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



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### HIGHLIGHTS

- 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.

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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- $\Delta^9$ -tetrahydrocannabinol (THC-COOH) in wastewater-based epidemiology has presented several challenges and

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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 organized with eight laboratories that tested the performance of the proposed procedure. Results were found satisfactory with z-scores  $\leq 2$ .

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## 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  $\Delta^9$ -tetrahydrocannabinol (THC), but in WBE studies the urinary metabolite of THC, 11-nor-9-carboxy- $\Delta^9$ -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 [Tables 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 [Tables 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- $d_9$ .

**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 <sup>a</sup>	Lab 10 <sup>a</sup>
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 (2) 0.45 µm mixed cellulose acetate & cellulose nitrate	Filtration (1) 1 µm glass fiber filter A/E PES membrane filter	Filtration 0.2 µm Whatman PTFE syringe filter	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 <sup>b</sup>	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 Ref. [20]	Unpublished [10]		Adaptation from Ref. [21]	[22]	Adaptation from Ref. [23]	Unpublished
Instrumental variability <sup>c</sup> (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 <sup>c</sup> (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)

n.a. not applicable.

<sup>a</sup> Labs 9 and 10 did participate in the interlaboratory study but provided results in preliminary experiments.

<sup>b</sup> QqQ: triple quadrupole; LTQ-FT Orbitrap: linear ion trap-Fourier transform Orbitrap; QTOF: quadrupole-time-of-flight.

<sup>c</sup> Instrumental variability was performed using a standard solution of 50 ng/L in solvent.

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<sub>2</sub> 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 Tables 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 Tables 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<sup>-1</sup>) 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<sup>-1</sup> 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<sup>-1</sup> 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.

### 2.3.3. Filtration

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

$$1 - (\text{average recovery filtered} / \text{average recovery nonfiltered})$$

Four laboratories provided results.

### 2.3.4. Sorption

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

### 2.3.5. Order of preparatory steps

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

### 2.3.6. Inter-laboratory study

From the preliminary experiments, a best-practice protocol was derived stating recommendations on the pre-analytical aspects of the analysis of THC-COOH in wastewater (see below). In order to test the performance of this protocol, an inter-laboratory study was 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<sup>-1</sup>), natural pH (7.5); Sample 3, spiked at high level (720 ng L<sup>-1</sup>), natural pH (7.5); and Sample 4, acidified to pH 2.5 and spiked at high level (720 ng L<sup>-1</sup>). The low level (72 ng L<sup>-1</sup>) and high level (720 ng L<sup>-1</sup>) were prepared by spiking 0.5 mL and 5 mL of a THC-COOH solution of 0.72 mg L<sup>-1</sup> (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.

## 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<sup>-1</sup> 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 ( $\leq 10\%$ , 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 (Tables SI–3). Results presented in Tables 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](http://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 Fig. 1, the average loss during filtration when sample pH was not adjusted (pH  $\approx$  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 =  $7e-4$ ).

### 3.1.4. Sorption

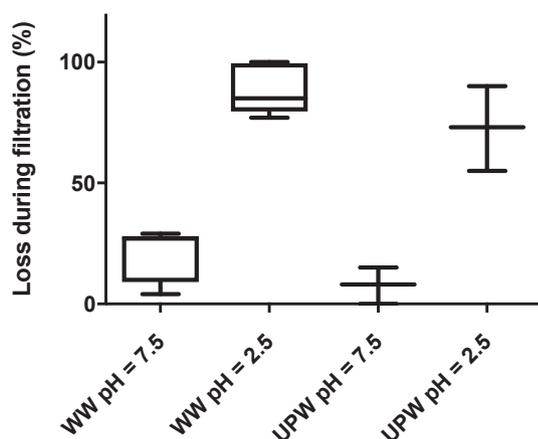
Results from the sorption experiments are shown in Figure SI-3 (.1 for wastewater matrix and .2 for ultrapure water). Sample pH appears to be a more important parameter than the type of sample container (glass or PP) used. Losses due to sorption to container walls occur more rapidly and to a higher extent at pH = 2.5, as the compound is in its non-charged hydrophobic form.

Altogether, the results from filtration, in-sample stability and sorption tests have identified pH as the variable having the most significant impact on the recovery of THC-COOH. This corroborated that, given the specific physico-chemical properties of THC-COOH, its behaviour is highly dependent on wastewater pH.

### 3.1.5. Order of preparatory steps

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

The conclusion is that sample acidification, if required by the selected enrichment protocols, should be performed only after the



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

sample filtration. Ideally, ILIS should be added before filtration to correct for any potential loss. The results of the preliminary experiments highlighted the influence of pH and the importance of the correct execution order of sample preparation steps before SPE, with sample acidification being critical. When consulting the SCORE inter-laboratory exercise participant laboratories [17], only 5% had performed their analysis using the order of steps identified as the optimal one in this study: 1st ILIS addition 2nd filtration 3rd pH adjustment (only if needed). Therefore, it was decided to perform an inter-laboratory study within the group in order to confirm this hypothesis before making any recommendation.

### 3.2. Inter-laboratory study

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

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

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

Z-scores were calculated to help in the identification of random or systematic errors. To do so, the difference between each individual lab's mean (*m*) and the group's mean (*M*) was subtracted, and then divided by the group's standard deviation. This computation provides a value that can be either positive or negative (when the mean is above or below the group's average, respectively), as a measure of the accuracy of each laboratory. The accepted cut-off value is  $z\text{-score} \leq |3|$ , whilst a value between 2 and 3 is considered questionable, in accordance with the IUPAC [27] terminology. Graphical results are presented in Fig. 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.

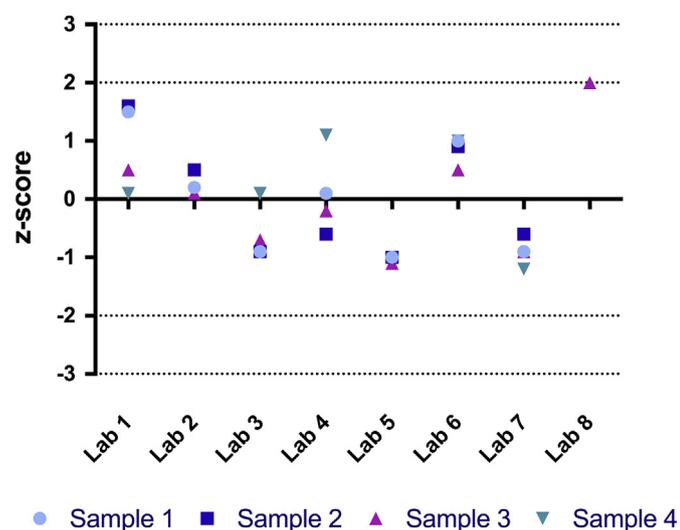
**Table 2**  
Group's mean (M) per sample expressed in  $\text{ng L}^{-1}$ , Recovery (R) expressed in absolute value ( $\text{ng L}^{-1}$ ) and percentage (%), and group's relative standard deviation (RSD%) in the inter-laboratory study.

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

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

<sup>a</sup> Modified order of analytical steps, the sample was acidified at KWR before being shipped frozen to the laboratories.

<sup>b</sup> After removal of laboratory 8 data.



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

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.

#### 4. Conclusions

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

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

Studies regarding THC-COOH sorption to biofilms and solid particles during in-sewer transport 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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#### Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.aca.2017.08.011>.

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