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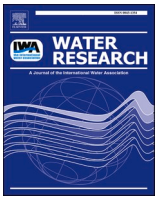

Analytical investigation of cannabis biomarkers in raw urban wastewater to refine consumption estimates

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ABSTRACT

Wastewater analysis of Δ^9-tetrahydrocannabinol (THC) biomarkers can provide essential information on trends in cannabis consumption. Although analysis is mostly focused on the aqueous phase, previous studies have illustrated the need of improving the measurements of raw influent wastewater (IWW) considering also suspended solids. This is important for cannabis biomarkers, because a substantial part of them is expected to be found in the suspended solids due to their more lipophilic character compared with other metabolites/drugs included in these types of studies. However, it remains open to which extent trend estimates might be affected by solely analysing the liquid phase. To investigate this aspect, robust analytical methodologies are required to measure both the liquid and solid phases of IWW. In this work, we firstly tested liquid-liquid extraction (LLE) for THC and its major metabolites (THC—OH, and THC—COOH). Using LLE, no filtration or centrifugation step was required for raw IWW analysis, and the three analytes were extracted from both the liquid and the solid phase simultaneously. In parallel, the raw IWW was centrifuged and the obtained solid and liquid phases were analyzed separately: the liquid phase by both LLE and solid phase extraction (SPE) for comparison of data, and the suspended solids by solid-liquid extraction (SLE). The separate analysis of both phases in a number of samples revealed that a significant amount of cannabis biomarkers (ranging from 42 to 90%) was found in the suspended solids. In addition, the total amount of cannabis biomarkers obtained by analysing raw IWW on the one hand, and by separate analysis of the liquid and the solid phases, on the other hand, was in good agreement. Data from this study show that the sole analysis of the liquid phase would lead to a notable underestimation of cannabis biomarkers concentrations in IWW.

1. Introduction

Cannabis is worldwide the most commonly consumed illicit drug (European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), 2017; United Nations Office on Drugs and Crime, 2021). The European drug report 2021 (European Monitoring center for Drugs and Drug Addiction (EMCDDA), 2021) indicates that cannabis is an established drug, and new forms of cannabis with high Δ^9-tetrahydrocannabinol (THC) content are now available on the illicit market such as cannabis oil/liquid taken orally or for vaping, edibles, drinks, concentrates (e.g., wax, shatter, budder) or tinctures (e.g., concentrated amounts ingested orally or taken under the tongue) (Goodman et al., 2020) which raises health concerns. Moreover, a range of products containing cannabis extracts with low levels of THC are sold legally and commercially (European Monitoring center for Drugs and Drug Addiction (EMCDDA), 2021). Alongside these market changes, the number of first-time cannabis treatment entrants is increasing (European Monitoring center for Drugs and Drug Addiction (EMCDDA), 2021). Therefore, careful monitoring of THC use is necessary to detect changes in consumption patterns and to understand shifts in the drug markets (Burgard et al.,

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2019). The most comprehensive approach would thus consist of a triangulation of data from different sources e.g. key informants, seizure data, population surveys, and city-based wastewater analysis.

Wastewater analysis of human biomarkers, also known as wastewater-based epidemiology (WBE), has been an effective tool to show temporal patterns of drug use such as cocaine, MDMA, methamphetamine, and amphetamine (Humphries et al., 2016; Ort et al., 2014). Moreover, the comparison of WBE data and sales statistics has shown to be an accurate and complementary tool to estimate nicotine and alcohol consumption (Lai et al., 2018). While the parent drugs were quantified for methamphetamine, amphetamine and MDMA, the consumption of nicotine, ethanol, and cocaine was estimated by analysing their main human metabolites (cotinine and hydroxy-cotinine; ethyl sulfate; and benzoylecgonine, respectively). Regarding cannabis, THC is the major psychoactive ingredient, which is metabolized by microsomal hydroxylation to the primary and intermediate metabolite, 11-hydroxy-THC (THC—OH). Subsequently, THC—OH is further metabolized by the enzyme alcohol dehydrogenase to 11-nor-9-carboxy-THC (THC—COOH), which is primarily quantified in wastewater and used to estimate cannabis consumption in WBE studies (Bijlsma et al., 2020).

While WBE has been successfully implemented for the monitoring of stimulants mentioned above, in the specific case of cannabis, the method is linked with various inconsistencies (Burgard et al., 2019) and several studies have identified important knowledge gaps related to the analytical determination of cannabis biomarkers in wastewater (Causanilles et al., 2017): (i) the possible sorption of biomarkers to suspended solids in wastewater or to the biofilm of the sewer system (Ramin et al., 2017, 2016) and as a consequence the potential partition of the different biomarkers in the solid and liquid phase of raw influent wastewater (IWW) (Ramin et al., 2017) and (ii) the metabolism and excretion rates of THC considering sex, race and routes of administration, and subsequently the derived excretion factors (Khan and Nicell, 2012).

Results of inter-laboratory exercises accomplished by the Sewage Analysis CORe group Europe (SCORE) revealed that, although laboratories were able to determine THC—COOH in methanol successfully, its accurate determination in the liquid phase of IWW was challenging (van Nuijs et al., 2018). Despite several improvements focusing on the analytical procedure (Causanilles et al., 2017), back-calculation of cannabis consumption in WBE suggested important deviations from consumption estimates obtained through conventional indicators (Bijlsma et al., 2021; Burgard et al., 2019; Causanilles et al., 2017). An important cause of these systematic deviations could be related to the lower polarity of the cannabis biomarkers in comparison with other drugs, which would favor their sorption onto suspended solids (Senta et al., 2013), as suggested by some authors (Burgard et al., 2019; Khan and Nicell, 2012; Pandopulos et al., 2020). Moreover, THC and its metabolites are excreted via feces in a much higher proportion than other drugs (Gracia-Lor et al., 2016). Hence, more emphasis needs to be placed on understanding cannabis biomarkers distribution between the liquid and suspended solids fraction.

The aim of this work is to use different analytical approaches for the determination and investigation of the three cannabis biomarkers (THC, THC—OH, and THC—COOH) in raw IWW. The analytical determination of THC—COOH is commonly performed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with a sample preparation consisting of a filtration or centrifugation step followed by preconcentration of the sample through solid-phase extraction (SPE) (Bijlsma et al., 2014, 2020; Causanilles et al., 2017). Other sample extraction procedures are also reported, such as liquid-liquid extraction (LLE) (González-Mariño et al., 2018; Pandopulos et al., 2020; Tscharke et al., 2016) and solid-phase microextraction (SPME) (Racamonde et al., 2012). In one study using direct injection, THC—COOH measurements were below the limit of detection in real samples (Berset et al., 2010), suggesting that a concentration step was necessary. In this research, both LLE and SPE were employed for the extraction of the liquid phase after centrifugation, while the suspended solids were analyzed separately by solid-liquid extraction (SLE). In parallel, the total raw IWW (without centrifugation or filtration) was analyzed by LLE for comparison. The present study allows a better understanding of the occurrence (e.g. partition between the liquid phase and the suspended solids) of cannabis biomarkers in IWW. Special attention was paid to THC—COOH as this is the biomarker commonly used in WBE studies for estimating cannabis consumption.

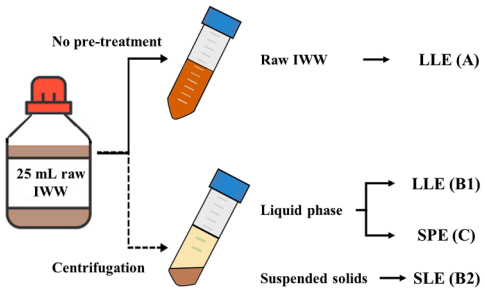

Fig. 1. Sample preparation for the analysis of the liquid and solid phase by different methods: liquid-liquid extraction (LLE), solid-phase extraction (SPE) and solid-liquid extraction (SLE).

2. Experimental

2.1. Chemicals and reagents

High purity analytical standards were purchased from Sigma-Aldrich (Cerilliant Corporation, TX, USA). The standards used were Δ^9-tetrahydrocannabinol (THC), 11-hydroxy-Δ^9-THC (THC—OH) and 11-nor-9-carboxy-Δ^9-THC (THC—COOH) with their respective isotope labelled internal standards (ILIS), THC-D_3, THC—OH-D_3 and THC—COOH-D_3.

Individual standard stock solutions were prepared at 100 mg/L or 10 mg/L in methanol (MeOH) and stored in amber glass vials at −20 °C. Multi-compound working solutions were prepared by appropriate dilution of the standard stock solutions in MeOH. The analytes working mix solution was prepared at 500 μg/L and the ILIS working mix solution was prepared at 200 μg/L. LC-MS grade MeOH, hexane (HX), ethyl acetate (EA), hydrochloric acid (HCl), formic acid (HCOOH), ammonium acetate (NH_4Ac), and sodium chloride (NaCl) were supplied by Scharlab (Barcelona, Spain). HPLC-grade water was obtained by purifying demineralized water using a Milli-Q system from Millipore (Bedford, MA, USA).

2.2. Sample collection and treatment

Seven daily samples (1 L) of influent wastewater (24-h composite, time-proportional with a time interval of 10 min) were collected from the wastewater treatment plant (WWTP) of Castellon, Spain. After collection, the samples were immediately transported to the laboratory and stored in the dark at −20 °C until analysis. Several extraction techniques were applied for sample treatment of entire raw IWW (unfiltered or non-centrifuged), and for the liquid phase and suspended solids separately. These techniques included LLE, SPE, and SLE. Fig. 1 shows the different extraction methods used.

2.2.1. Liquid-liquid extraction method (A and B1)

The LLE was applied for sample treatment of both raw IWW (Fig. 1, A) and the separated liquid phase (Fig. 1, B 1), using in both cases 25 mL sample. The sample was transferred to a 50 mL Falcon tube and 50 μL of the ILIS working mix solution (200 μg/L) was added to 25 mL of non-centrifuged IWW (A) or centrifuged IWW (B.1), vortexed for 30 s and let stand for two hours before extraction. Then, a spatula tip of NaCl was added and the sample was acidified to pH ~2 with HCl 1 M (400 μL),

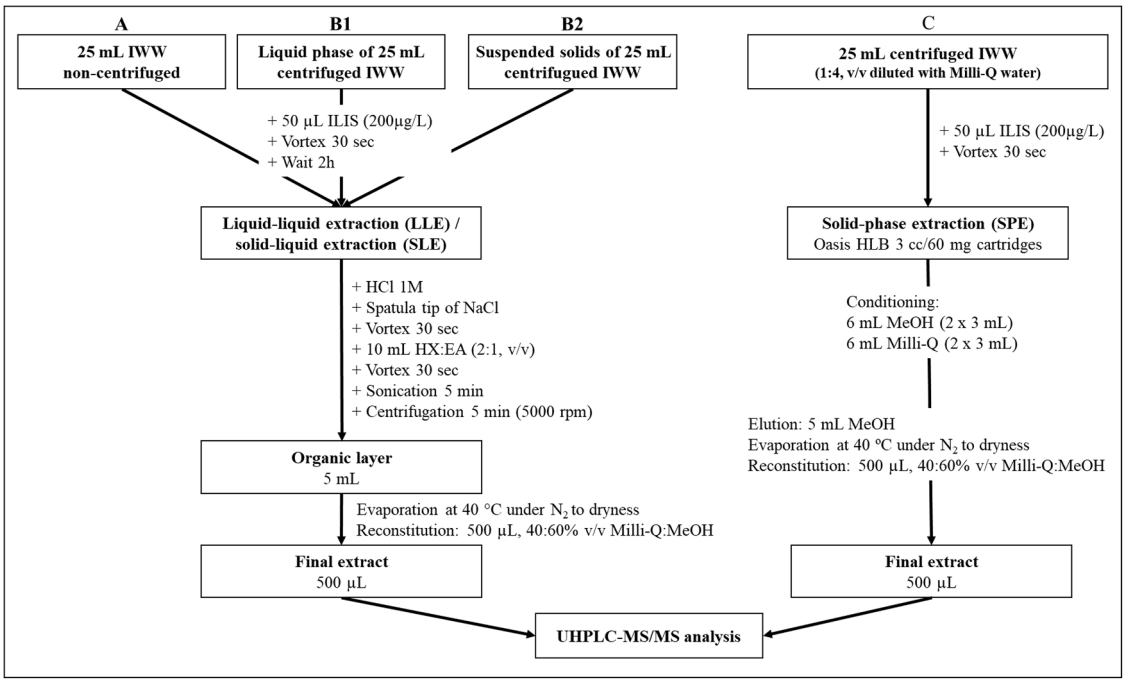

Fig. 2. Graphical workflow of the analytical procedure liquid-liquid extraction (LLE), solid-liquid extraction (SLE) and solid-phase extraction (SPE).

followed by 30 s vortexing. Subsequently, 10 mL of HX:EA (2:1, *v/v*) was added, the sample was vortexed for 30 s and sonicated for 5 min, and afterwards the content of the vessel was centrifuged at 5000 rpm for 5 min. A volume of 5 mL of the organic layer was transferred to a glass test tube and evaporated at 40 °C under a gentle stream of nitrogen to dryness. Extraction was executed once and the residue was reconstituted in a mixture of MeOH: Milli-Q water (3:2 *v/v*), and 5 µL of the final extract was injected into the LC-MS/MS system.

2.2.2. Solid-liquid extraction method (B2)

For the SLE of suspended solids (Fig. 1, B.2), 25 mL of raw IWW was centrifuged at 5000 rpm for 5 min in a Falcon tube; the liquid phase was removed and 50 µL of the ILIS working mix solution (200 µg/L) was added to the pellet containing the suspended solids. The sample was then vortexed for 30 s and after two hours at room temperature, a spatula tip of NaCl and 100 µL of HCl 1 M were added to the Falcon tube. The sample was vortexed during 30 s followed by adding 10 mL of HX: EA (2:1, *v/v*). Subsequently, the content was mixed by vortexing for 30 s and sonicated for 5 min. Finally, the content was centrifuged at 5000 rpm for 5 min, and 5 mL of the organic layer was transferred to a glass test tube and evaporated at 40 °C under a gentle stream of nitrogen to dryness. The residue was reconstituted in a mixture of MeOH: Milli-Q water (3:2 *v/v*), and 5 µL of the final extract was injected into the LC-MS/MS system.

2.2.3. Solid-phase extraction method (C)

First, the comparison of Strata X (60 mg, 3 mL) and Oasis HLB (60 mg, 3 mL) cartridges in terms of recoveries was performed by spiking 25 mL of Milli-Q water at two different levels (100 and 800 ng/L) based on a previously developed in-house method for the determination of THC—COOH in wastewater (Bijlsma et al., 2014). The Oasis HLB cartridges were selected and applied to the separated liquid phase (Fig. 1, C) . Briefly, 25 mL centrifuged IWW was diluted with 75 mL Milli-Q water (leading to 100 mL of four-fold diluted centrifuged IWW), and 50 µL of the ILIS working mix solution (200 µg/L) was added before SPE with Oasis HLB cartridges (3 mL, 60 mg). The cartridges were conditioned by washing and rinsing with 6 mL of MeOH and 6 mL of Milli-Q water. After conditioning, the samples were percolated through the cartridges by gravity (flow rate of ~ 3 mL/min), and vacuum dried for approximately 35 min. The analytes were eluted with 5 mL of MeOH and the extract was evaporated to dryness at 40 °C under a gentle stream of nitrogen. Finally, the residue was reconstituted in 0.5 mL MeOH:Milli-Q water (3:2, *v/v*) and 5 µL was injected into the LC-MS/MS system. A schematic overview of the sample treatment protocol is shown in Fig. 2.

2.3. Instrumentation

Ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) sample analysis was performed using a Waters Acquity H—Class UPLC system (Waters Corporation, MA, USA) coupled to a triple quadrupole mass spectrometer (Xevo TQS, Waters, Manchester, UK) equipped with an electrospray ionization source (ESI) operated in positive ionization mode. Chromatographic separation was carried out using an Acquity UPLC BEH C18 column (1.7 µm, 50 × 2.1 mm) from Waters (Manchester, UK) at a flow rate of 0.3 mL/min. Column temperature was kept at 40 °C and the sample manager was kept at 10 °C. The mobile phase consisted of a gradient of A: Milli-Q water with 0.01% HCOOH and 5 mM NH_4Ac and B: MeOH as follows: 0 min 60% B, 3.5 min 95% B, 5.0 min 95% B, 5.1 min 60% B until 7 min for re-equilibration of the column for the next injection. Injection volume was 5 µL. Cone and desolvation gas flow were set to 250 L/h and 1200 L/h, respectively. For the operation of MS/MS mode, collision gas was argon 99.995% (Praxair, Madrid, Spain) set to 0.15 mL/min. The source temperature was kept at 150 °C, desolvation temperature at 650 °C and capillary voltage was established at 1.5 kV. Dwell times were established at 15 ms. Selected transitions, cone voltages and collision energies can be found in Table S-1. All data were acquired and processed using MassLynx v4.1 software (Waters, Manchester, UK).

2.4. Stability experiments

The in-sample stability of THC, THC—OH and THC—COOH was tested at three temperatures (−20 °C, 4 °C and 20 °C) over 30 days at 0 h, 6 h, 12 h, 24 h, 3 d, 7 d, 14 d, and 30 d after the method validation. For each storage temperature, 2 bottles of 1000 mL of non-centrifuged raw IWW (one “blank” and one spiked at 1 µg/L with a mix of the three

Table 1
Liquid-liquid extraction method validation in raw influent wastewater ($n = 5$).

Compound	LOQ (ng/L)	LOD (ng/L)	Conc. in "blank" (ng/L)	Recovery (RSD,%)		q1/Q ratio deviation (%)		q2/Q ratio deviation (%)	
				100 ng/L	800 ng/L	100 ng/L	800 ng/L	100 ng/L	800 ng/L
THC	10	3	51	62 (10)	65 (4)	3	7	28	1
THC—OH	5	2	104	*	80 (9)	3	0.3	4	3
THC—COOH	3	1	246	*	73 (10)	10	7	1	2

* Not estimated due to the high concentration of the analyte in the spiked "blank" sample.

analytes) and 2 bottles of 1000 mL of centrifuged IWW (one "blank" and one spiked at 1 μg/L with a mix of the three analytes) were prepared and the ILIS mix solution was added in all bottles at 1 μg/L. Then, samples were homogenized and distributed in 96 conical tubes (12 for each time frame), containing 10 mL of sample. LLE was performed for the non-centrifuged and centrifuged IWW at the three temperatures tested. For experiments at −20 °C, thawing was done by adding mechanical shaking at body temperature (holding in hands) After LLE, the extracts were stored at −20 °C in a vial until LC-MS/MS analysis. Fig. S-1 shows the procedure applied for the stability experiments.

2.5. Method validation

Method performance was evaluated with authentic IWW samples in terms of linearity, limits of detection (LODs), limits of quantification (LOQs), accuracy (in terms of recovery), and precision (intra-day precision expressed as relative standard deviation (RSD)) taking into account the SANTE guideline (SANTE/12682/2019, 2019). Linearity was studied by the preparation of calibration curves in Milli-Q water with 10% MeOH, using linear regression ($r^2 > 0.99$) with concentrations ranging from 50 to 100,000 ng/L. LODs and LOQs were estimated by analyzing spiked IWW at 100 ng/L and back-calculating based on a signal-to-noise (S/N) ratio of 3:1 and 10:1, respectively. When the analytes were present in the "blank" at concentrations higher than the spiked level, the LOD and LOQ were calculated from the signal in this "blank" sample without spiking. Accuracy, and intra-day precision were evaluated using spiked IWW samples ($n = 5$, from different origins/locations) at two concentration levels (100 and 800 ng/L) quantified after ILIS correction. Recoveries were considered satisfactory when they ranged between 70% and 120%, with RSD values lower than 20%. Identification of the compounds in samples was ensured when the ion ratio deviations between the quantification (Q) and confirmation (q1 and q2) transitions (q/Q ratios) were below 30%, and the chromatographic retention time was ± 0.1 min, in relation to the reference standards (SANTE/12682/2019, 2019). Due to the impossibility of obtaining real "blank IWW samples", as all of the target analytes are usually present in IWW, samples were initially analyzed without the spiking of the analytes and the quantified amount of the analytes was subtracted from the measured concentration in spiked IWW.

3. Results and discussion

3.1. Stability experiments

Stability experiments were performed once the LLE and SLE methods were validated. The results obtained in the in-sample stability experiments are summarized in Figs. S2-S4. Concentration at time zero is considered as 100% of recovery. In the case of non-centrifuged IWW, THC and THC—OH were found to be stable (deviations below 30% during the entire experiment) at −20 °C, 4 °C, and 20 °C for up to one month as reported by Causanilles et al. (Causanilles et al., 2017). Oppositely, an increase in recovery (up to 140%) was observed for THC—COOH at all temperatures (Fig. S-2). Desorption of THC—COOH present in the suspended solids is the most probable reason for the observed increase in concentration, yet an interconversion of compounds, due to the transformation of THC—OH to THC—COOH by oxidation might also occur, although very unlikely (Ramin et al., 2017). However, the obtained data cannot support any of these hypotheses since a mixed spiking solution was used, and the real solid used is not an authentic blank due to the presence of all three compounds.

The stability data of the analytes in centrifuged IWW presented more variability. All compounds were stable up to 1 month at 4 °C and −20 °C, as reported previously (Causanilles et al., 2017; González-Mariño et al., 2018; Heuett et al., 2015). However, in the case of THC—COOH (Fig. S-2) and THC—OH (Fig. S-3), notable losses were observed when stored at 20 °C after two weeks. The adsorption onto hydroxyl sites present on the surface of glassware might play an important role in this process (Baker and Kasprzyk-Hordern, 2011), but no clear explanation was found for the analytes behavior at 20 °C. Thus, the best option seems to store the samples at 4 °C or −20 °C to ensure their stability.

These results illustrate the relevance of appropriate storage conditions of the samples, with the recommendation of storing the centrifuged IWW at −20 °C or 4 °C if analysis cannot be performed within 14 days after sample reception. If analysis is performed within 14 days, the samples could be stored at 20 °C without significant loss of analytes. Non-centrifuged samples could be stored during one month at any of the three temperatures tested. Nevertheless, further research is necessary by spiking samples individually with each analyte to clarify the possible interconversion mentioned above.

3.2. Analysis of raw IWW (A)

Previous publications reporting the use of LLE for THC—COOH extraction from IWW (González-Mariño et al., 2018; Pandopulos et al., 2020; Tscharke et al., 2016) were used as a guide in this study. The tested solvent mixture was HX and EA, since HX has been reported as appropriate to extract THC, and EA or HX:EA (1:1, *v/v*) to extract THC—OH and THC—COOH from ultra-pure water (González-Mariño et al., 2018). EA has also been reported for the extraction of THC—COOH from wastewater (Pandopulos et al., 2020). In the present work, HX:EA (2:1, *v/v*) was chosen as the extraction solvent, as it resulted in the best recoveries with the lowest RSDs (Table S-2).

The addition of NaCl to the sample was also evaluated. Although no significant differences were found in the recovery of analytes from IWW (RSD < 16%), the addition of NaCl was eventually applied since IWW samples are highly variable, and previous research recommended the addition of NaCl to improve recovery by "salting-out" target analytes and to prevent the formation of emulsion in the LLE process (González-Mariño et al., 2018; Pandopulos et al., 2020). The waiting time between the addition of ILIS to the IWW and the addition of approximately 400 μL of 1 M HCl to neutralize THC—COOH, allowing the extraction of this compound in a neutral uncharged form, was also evaluated at 20 min, 2 h, and overnight (14 h), obtaining the most reproducible results when the ILIS was added and waiting for 2 h before starting the LLE process. The RSDs ranged from 11 to 23% in the ILIS abundance after 20 min waiting, from 1 to 11% after 2 h, and from 20 to 23% overnight.

Data obtained in the validation of the LLE procedure applied to raw IWW (non-centrifuged) are shown in Table 1. It was not possible to obtain real "blank" IWW, because of the frequent occurrence of these three biomarkers in wastewater. This fact impacted the validation process, especially at low analyte concentrations (i.e., at 100 ng/L spiked

Table 2
Liquid-liquid extraction and solid-phase extraction method validation in the liquid phase of influent wastewater ($n = 3$).

	Compound	LOQ (ng/L)	LOD (ng/L)	Conc. in "blank" (ng/L) [a]	Recovery (RSD,%)		q1/Q ratio deviation (%)		q2/Q ratio deviation (%)	
					100 ng/L	800 ng/L	100 ng/L	800 ng/L	100 ng/L	800 ng/L
LLE	THC	10	3	–	70 (6)	72 (3)	7	6	9	4
	THC—OH	5	2	35	71 (4)	78 (5)	5	3	2	1
	THC—COOH	3	1	183	*	69 (6)	10	8	3	1
SPE	THC	20	6	–	84 (9)	82 (4)	5	9	14	22
	THC—OH	12	4	35	95 (3)	90 (1)	22	19	7	10
	THC—COOH	26	8	183	*	98 (6)	4	5	26	13

[*] Not estimated due to the high concentration of the analyte in the "blank" sample.
[a] Average value of the "blank" concentration obtained by SPE and LLE extraction methods.

Table 3
Determination of THC—COOH by liquid-liquid extraction and solid-phase extraction in seven centrifuged influent wastewater samples from a one-week sampling.

Sample	THC—COOH (ng/L)		Deviation SPE/LLE (%)
	LLE	SPE	
IWW 1	239	336	+41
IWW 2	270	328	+22
IWW 3	262	308	+18
IWW 4	221	208	−6
IWW 5	327	348	+6
IWW 6	281	356	+27
IWW 7	332	308	−7

level, which was similar or even lower than the concentration of the analyte present in the "blank" IWW used for validation). Recoveries were around 70% for the three compounds with intra-day precision, expressed as RSD ≤ 10%. All analytes could be fully identified in the sample with two confirmatory transitions (q1, q2) and low deviations (≤28%) in the q/Q ratios in relation to the reference standard average values.

3.3. Analysis of the liquid phase (B1 and C)

In this study, the liquid phase of the centrifuged IWW samples was extracted using both LLE (B1) and SPE (C) separately and the performance of the two approaches was compared (Fig. 2). In the case of the SPE, two sorbents i.e., Strata X (60 mg, 3 mL) and Oasis HLB (60 mg, 3 mL) often applied in multi-residue methods, were tested for spiked Milli-Q water samples (Table S-3). Oasis HLB cartridges led to good recoveries (82 - 130%) and were selected for subsequent experiments in IWW. In parallel, LLE was also tested for the extraction of the liquid phase of IWW, and both procedures were finally validated (Table 2).

Accuracy was consistently below 100%, with SPE recoveries being slightly higher at both validated levels. Precision was satisfactory, with RSD ≤ 10% in all cases. LOQs (from 3 to 10 ng/L in the case of LLE and from 12 to 26 ng/L in the SPE) and LODs were lower for the LLE procedure for all compounds studied (Table 2).

In order to obtain more data to compare both procedures, seven consecutive daily samples were processed using both methods. Concentrations of THC—COOH showed deviations < 30% in 6 out of the 7 samples analyzed when comparing data for both methods (Table 3). Despite the, in general, slightly higher recoveries when employing SPE, the LLE procedure (25 mL of sample extracted with 10 mL of HX:EA (2:1, *v/v*)) was considered as a good alternative for the analysis of these compounds, taking into account the higher cost of SPE and the more time-consuming steps (i.e. conditioning, sample loading, washing, and elution).

3.4. Analysis of the suspended solids (B2)

The SLE method for suspended solids was tested in terms of extraction system, solvent, and extraction time. Three different extraction systems were tested, including vortex-assisted (1 min), rotatory-assisted (time=10, 20 and 30 min), and ultrasonic-assisted extraction (time=10, 20 and 30 min). Based on the data summarized in Table S-4, ultrasonic-assisted extraction for 10 min led to the highest extraction of cannabis biomarkers and therefore it was chosen for subsequent experiments. Next, ultrasonic-assisted extraction was performed with different ratios of HX:EA, including 1:1 (*v/v*), 2:1 (*v/v*), 3:1 (*v/v*), and 1:2 (*v/v*), all tested at different time frames ($t = 2$, 5 and 10 min). The best results in terms of extraction efficiency were obtained with HX:EA (2:1, *v/v*) during 5 min, which was finally selected as the optimal procedure for the extraction of cannabis biomarkers from suspended solids (Table S-5).

The validation of the SLE procedure was subjected to practical challenges because of difficulties to accurately weigh or measure the amount of solids in each sample aliquot used for validation. To try to overcome this, the samples subjected to validation were shaken vigorously until all solid particles were floating homogeneously and subsequently 25 mL aliquots were collected and centrifuged. After that, the liquid phase was removed as much as possible, leaving the pellet (suspended solids) at the bottom of the Falcon tube to proceed with the extraction and validation. In this way, RSDs were below 20% in all cases, and recoveries were close to 100% for the three cannabis biomarkers (Table 4).

Table 4
Solid-liquid extraction method validation in the suspended solids of 25 mL influent wastewater ($n = 3$).

Compound	LOQ (ng)	LOD (ng)	Conc. in "blank" (ng/L) [a]	Recoveries at 20 ng (RSD,%)
THC	0.22	0.07	79	97 (12)
THC—OH	0.21	0.07	175	101 (16)
THC—COOH	0.18	0.06	338	106 (15)

[a] Calculated from the concentration extracted from the entire pellet and the volume of raw IWW sample (25 mL).

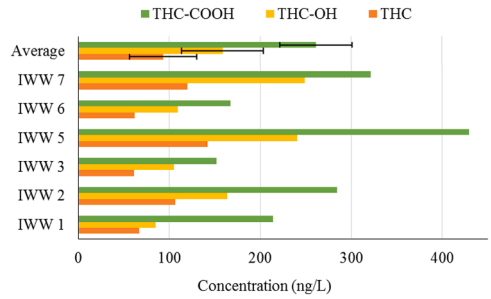

Fig. 3. Concentration of the cannabis biomarkers in the suspended solids of six influent wastewater samples individually and average of the six samples with error bars (i.e., standard deviation).

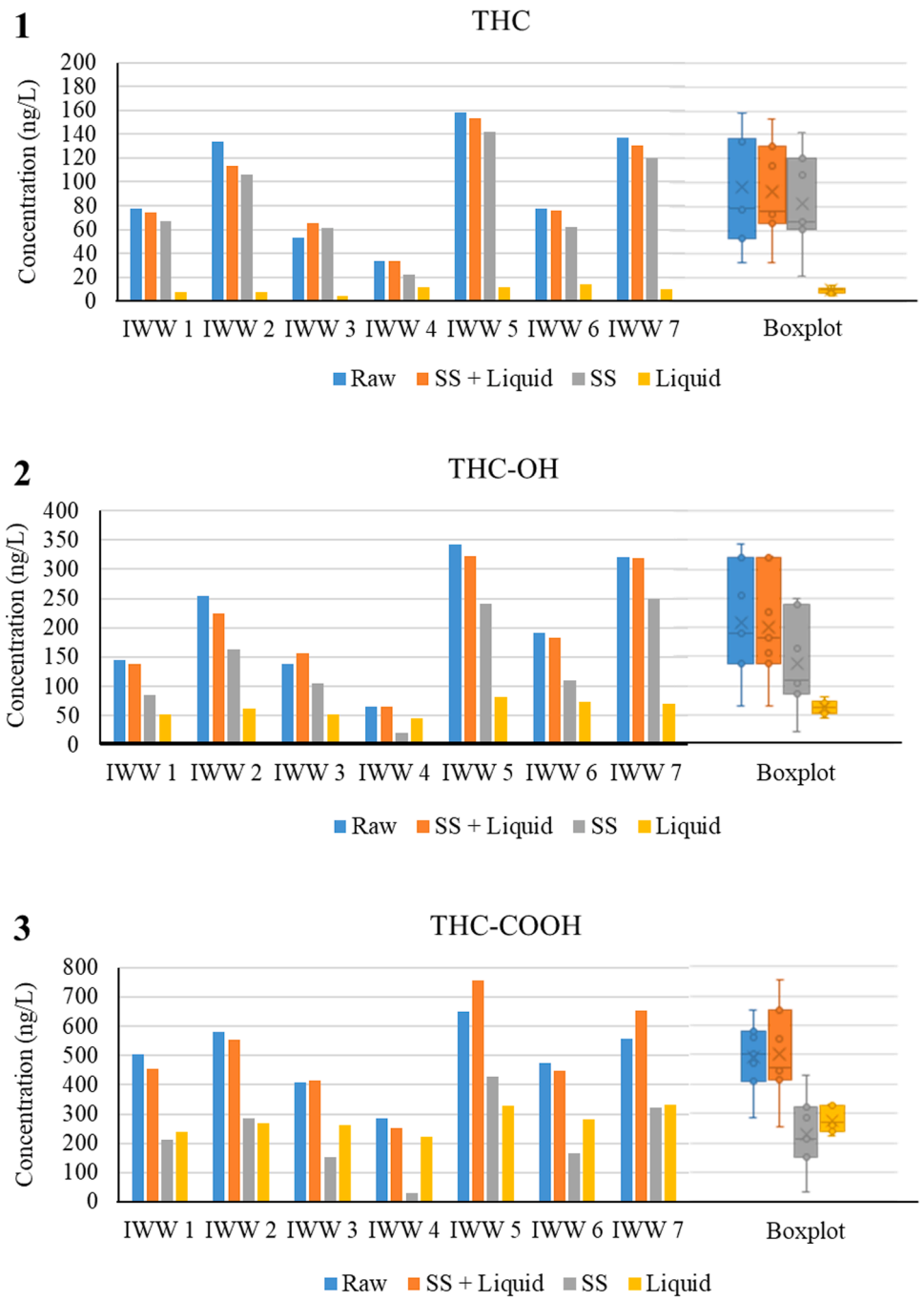

Fig. 4. Concentrations of (1) THC, (2) THC—OH and (3) THC—COOH in different phases of seven influent wastewater samples and boxplots of each phase ($n = 7$). Comparison of the whole raw influent wastewater (blue bar), the liquid phase (yellow bar), the suspended solids (SS) (gray bar), the sum of SS and the liquid phase analyzed separately (orange bar).

This method was applied to the suspended solids of the seven samples mentioned in Section 3.3 of which the results are shown in Fig. 3. The amount of biomarker (ng) measured in the solids was converted to ng/L bearing in mind that the suspended solids were taken from 25 mL of IWW. By expressing the concentration in ng/L, the comparison between data from LLE and SLE was easier. It can be seen that the three cannabis metabolites were present in all samples; the predominant compound being THC—COOH, followed by THC—OH and THC. These findings are in agreement with the low polarity of the compounds, which are consequently substantially sorbed onto the solid phase of IWW. It should be noted that low concentrations, particularly for THC—OH and THC—COOH, were found in the solids of sample IWW4, which was characterized by a low content of suspended solids (visual observation). These two values were confirmed as outliers (test Q Dixon) and IWW4 was removed from the dataset to obtain the percentage of each biomarker present in the suspended solids.

It can be concluded that analyzing only the liquid phase of IWW (e.g. after centrifugation or filtration), independently of whether SPE or LLE is applied for that analysis, would imply that only a fraction of the cannabis biomarkers is measured.

3.5. *Analysis of cannabis biomarkers in IWW*

The seven IWW samples under investigation were also analyzed by

LLE without previous centrifugation (i.e. analysis of the raw IWW including the liquid phase and suspended solids), one analysis per sample (Fig. 4). This allows to compare the total amount of biomarkers obtained by LLE of the whole raw wastewater with the sum of the suspended solids and liquid phase biomarkers analyzed separately (data given in previous sections). The obtained data show a good agreement using both approaches (deviation <5% in 70% of the results and <20% in the remaining data). These results support the hypothesis that data obtained analyzing the raw IWW by LLE without previous removal of the suspended solids, are similar to the sum of biomarkers in the suspended solids and in the liquid phase (either extracted by SPE or LLE) analyzed separately.

Taking into consideration the data obtained in this study, two approaches could be implemented in future studies to further improve the knowledge on cannabis biomarker concentrations in IWW:

- to analyze the raw IWW by LLE without separating the liquid and solid phase. This would imply the use of an extra aliquot of the sample and a dedicated LLE extraction procedure, in addition to that used for the conventional multi-residue analysis of other illicit drugs that it is normally applied in WBE and based on SPE after filtration or centrifugation. At this point, an issue is to maintain the homogenity of the samples in order to obtain representative aliquots containing suspended solids. Vigorous shaking of the samples is essential until all solid particles are floating homogeneously in the liquid, and subsequently collecting 25 mL aliquots for analysis. The limited information available in relation to urinary and fecal excretion rates, and the representative sampling for solids (including an alteration of suspended solids during freeze/thaw cycle of the samples) are currently bottlenecks and, therefore these issues should be studied in more depth in the future. In this context, quantifying the total suspended solids in the sample collected could give more insight. Moreover, more clinical studies focussed on obtaining accurate urinary and fecal excretion rates seem necessary if more quantitative estimates beyond relative trends should be assessed.
- to perform analysis of only the liquid phase of IWW by either SPE or LLE (following centrifugation/filtration of the sample to remove the suspended solids). As this procedure does not include the fraction in the solid phase, a correction factor could be applied to the measured concentration in order to provide a better estimation of the total biomarker concentration in raw IWW. The suitability and robustness of such a correction factor should be evaluated analysing a large number of wastewater samples collected from different locations with different compositions and characteristics to assess spatial and temporal variation. These experiments will be performed in the near future as this approach appears as a good option for most multi-residue, multi-class analysis, where THC—COOH is determined together with other illicit drugs following a common sample treatment (typically SPE);

The obtained results indicate that an important amount of cannabis biomarkers is present in the suspended solids, a fact that should be taken into account when performing and interpreting wastewater analysis on cannabis biomarkers. Despite the important contribution of suspended solids to the total measured analytes in the sample, there is still a knowledge gap in the possible adsorption or desorption processes that may exist between the suspended solids and the liquid phase. Preliminary sorption experiments have been performed in Milli-Q water containing an amount of suspended solids, but no conclusive results have been obtained yet. Further research is planned to apply the use of THC—COOH-D_9 as an analogue to study the behaviour and distribution of THC—COOH between both phases in different IWW. With the current knowledge on this issue, WBE back-calculation to estimate cannabis consumption is subjected to high uncertainty. A strategy based on concentrations measured in the liquid phase only using the THC—COOH urinary excretion factor does not seem the best option until more information is available with regards to the possible adsorption or desorption processes.

4. Conclusions

In this research, an analytical method has been developed for measuring cannabis biomarkers (THC, THC—OH, and THC—COOH) in wastewater, with a focus on their occurrence and distribution in the liquid phase and suspended solids. Data from this paper show that LLE of raw IWW (unfiltered or non-centrifuged) allowed obtaining cannabis biomarker concentrations in both the liquid and solid phase. The separate analysis of the liquid and solid phase revealed that a high percentage of the compounds present in influent wastewater corresponded to the solid phase (on average 90% THC, 69% THC—OH, 42% THC—COOH). To date, the most common analytical protocol for cannabis biomarkers analysis in IWW, consisting of the application of SPE, only considers the liquid phase and does not consider their presence in the suspended solids. This, consequently, leads to an underestimation of the total biomarker amount present in IWW. The analysis of the IWW without separation of the solid phase by LLE offers more realistic information on the biomarker concentration in IWW than analysis of only the liquid phase. The results in this study allowed to identify the need for future research where the following points should be addressed i) assess sampling uncertainty related to solids ii) partition of the cannabis biomarkers between liquid and solid phases during in-sewer transport and sample storage, and iii) obtaining accurate urinary and fecal excretion rates. By answering these knowledge gaps more insight will be obtained in how to use WBE as a tool to monitor cannabis consumption. Until these issues are sufficiently clarified, the best option at the moment seems to evaluate trends in daily loads (e.g. expressed in mg/day/1.000 people) rather than estimating consumption after back-calculation based on human excretion rates.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2022.119020.

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