly for samples 1, 2 and 4, as commented above. However, an unambiguous explanation could not be found for this performance.

Recoveries of THC-COOH, defined as the difference between the group's mean for the spiked samples subtracted by the blank sample (see Table 2), were satisfactory (64-112%), with good accuracy from the participating labs for samples 2 and 3, confirming the correct use of the recommended protocol. The mean recovery (52%) observed for the acidified sample 4 demonstrated the negative influence that acidification of the sample may have on recovery.

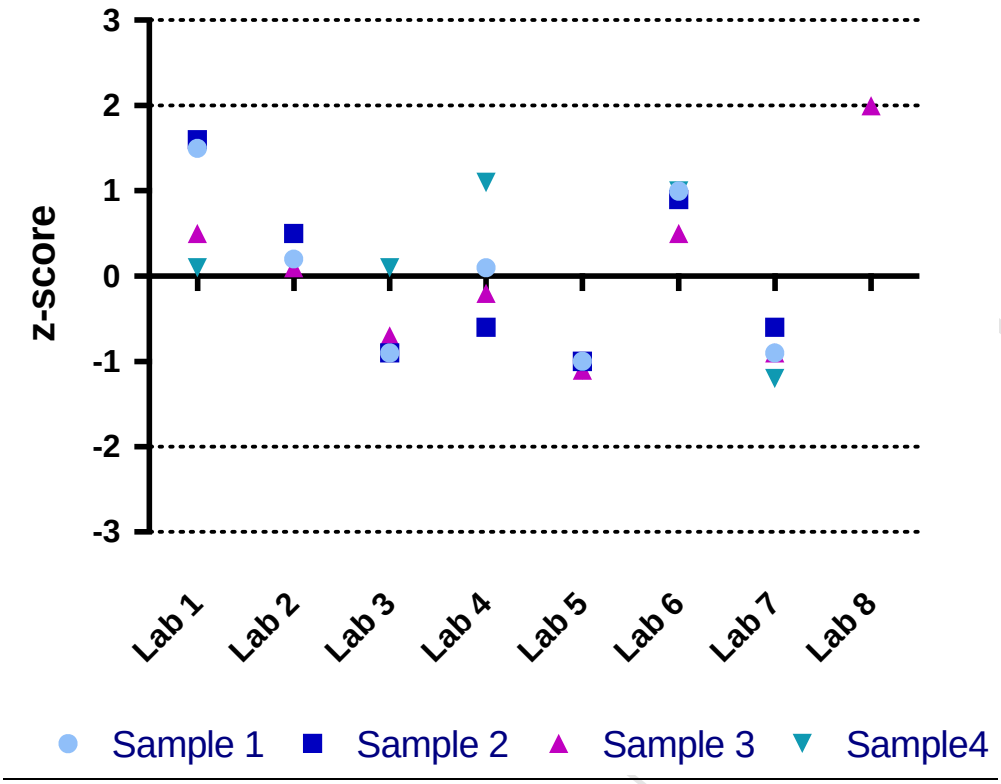
Table 2. Group's mean (M) per sample expressed in ng L^{-1}, Recovery (R) expressed in absolute value (ng L^{-1}) and percentage (%), and group's relative standard deviation (RSD%) in the inter-laboratory study.

	M	**R**	**RSD (%)**	**n**
Sample 1 – WW blank	814 [b]	-	28% [b]	7 [b]
Sample 2 – WW blank + 72 ng L^{-1}	860 [b]	46 (64%)	27% [b]	7 [b]
Sample 3 – WW blank + 720 ng L^{-1}	1527	807 (112%)	34%	8
Sample 4[a] – WW blank acidified + 720 ng L^{-1}	442 [b]	-372 (-52%)	50% [b]	6 [b]

[a] Modified order of analytical steps, the sample was acidified at KWR before being shipped frozen to the laboratories.

[b] after removal of laboratory 8 data

R = sample x (x=2,3,4) – sample 1 (WW blank)



363

364 Figure 2. Inter-laboratory study z-scores per laboratory and sample, calculated as the
365 difference between each individual lab's mean (m) and the group's mean (M) divided by the
366 group's standard deviation.

367 *4. Conclusions*

368 The estimation of cannabis use through wastewater analysis is of high interest. Previous
369 studies have identified several important knowledge gaps as well as analytical challenges. This
370 means that previously published results should be considered with care, as results could have
371 been underestimated.

372 The results obtained in the current study can be used to define the way forward towards more
373 accurate determination of THC-COOH in wastewater. The adjustment of pH has been identified
374 as a critical step in sample processing. If necessary, samples should be acidified after filtration
375 and only after the ILIS have been added to correct for possible losses. Although the results
376 among all labs varied by approximately 30% and therefore higher than optimal, the proposed
377 protocol was successfully tested, and can, therefore, be recommended for future WBE
378 applications.

379 Studies regarding THC-COOH sorption to biofilms and solid particles during in-sewer transport
380 would be needed (i) to further reduce uncertainties, as they have already been done for other

illicit substances [7, 8, 23, 28, 29], as well as (ii) to better understand the cannabis excretion profile in order to achieve a more accurate back-calculation of its consumption.

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- Improvement in the estimation of cannabis consumption through wastewater analysis
- The order of sample treatment steps is crucial for the determination of THC-COOH
- Acidification of the wastewater samples should be avoided
- Results of inter-laboratory exercise support the recommended protocol

Supplementary information

Improving wastewater-based epidemiology to estimate cannabis use: focus on the initial aspects of the analytical procedure

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15 pages

4 tables

6 figures

8 references

Table SI-1. Physico-chemical properties of some illicit drugs and metabolites.

Compound	Formula	pK_a		LogP	
		Experimental	Calculated	Experimental	Calculated
Amphetamine[1]	C9H13N	10.1	9.9	1.8	1.8
Methamphetamine[1]	C10H15N	10.1	10.4	2.1	2.2
MDMA[1]	C11H15NO2	9.4	10.3	n.a.	2.1
Cocaine[1]	C17H21NO4	8.6	8.9	2.3	2.3
Benzoylecgonine[1]	C16H19NO4	n.a.	10.8, 3.3	−1.3	2.3
THC[2]	C21H30O2	n.a.	9.3	n.a.	5.9
THC-COOH[2]	C21H28O4	n.a.	4.2	n.a.	5.1

[1] Baker et al. 2011 [1]

[2] ChemAxon software-calculated values [2]

n.a. not available

41 **Table SI-2**. Full details of the analytical methodology used by each participant laboratory: sample treatment, LC conditions, MS parameters

Lab #	**Lab 1**	**Lab 2**	**Lab 3**	**Lab 4**	**Lab 5**	**Lab 6**	**Lab 7**	**Lab 8**	**Lab 9** [(1)]	**Lab 10** [(1)]
ILIS	THC-COOH-d_3	THC-COOH-d_3	THC-COOH-d_3	THC-COOH-d_3	THC-COOH-d_3	THC-COOH-d_3	THC-COOH-d_3	THC-COOH-d_3	THC-COOH-d_9	THC-COOH-d_3
Filtering material	1.6 µm Glass fiber filter	0.2 µm RC filter (syringe)	No filtering Sample diluted 4x	No filtering. Sample centrifuged at 3000 rpm for 10 min and the supernatant used for analysis	(1) Whatman No. 41 filter paper (2) 0.2 µm PTFE syringe filter	2.7 µm Whatman, glass-fiber filter	(1) 1.6 µm glass microfiber filter GF/A (2) 0.45 µm mixed cellulose acetate & cellulose nitrate	(1) 1 µm glass fiber filter A/E (2) 0.2 µm PES membrane filter	0.2 µm Whatman PTFE syringe filter Primo 1 mL syringe	(1) 1.6 µm glass microfiber filter GF/A (2) 0.45 µm mixed cellulose acetate & cellulose nitrate filter
pH at extraction	Natural	Natural	Natural , except the acidified sample (pH 3-4)	Natural	Natural	Acid (pH=2)	Acid (pH 4.5)	Natural	n.a.	n.a.
SPE material	Oasis HLB	Phenomenex Strata-XC (3cc, 60mg)	Oasis HLB (3cc, 60mg)	Oasis HLB (3cc, 60mg)	Oasis HLB (6cc, 200 mg)	Oasis MCX (6cc, 150mg), extra clean up with Strata NH_2 (3cc, 200mg)	Oasis MCX (6cc, 150 mg)	Oasis HLB (6cc, 150 mg)	n.a.	n.a.
SPE protocol: Conditining	MeOH + ultrapure water	MeOH + 25mM $NH_4CH_3CO_2$	MeOH + ultrapure water	MeOH + ultrapure water	MeOH + ultrapure water	MeOH + ultrapure water + 25mM H_3PO_4	MeOH 5% NH_4OH + ultrapure water (pH 4.5)	MeOH + ultrapure water	n.a.	n.a.

SPE protocol: Sample load	50 mL sample	5 mL sample	100 mL of "sample" (25mL sample + 75mL ultrapurewater)	50ml of supernatant	100 mL of sample	125mL of sample	100 mL sample adjusted at pH 4.5	100 mL of sample	n.a.	n.a.
SPE protocol: Wash	no	85/15water/acetonitrile	no	ultrapure water	ultrapure water + 50% MeOH	ultrapure water	ultrapure water pH 4.5	ultrapure water	n.a.	n.a.
SPE protocol: Drying	yes	yes	yes	yes	yes	yes	yes	yes	n.a.	n.a.
SPE protocol: Elution	8 mL MeOH	2mL MeOH + 2 mL 85/15 ethyl acetate/isopropyl alcohol	5mL MeOH	5ml MeOH	MeOH	6mL MeOH	4 mL MeOH 5% NH4OH	8mL MeOH	n.a.	n.a.
SPE protocol: Extra clean-up	no	no	no	no	no	Conditionin: 1% HCOOH in MeOH Loading: MCX extract (MeOH) acidified with 60µL HCOOH) Additional elution: 4mL 1% HCOOH in MeOH	no	no	no	no

42

SPE protocol: Evaporation	to dryness, at 35 °C	to dryness, at 40 °C	to dryness, at 35 °C	to dryness, at 40 °C	to ~ 0,5 mL, at 40 °C.	to dryness, at 40 °C	to ~ 0,5 mL	to 250 µL, addition of 250 µL of ultrapure water, second evaportation to 250 µL.	n.a.	n.a.
SPE protocol: Reconstitution	100 µL ACN + 100 µL 5 mM NH_4CH_3CO in ultrapure water	1000 µL 5mM NH_4HCO_2	100 µL MeOH + 900uL H_2O	1mL MeOH and diluted 1/10 with MeOH due to matrix effects	1 mL with MeOH	500 µL H_2O:MeOH= 8:2 with addition of 0.1% acetic acid	1 mL with MeOH	0.5 mL water:MeOH , 90:10	n.a.	n.a.
Time between samples received and analysis	Samples received on 6/9/16; stored at -20 °C until analysis on 22/9/16	Samples received on 7/9/16; stored at -20°C until analysis on 22/9/16	Samples received on 6/9/16; stored at -20°C until analysis on 16/9/16	Samples received on 6/9/16; stored at -20°C until analysis on 11/10/16	Samples received on 7/9/16; stored at 4°C until analysis on 23/9/16	Samples received on 6/9/16; stored at -20 °C until analysis on 26/9/2016.	Samples received on 7/9/16; stored at -20°C until analysis on 22/9/16	Samples received on 6/9/ 16; stored at -20°C until analysis on 18/11/16	n.a.	n.a.
Analytical instrument	Agilent 6410 (QqQ)	Agilent 1260 LC with a 6460 triple quad ms (QqQ)	Waters Xevo triplequad (QqQ)	Waters Xevo TQS Micro (QqQ)	Sciex Triple Quad 6500+ LC-MS/MS System (QqQ)	ThermoTSQ Quantum AM (QqQ)	Varian LC - Varian 320-MS (QqQ)	LTQ-FT-Orbitrap (Thermo Electron, Bremen, Germany)	Applied Biosystems 5500 QTrap linear ion trap triple quadrupole mass spectrometer (Sciex, Darmstadt/Germany)	Agilent LC – Agilent 6550 iFunnel Q-TOF

									(QqQ)	
Mobile phase composition	A: Ultrapure water 5 mM ammonium acetate; B: Acetonitrile	A: Ultrapure water 5 mM ammonium acetate; B: Methanol	A: Ultrapure water 5 mM ammonium acetate + 0.01% formic acid; B: MeOH	A: Ultrapure water 5 mM ammonium acetate + 0.01% formic acid; B: MeOH	A: Ultrapure water 5 mM ammonium formate with 0.01 % formic acid; B: Acetonitrile 0.01 % formic acid	A: Ultrpure water 0.1 % acetic acid; B: MeOH 0.1 % acetic acid	A: Ultrapure water 5 mM ammonium acetate; B: MeOH 5 mM ammonium acetate	A: Ultrapure water 0.05 % formic acid; B: MeOH 0.05 % formic acid	A: Ultrapure water 5 mM ammonium formate buffer at pH 3; B: MeOH 0.5% of a 1 M ammonium formate	A: Ultrapure water 5 mM NH_4HCO_2; B: Acetonitrile
Ionization mode	negative	negative	positive	positive	positive	negative	negative	positive	positive	negative
Transitions	THC-COOH Quantifier: 343>299 Qualifier: 343>245	THC-COOH Quantifier: 343>299 Qualifier: 343>245 THC-COOH-d_3 Quantifier: 346>302 Qualifier: 346>248	THC-COOH Quantifier: 345 >193 Qualifier: 345 > 299	THC-COOH Quantifier: 345.3 >299.2 Qualifier: 345.3 > 327.3 THC-COOH-d_3 Quantifier: 346.1>302.1	THC-COOH Quantifier: 345.2 >193.2 Qualifier: 345.2 > 299.2 THC-COOH-d_3 Quantifier: 348.2>302.2	THC-COOH Quantifier: 343 > 245 Qualifier: 343 > 299 THC-COOH-d_3 Quantifier: 346 > 248	THC-COOH Quantifier: 343.2 > 299 Qualifier: 343.2 > 245 THC-COOH-d_3 Quantifier: 346.2 > 302	THC-COOH $[M+H]^+$ 345.2060 qualifiers: 345 > 327 THC-COOH-d_3 $[M+H]^+$ 348.2249	THC-COOH Quantifier: 345.1 > 299.2 Qualifier: 345.1 > 193.1 THC-COOH-d_9 Quantifier: 354.1 > 336.2	THC-COOH $[M+H]^+$ 343.1915 Quantifier: 299.2017 Qualifiers: 245.1547 191.1078 325,1809 THC-COOH-d3 $[M+H]^+$ 346.2103 Quantifier: 302.2205 Qualifiers: 248.1735 194.1266
Reference	[3]	Unpublished	[4]	Adaptation from [4]	Unpublished	[5]	Adaptation from [6]	[7]	Adaptation from [8]	Unpublished
Instrument	6% (n=6)	2% (n=6)	7% (n=6)	3% (n=6)	1% (n=5)	5% (n=6)	4% (n=6)	2% (n=6)	10% (n=5)	8% (n=6)

al variability[3] (Intra-day, RSD (%))										
Instrumental variability[3] (Inter-day, RSD (%))	11% (n=6)	3% (n=6)	7% (n=6)	3% (n=6)	2% (n=5)	7% (n=6)	5% (n=6)	4% (n=3)	6% (n=3)	7% (n=6)

43 [(1)] Labs 9 and 10 did not participate in the interlaboratory study but provided results in preliminary experiments

44 [(2)] QqQ: triple quadrupole; LTQ-FT Orbitrap: linear ion trap-Fourier transform Orbitrap; QTOF: quadrupole-time-of-flight

45 [(3)] Instrumental variability was performed using a standard solution of 50 ng/L in solvent

46 n.a. not applicable

47

Table SI-3. Loss during filtration (expressed as %) with standard deviation (n=3)

	Wastewater				Ultrapure water			
Filter material	**pH = 7.5**	**sd (n=3)**	**pH = 2.5**	**sd (n=3)**	**pH = 7.5**	**sd (n=3)**	**pH = 2.5**	**sd (n=3)**
Glass fibre + PES	27	0.1	100	0.1	-8	0,1	73	0,2
Glass fibre+ cellulose nitrate and acetate	30	0.6	82	0.1	15	0,3	90	0,1
Glass fibre (45 mm)	27	0.2	77	0.1	8	0,2	55	0,1
RC (syringe filter)	4	0.03	85	0.1	-		-	
PES syringe (syringe filter)	14	0.04	99	0.2	-		-	

Table SI-4. Mean (m) of replicates (expressed in ng L^{-1}) and standard deviation (sd) (n=3) per sample and participant laboratory in the inter-laboratory study.

		Lab 1	**Lab 2**	**Lab 3**	**Lab 4**	**Lab 5**	**Lab 6**	**Lab 7**	**Lab 8**
Sample 1	m	1158	860	604	848	588	1040	602	1210
	sd	94	92	11	10	13	81	97	308
Sample 2	m	1226	983	665	727	629	1055	732	1434
	sd	15	65	22	67	20	51	137	267
Sample 3	m	1762	1580	1148	1413	975[a]	1759	1043	2540
	sd	56	79	32	19	-	103	95	133
Sample 4	m	458	N/D	472	695	193	663	174	2532
	sd	77	-	111	74	7	82	25	390

N/D: non detected (below LOD)

[a] n=1

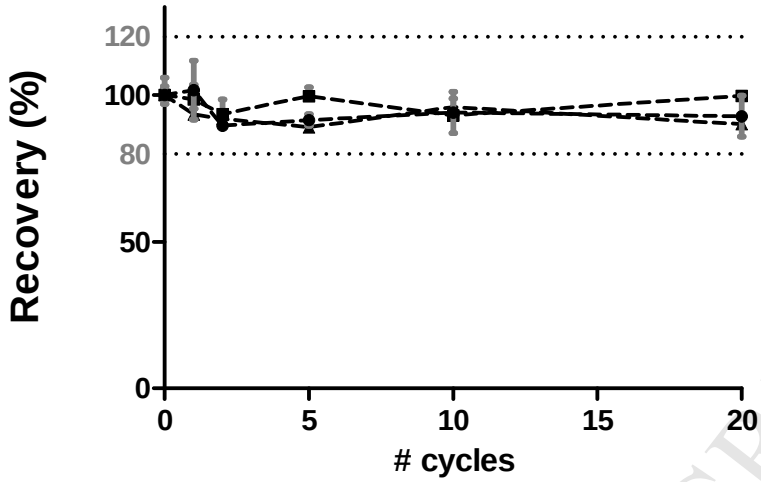


Figure SI-1.1. Average THC-COOH recovery (in wastewater matrix) after n cycles of freezing-thawing relative to the 1st cycle. Error bars represent the standard deviation (n=3). Dotted lines at y=80 and 120%. Legend: Lab 1: circle ●; Lab 2: square ■; Lab 3: triangle ▲.

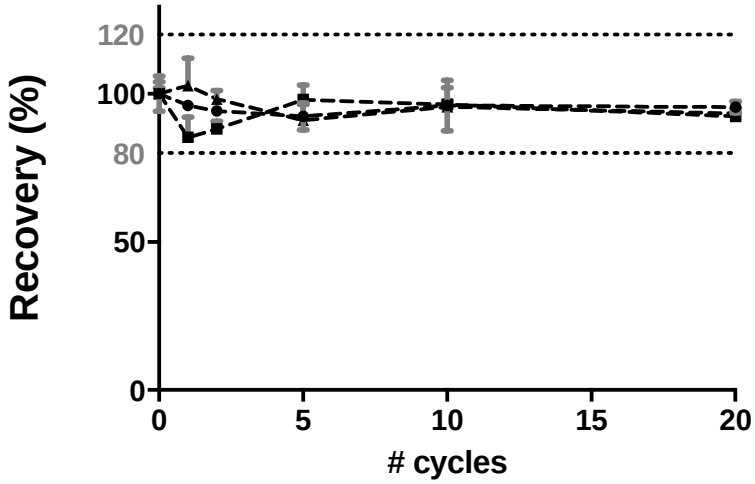
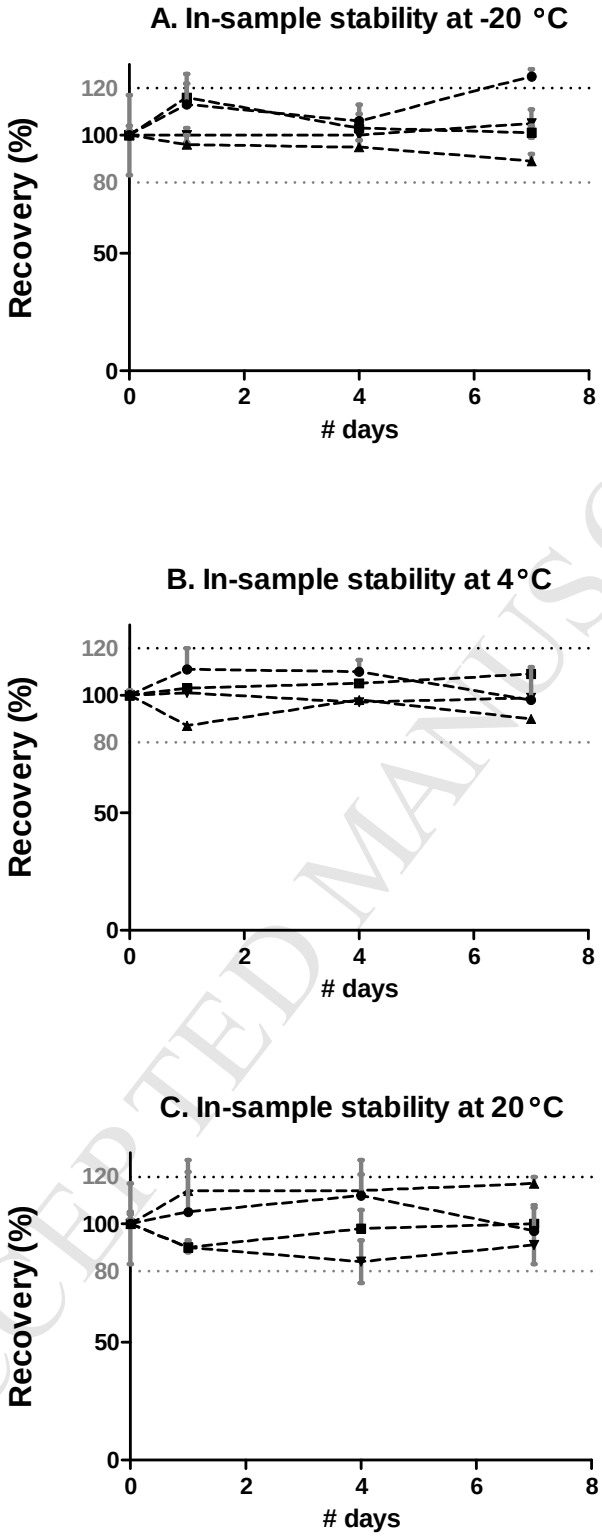


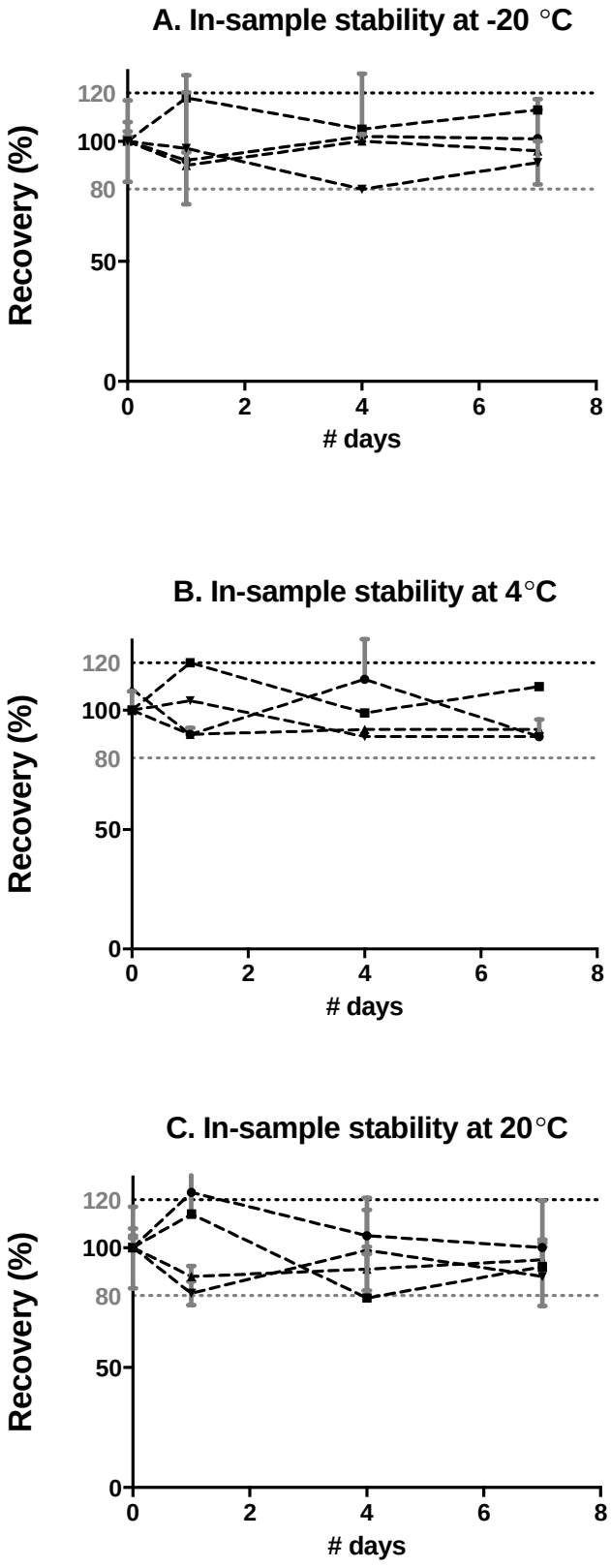
Figure SI-1.2. Average THC-COOH recovery (in ultrapure water matrix) after n cycles of freezing-thawing relative to the 1st cycle. Error bars represent the standard deviation (n=3). Dotted lines at y=80 and 120%. Legend: Lab 1: circle ●; Lab 2: square ■; Lab 3: triangle ▲.



66

67 **Figure SI-2.1**. Stability of THC-COOH in wastewater stored at different temperatures. Data are
68 expressed as recovery relative to day 0. A at -20°C, B at 4°C, C at 20°C. Lab 1: circle ●; Lab 2: square
69 ■; Lab 3: triangle ▲; Lab 4:triangle upside down ▼

70



71

72 **Figure SI-2.2**. Stability of THC-COOH in ultrapure water stored at different temperatures. Data are
73 expressed as recovery relative to day 0. A at -20°C, B at 4°C, C at 20°C. Lab 1: circle ●; Lab 2: square
74 ■; Lab 3: triangle ▲; Lab 4:triangle upside down ▼

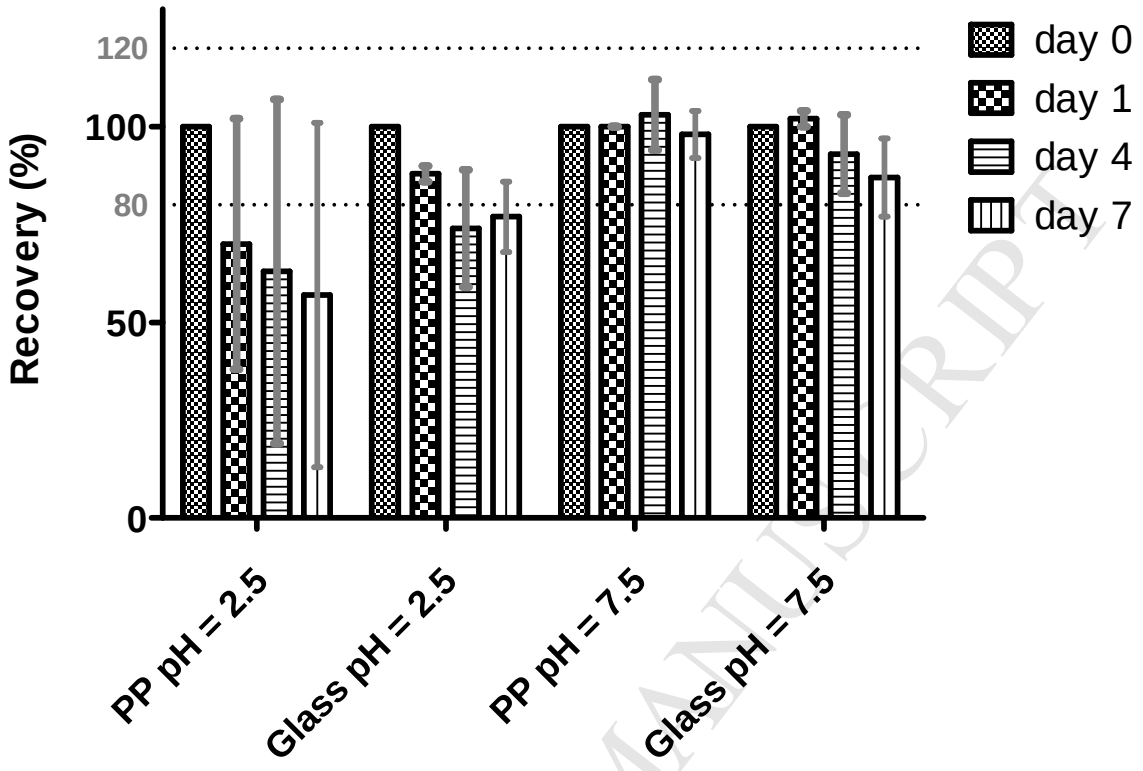
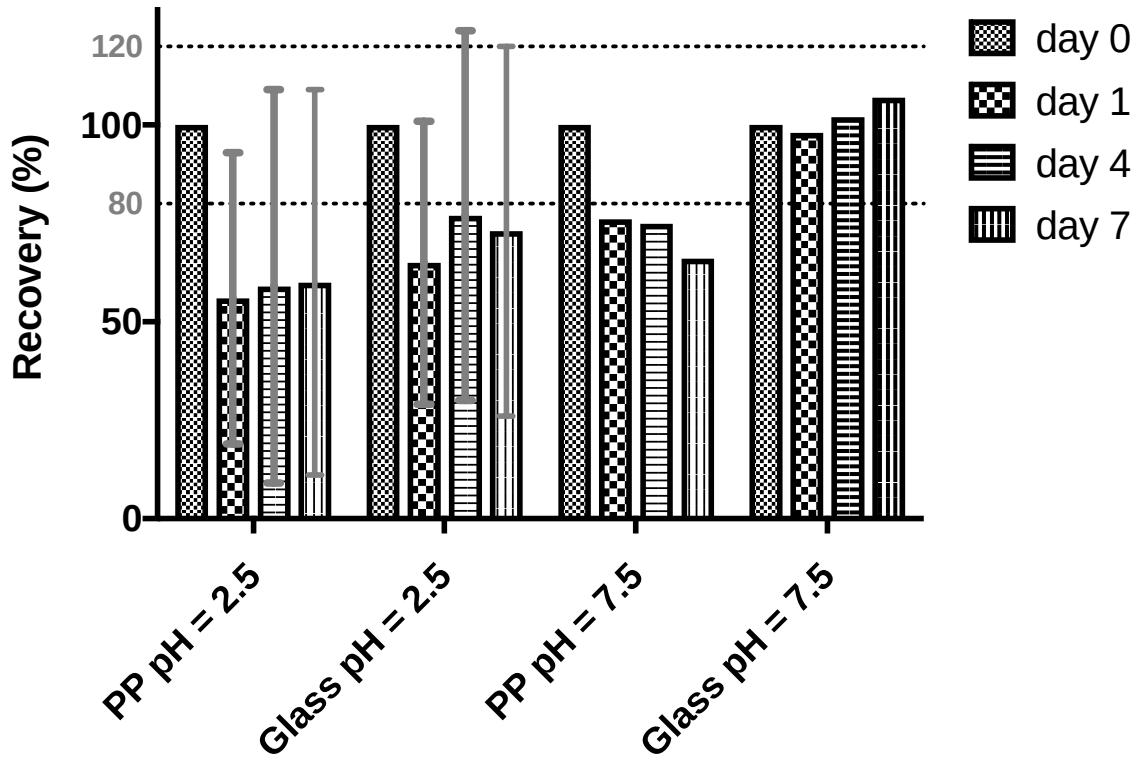


Figure SI-3.1. Influence of pH on sorption to polypropylene or glass container walls of THC-COOH spiked in wastewater. Data collected during a period of 7 days and expressed as recovery relative to day 0.



81

82 **Figure SI-3.2**. Influence of pH on sorption to polypropylene or glass container walls of THC-
83 COOH spiked in ultrapure water. Data collected during a period of 7 days and expressed as
84 recovery relative to day 0.

85

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