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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 focussed on the aqueous phase, previous studies have illustrated the need of improving the measurements of raw influent wastewater (IWW) considering also suspended solids. Especially for cannabis biomarkers, a substantial part of them is expected to be found to solids in wastewater due to their hydrophilic character. 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 phase 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 and the three analytes were extracted from both the liquid and the solid phase simultaneously. In parallel, the same samples were centrifuged and analysed as follows: the liquid phase separately by both LLE and solid phase extraction (SPE) and the suspended solids by solid-liquid extraction (SLE). The separate analysis of the liquid and solid phase in different IWW samples revealed that a significant amount of cannabis biomarkers (ranged from 42 to 90%) could be found in the suspended solids. In addition, the total amount of cannabis biomarkers observed by analysing raw IWW on the one hand, and by separate analysis of the liquid and the solid phase 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 (50%) of cannabis biomarkers determination in IWW.
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Water Research
Editorial Office

Castellon, 4th of March 2022

Dear Editor,

Please, find enclosed our paper entitled "Analytical investigation of cannabis biomarkers in raw urban wastewater to refine consumption estimates", which we submit for your consideration for publication in Water Research

Wastewater analysis of Δ9-tetrahydrocannabinol (THC) biomarkers can provide essential information on trends in cannabis consumption in wastewater-based epidemiology (WBE) studies. However, it remains unclear to which extent estimates may be affected by solely analysing the liquid phase.

In this study, we analyzed Δ9-tetrahydrocannabinol (THC) and its major metabolites (THC-OH and THC-COOH) from both the liquid and the solid phase of wastewater samples. This information was complemented by results obtained from the analyte stability study in samples. Data from this paper revealed that a significant amount of cannabis biomarkers (ranged from 42 to 90%) were found in suspended solids, and therefore the sole analysis of the liquid phase would lead to a notable underestimation (50%) of cannabis biomarkers present in influent wastewater. Thus, an important knowledge gap in the application of WBE for cannabis use estimation has been identified and carefully studied in this work.

The results obtained in this work allow a better understanding of the occurrence and partition of cannabis biomarkers between the liquid phase and the suspended solids in influent wastewater, and will help to improve surveillance of cannabis consumption at future events. This study is an important piece of the puzzle that give more insight in how to use WBE as a tool to monitor cannabis consumption. In addition, several key issues have been identified for future WBE research, i.e. assessing sampling uncertainty, evaluation of the cannabis biomarkers behavior during in-sewer transport, obtaining accurate urinary and fecal excretion rates.

We feel our paper fits well for publication in Water Research, and will be of interest especially for those researchers working on analysis of drugs in (wastewater).

We look forward to your evaluation of our manuscript. Please notify me if I may be of assistance.

Yours sincerely,

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Declaration of interests

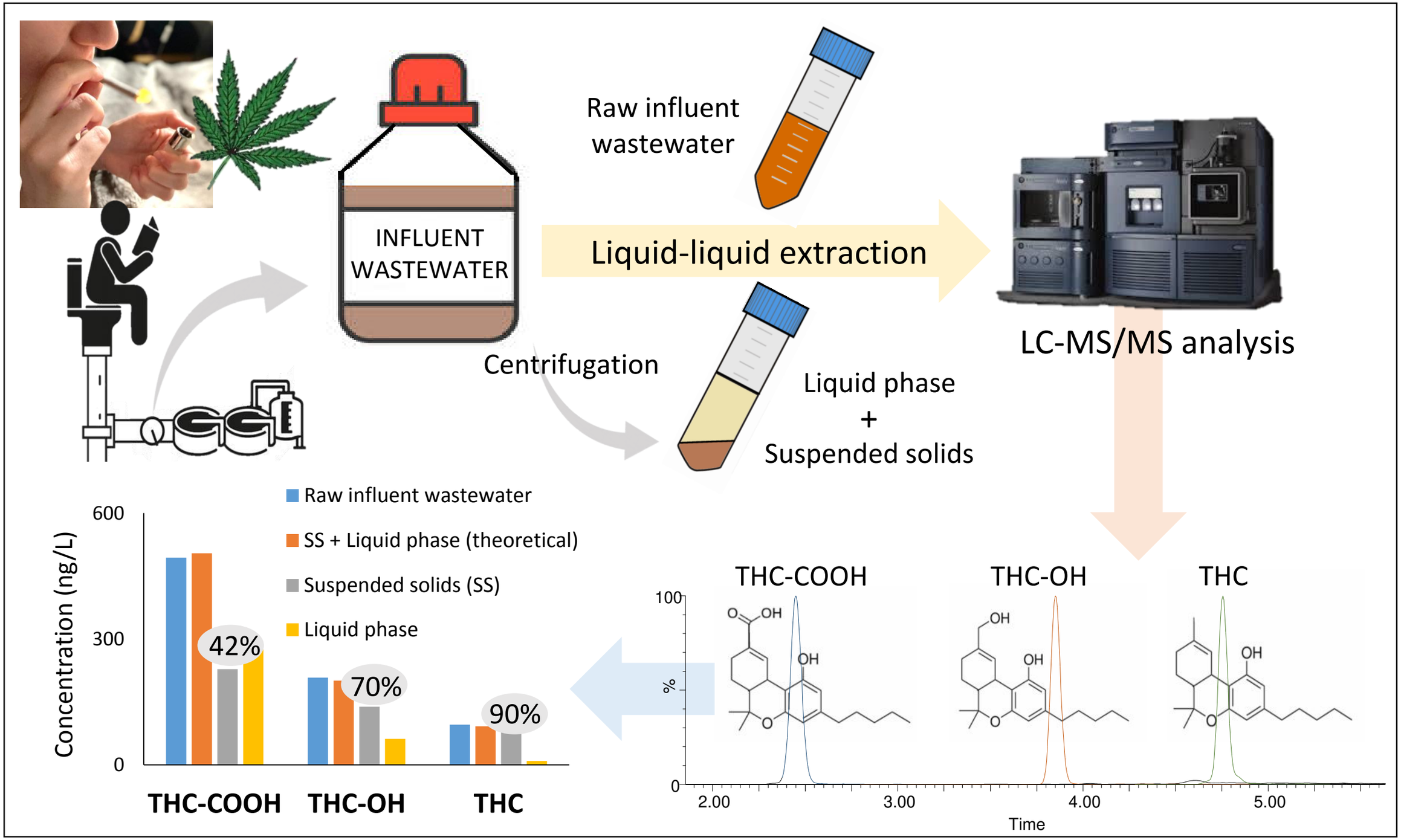
☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

HIGHLIGHTS

- Improvements in measuring cannabis biomarkers in raw influent wastewater
- Liquid-liquid extraction as an alternative to conventional solid phase extraction
- Significant amount of cannabis biomarkers is in the suspended solids of wastewater
- Partition of cannabis biomarker in the liquid and solid phase of influent wastewater
- More knowledge on how to use wastewater-based epidemiology for monitoring cannabis

Graphical Abstract



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 focussed on the aqueous phase, previous studies have illustrated the need of improving the measurements of raw influent wastewater (IWW) considering also suspended solids. Especially for cannabis biomarkers, a substantial part of them is expected to be found to solids in wastewater due to their hydrophilic character. 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 phase 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 and the three analytes were extracted from both the liquid and the solid phase simultaneously. In parallel, the same samples were centrifuged and analysed as follows: the liquid phase separately by both LLE and solid phase extraction (SPE) and the suspended solids by solid-liquid extraction (SLE). The separate analysis of the liquid and solid phase in different IWW samples revealed that a significant amount of cannabis biomarkers (ranged from 42 to 90%) could be found in the suspended solids. In addition, the total amount of cannabis biomarkers observed by analysing raw IWW on the one hand, and by separate analysis of the liquid and the solid phase 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 (50%) of cannabis biomarkers determination in IWW.

Keywords: Δ^9-tetrahydrocannabinol, THC metabolites, liquid-liquid extraction, LC-MS/MS, suspended solids, wastewater-based epidemiology

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," n.d.). The European drug report 2021 (European Monitoring Centre 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 Centre for Drugs and Drug Addiction (EMCDDA), 2021). Alongside these market changes, the number of first-time cannabis treatment entrants is increasing (European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), 2021). Therefore, careful monitoring of THC use is necessary to detect changes in consumption patterns and to understand the shifts in the drug markets (Burgard et al., 2019). The most comprehensive approach would thus consist of the 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 within- and between-week or years 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, nicotine, ethanol, and cocaine

consumption were estimated by the quantification of 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 (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 suffers from 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., 2017a): (i) the possible sorption of the biomarkers to the 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 considering gender, 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., 2017b), back-calculations 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., 2017b). An important cause of these systematic deviations could be

related to the lower polarity of the cannabis biomarkers in comparison with other drugs (e.g. pKa of THC ~ 10.6 and THC-COOH ~ 4.2), which would favour 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., 2020a). 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 previous sample treatment consisting of a filtration or centrifugation step followed by pre-concentration of the sample through solid-phase extraction (SPE) (Bijlsma et al., 2020, 2014; Causanilles et al., 2017a). Other sample extraction alternatives are also reported, such as liquid-liquid extraction (LLE) (González-Mariño et al., 2018; Pandopulos et al., 2020b; Tscharke et al., 2016) and solid-phase microextraction (SPME) (Racamonde et al., 2012). In one study using direct injection, THC-COOH was 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. This 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.

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 ºC. 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), and sodium chloride (NaCl) were supplied by Scharlab. 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

Influent wastewater samples (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 the sample treatment of entire raw IWW and the liquid phase, and the suspended solids separately. These techniques included LLE, SPE, and SLE. **Figure 1** shows the different extraction methods used.

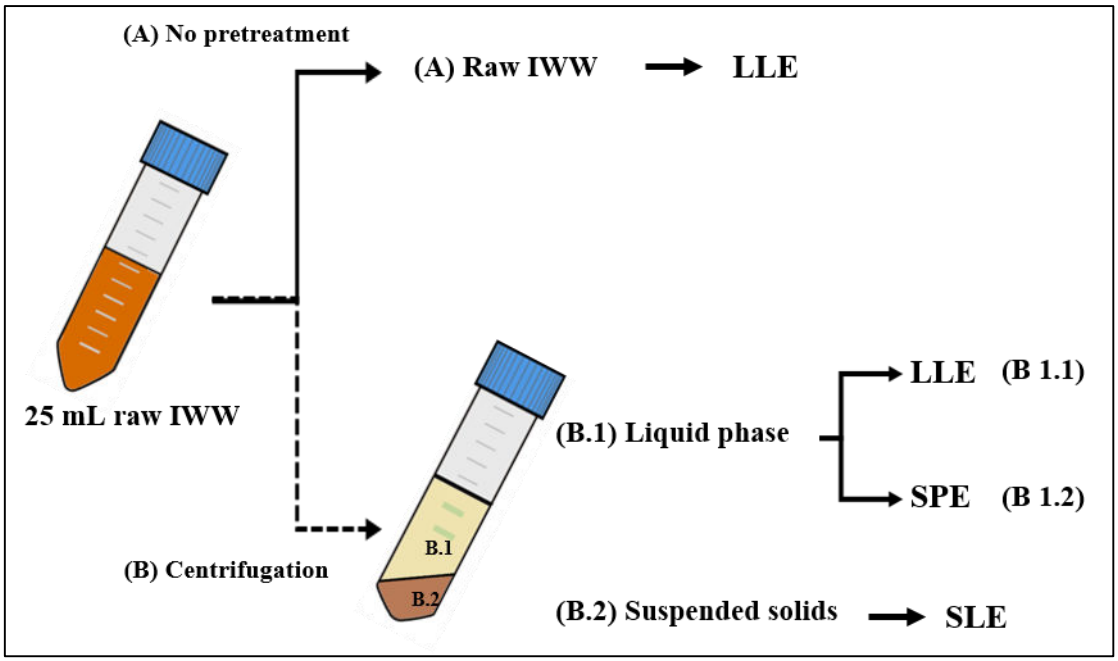

Figure 1. Sample preparation for the analysis of the liquid and solid phase by different methods: liquid-liquid extraction (LLE), solid-phase extraction (SPE) and solid-liquid extraction (SLE).

2.2.1. Liquid-liquid extraction method

The LLE was applied for sample treatment of raw IWW (Figure 1, A) and the separated liquid phase (Figure 1, B 1.1). 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 sec 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), followed by 30 sec vortexing. Subsequently, 10 mL of HX:EA (2:1, *v/v*) was added, it was vortexed for 30 sec, sonicated 5 min and 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. Extraction was executed once and the residue was reconstituted in a mixture of

300 µL MeOH and 200 µL Milli-Q water and the final extract was transferred to a vial for LC-MS/MS analysis.

2.2.2 Solid-liquid extraction method

For the SLE of the analytes in the suspended solids (Figure 1, B.2), 25 mL of non-centrifuged 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 suspended solids (pellet), vortexed for 30 sec and let stand for two hours before extraction. Then, a spatula tip of NaCl and 100 µL of HCl 1 M were added to the Falcon tube and vortexed during 30 sec followed by adding 10 mL of HX:EA (2:1, *v/v*). Subsequently, the content of the vessel was mixed by vortexing for 30 sec 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. The residue was reconstituted in a mixture of 300 µL MeOH and 200 µL Milli-Q water, and the final extract was transferred to a vial for LC-MS/MS analysis.

2.2.3 Solid-phase extraction method

SPE applied to the separated liquid phase (Figure 1, B 1.2) was based on a previously developed in-house method (Bijlsma et al., 2014). 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 15 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 Milli-Q water:MeOH: (40:60, *v/v*) and

transferred to a vial for LC-MS/MS analysis. A schematic overview of the sample treatment protocol is shown in **Figure 2**.

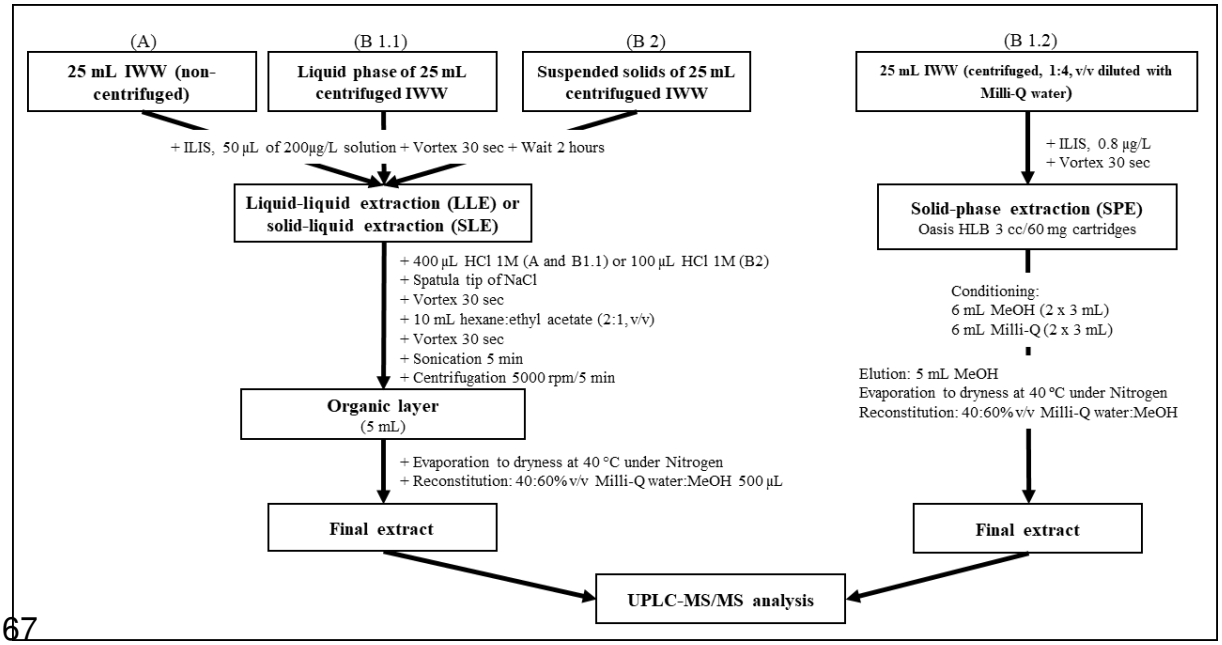

Figure 2. Graphical workflow of the analytical procedure liquid-liquid extraction (LLE), solid-liquid extraction (SLE) and solid-phase extraction (SPE).

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 x 2.1 mm) from Waters 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. Mobile phase consisted of a gradient of A: Milli-Q water 0.01% HCOOH, 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. 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 observed in **Table S-1**. All data were acquired and processed using MassLynx v4.1 software (Waters, Manchester, UK).

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 (inter-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, using linear regression ($r^2 > 0.9900$) with concentrations ranging from 50 to 100000 ng/L. LODs and LOQs were estimated by analyzing spiked IWW at 100 ng/L based on a signal-to-noise (S/N) ratio of 3 and 10, respectively. Accuracy and intra-day precision were evaluated using spiked IWW samples (n=5, from different origin) at two concentration levels (100 and 800 ng/L) quantified after ILIS correction. Recoveries were considered satisfactory when they ranged between 60% and 120%, with RSD values lower than 20% (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.

206 **2.6. Stability experiments**

207 The in-sample stability of THC, THC-OH and THC-COOH was tested at three
208 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,
209 and 30 d. For each storage temperature, 2 bottles of 100 mL of non-centrifuged IWW
210 (one "blank" and one spiked at 1 µg/L with a mix of the three analytes) and 2 bottles of
211 100 mL of centrifuged IWW (one "blank" and one spiked at 1 µg/L with a mix of the
212 three analytes) were prepared and the ILIS mix solution was added in all bottles at 1 µg/L.
213 Then, samples were homogenized and distributed in 96 conical tubes (12 for each time
214 frame), containing 10 mL of sample. LLE was performed for the non-centrifuged and
215 centrifuged IWW at the three temperatures tested. For experiments at -20 ºC, thawing was
216 done by adding mechanical shaking and heating with human temperature (holding in
217 hands). After LLE, the extracts were stored at -20 °C in a vial until LC-MS/MS analysis.
218 **Figure S-1** shows the procedure applied in the stability experiments.

219

220

221 **3. Results and discussion**

222 **3.1. Stability experiments**

223 The results obtained in the in-sample stability experiments are summarized in **Figures**
224 **S2-S4**. Concentration at time zero is considered as 100% of recovery. In the case of non-
225 centrifuged IWW, the three compounds were generally stable at -20 °C, 4 °C, and 20 °C
226 for up to one month as reported by *Causanilles et al* (Causanilles et al., 2017b).
227 Oppositely, a slight increase in recovery (up to 140%) was observed for THC-COOH at
228 all temperatures (**Figure S-2**). Desorption of THC-COOH present in the suspended solids
229 is the most probable reason for the increase in concentration, yet an interconversion of
230 compounds, due to the transformation of THC-OH to THC-COOH by oxidation might
231 also occur although less likely (Ramin et al., 2017). However, the obtained data cannot
232 support any of these hypotheses since a mixed spiking solution was used, and the real
233 solid used is not an authentic blank due to the presence of all three compounds on it.

234 The stability data of the analytes in centrifuged IWW presented more variability. All
235 compounds were stable up to 1 month at 4 °C and -20 ºC, as reported previously
236 (Causanilles et al., 2017a; González-Mariño et al., 2018; Heuett et al., 2015). However,
237 in the case of THC-COOH (**Figure S-2**) and THC-OH (**Figure S-3**), notable losses were
238 observed when stored at 20 ºC after two weeks. These results illustrate the relevance of
239 appropriate storage conditions of the samples, with the recommendation of storing the
240 samples at -20 ºC, if analysis cannot be performed within 14 days after sample reception.
241 If analysis is performed within 14 days, the samples could be stored at 4 ºC without
242 significant loss of analytes. Anyway, further research is necessary by spiking samples
243 individually with each analyte to clarify the possible interconversion mentioned above.

244

3.2. Liquid-liquid extraction of raw IWW

Previous publications reporting the use of LLE for THC-COOH extraction from IWW (González-Mariño et al., 2018; Pandopulos et al., 2020b; Tscharke et al., 2016) were used as a guide in the performed 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 extraction of THC-COOH from wastewater (Pandopulos et al., 2020b). In the present work, HX:EA (2:1, *v/v*) was chosen as the extraction solvent, as it resulted in the best recoveries (**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, 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 (Causanilles et al., 2017b; Pandopulos et al., 2020a). The waiting time between the addition of ILIS to the IWW and the addition of HCl (1 M) was also evaluated at 20 min, 2 h, and overnight (14 h), obtaining the most reproducible results when the ILIS was added and let stand minimum for 2 h before starting the LLE process.

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 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 low RSDs (≤10%), and 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.

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	*	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.

3.3. Analysis of the liquid phase

In this study the liquid phase of the centrifuged IWW samples was extracted using both LLE and SPE separately and the performance of the two approaches was compared (**Figure 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 non-spiked and spiked IWW samples. Oasis HLB cartridges led to good recoveries (82 - 130%) and were selected for subsequent experiments (**Table S-3).** In parallel, LLE was also tested for the extraction of the liquid phase of IWW, and both procedures were finally validated (**Table 2).**

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)	Concentration 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.

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 in LLE for all compounds studied (**Table 2**).

In order to obtain more data to compare both procedures, seven consecutive samples (*i.e.,* in whole week) were processed using both methods. Concentrations of THC-COOH showed deviations < 30% in 6 out of the 7 samples analyzed when comparing data from 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).

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

3.4. Analysis of suspended solids

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 solid in each sample aliquot used for validation. To try to overcome this situation, the samples subjected to validation were shaken vigorously until all solid particles were floating homogeneously in the liquid

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 below 20% were obtained 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)	Concentration 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 mass extracted from the entire pellet and the volume of raw IWW sample (25 mL).

This method was applied to the suspended solids of the seven samples mentioned in Section 3.3 of which the results are shown in **Figure 3**. The amount of biomarker quantified in the suspended solids of 25 mL of IWW was converted in ng/L. 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 were removed to obtain the average of each biomarker present in the suspended solids.

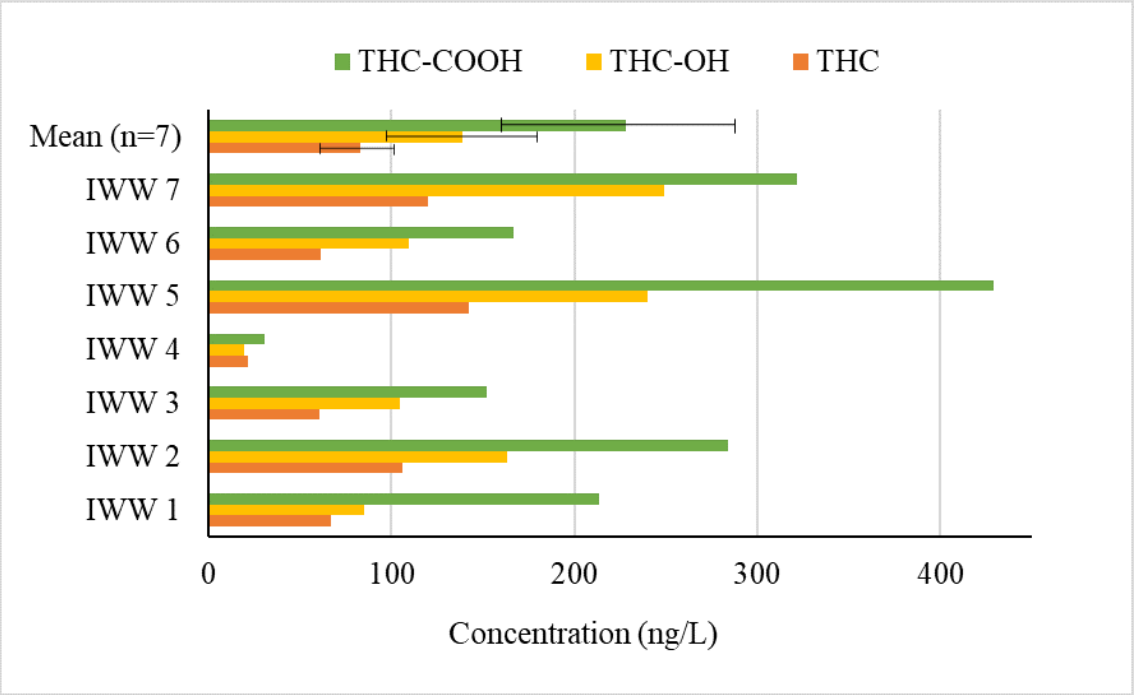

Figure 3. Cannabis biomarkers in the suspended solids of seven influent wastewater samples.

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. 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 wastewater analysis on cannabis biomarkers.

3.5. Analysis of raw 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) (**Figure 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 rather similar to the sum of biomarkers in the suspended solids and in the liquid phase (either extracted by SPE or LLE) analyzed separately.

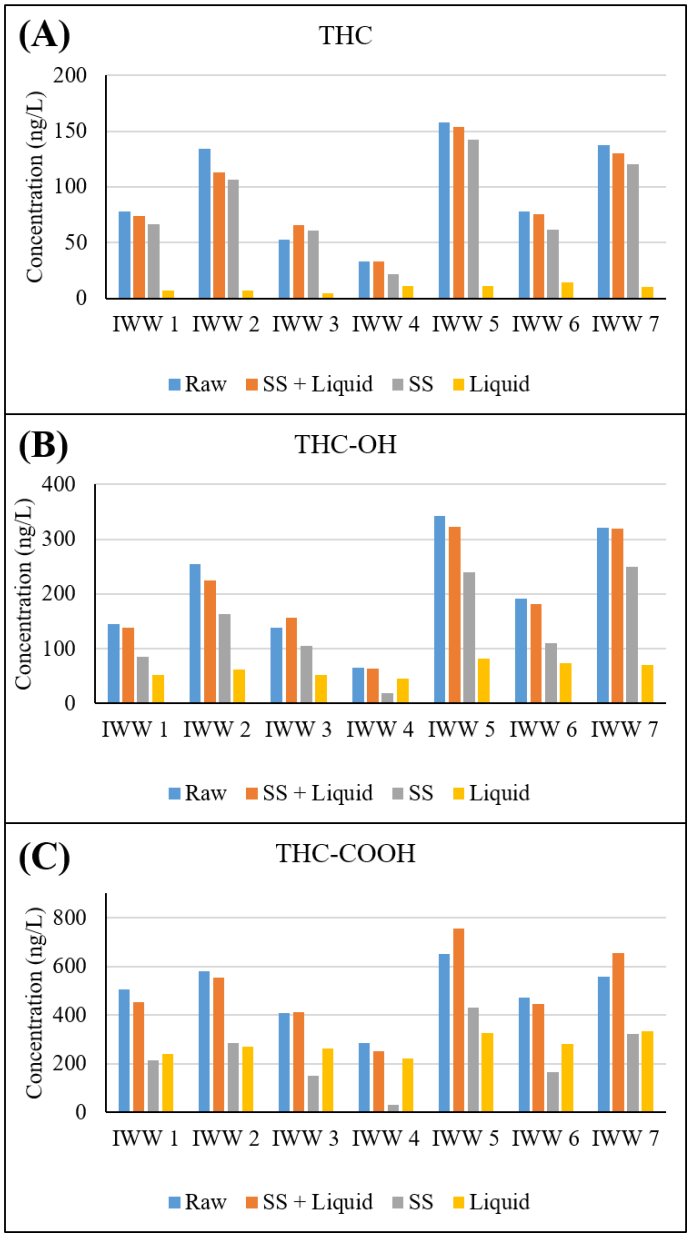

Figure 4. Analysis of (A) THC, (B) THC-OH and (C) THC-COOH in different phases of influent wastewater. Comparison of the whole raw influent wastewater (blue bar), the

liquid phase (yellow bar), the suspended solids (SS) (grey bar) and the sum of SS and the liquid phase analyzed separately (orange bar).

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:

(i) to analyze the raw IWW by LLE without separating the sample into the liquid and solid phase by filtration or centrifugation. This would imply the use of an extra aliquot of the sample and a dedicated LLE extraction procedure, in addition to that used for conventional analysis of other illicit drugs that it is normally based on SPE. The limited information available in relation to urinary and fecal excretion rates and representative sampling for solids are currently bottlenecks and, therefore this issue should be studied in more depth. In this context, a parameter such as turbidity to evaluate the suspended solids in the sample collected could give more insight.

(ii) to perform analysis of 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. However, the suitability and robustness of such a correction factor should be evaluated. This would include the analysis of a large number of wastewater samples collected from different locations with different composition 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);

392 Despite the important contribution of suspended solids to the total measured analytes in
393 the sample, there is still a knowledge gap in the possible adsorption or desorption
394 processes that may exist between the suspended solids and the liquid phase. Preliminary
395 sorption experiments have been performed in Milli-Q water containing an amount of
396 suspended solids, but no conclusive results have been obtained until now. Further
397 research is planned applying the use of THC-COOH-D_9 as an analogue to study the
398 behaviour and distribution of THC-COOH between both phases in different IWW.
399

4. Conclusions

In this research, an analytical method has been developed for cannabis biomarkers 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 allowed obtaining cannabis biomarker concentrations in both the liquid and solid phase. The separate analysis of the liquid and solid phase (e.g. after centrifugation of raw IWW) revealed that a high percentage of the compounds present in influent wastewater corresponded to the solid phase (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 do not consider their presence in the suspended solids. This, consequently, leads to an underestimation of the total biomarker amount present in IWW. Thus, 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. Hence, a better comprehension in the complexity of measuring cannabis biomarkers in IWW is given. Moreover, the preliminary results 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.

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