A new application of the Switchable Hydrophilicity Solvent-Based Homogenous Liquid–Liquid Microextraction to analyze synthetic cannabinoids in plasma by LC-MS/MS

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ABSTRACT

Synthetic cannabinoids are still a growing trend among drug users and consist of a group of hundreds of highly potent compounds. To investigate the use of such substances, sample preparation of biological matrices is a crucial step prior to instrumental analysis. Although different efficient extraction techniques have been proposed for that aim, they usually do not fit eco-friendly guidelines that have been gaining popularity in recent years, such as Green Analytical Toxicology. This work uses describes for the first time the use of switchable hydrophilicity solvent-based homogenous liquid–liquid microextraction (SHS-HLLME) for synthetic cannabinoids. This is a green technique that replaces highly toxic organic reagents for switchable hydrophilicity solvents (SHS), substances that can be either water-miscible or immiscible depending on their protonation. Thus, by simply adjusting the pH of the system, these SHS can be used as extraction solvents. A full optimization study including type of SHS, volume of protonated SHS, volume of NaOH, salting-out effect, and extraction time was perfomed. The optimized procedure consisted of precipitating the proteins of 300 µL of plasma with 300 µL of acetonitrile followed by centrifugation; evaporation of the organic solvent under N_2 stream; addition of 500 µL of the protonated DPA, DPA-HCl (6 M) (1:1, v/v); addition of 500 µL of NaOH (10 M); and finally centrifugation and evaporation. Validation results showed determination coefficients ≥ 0.99 for the 0.1-10 ng/mL linear range; 0.01-0.08 ng/mL as limit of detection; 0.1 ng/mL as limit of quantitation; accuracy and imprecision were within acceptable ranges; matrix effect, recovery, and process efficiency ranged from -55.6% to 185.9%, 36% to 56.7%, and 18.5% to 148.4%, respectively. The SHS-HLLME herein described was fully optimized providing satisfactory recoveries of 31 synthetic cannabinoids at low concentrations requiring only 300 µL of plasma. In addition, the validation results showed that the technique is a reliable eco-friendly alternative for clinical and toxicological analysis.

Keywords:

Synthetic Cannabinoids; New Psychoactive Substances; Switchable Hydrophilicity Solvent-Based Homogenous Liquid–Liquid Microextraction; SHS-HLLME; Switchable hydrophilicity solvents; Green Analytical Toxicology.

1. Introduction

Synthetic cannabinoid agonist receptors (SCRA), or simply synthetic cannabinoids, were first synthesized in the 1970s with the goal of understanding the endocannabinoid system [1, 2]. In the early 2000s, however, these synthetic compounds were found in the illicit drug market with the first reports dating to 2008 with JWH-018 in Austria and Germany [3]. This was the beginning of an unprecedented phenomenon that eventually flooded the illicit drug market with hundreds of novel chemicals, which were soon named New Psychoactive Substances (NPS). A total of 1,182 have been reported so far and are grouped according to their pharmacological effects or chemical structure [4].

These synthetic substances that mimic the effects of Δ-9-tetrahydrocannabinol (THC) are artificially added to herbs and sold to drug users as alternatives to cannabis, although this has not been the only presentation reported [5, 6]. In addition, synthetic cannabinoids became one of the most important groups of NPS due to a large number of substances and chemical variety. Up to December 2022, 224 SCRA were being monitored by the European Union Early Warning System and this group has been among the most detected NPS in the last decade [4]. As new compounds continue to appear in the drug market, slight to more drastic changes in their chemical structures have been observed, as shown in Fig. 1. Due to such differences, NPS tend to circumvent the existing legislation and can be legally commercialized – reason why they are also referred to as “legal highs”. This chemical complexity makes it difficult for professionals and analytical methodologies to keep up-to-date to deal with this threat. Thus, with the increasing number of novel chemically diverse compounds flooding the illicit drug market, the development of new analytical techniques suited to analyze such substances is warranted.

The development of techniques aiming to analyze SCRA on biological specimens, such as blood [7, 8], serum [9], plasma [10, 11], urine [11-15], oral fluid [16-18], and hair [19, 20], have been reported. To that end, sample preparation is mandatory for analyte extraction and preconcentration prior to analysis. In fact, this is the main challenge during the development of any analytical method addressed to drugs of abuse on biological samples, especially SCRA due to the low concentration that they are found associated with matrix complexity. Thus, solid-phase extraction [11, 15] and liquid-liquid extraction (LLE) [9, 17, 18] have been the traditional sample preparation procedures for drug analysis in decades, which have also been applied for SCRA. However, these techniques have many disadvantages and the use of alternatives have been

explored and encouraged [21]. For instance, methods using magnetic solid-phase extraction [10], micro-solid-phase extraction with molecularly imprinted polymers [14], dispersive liquid–liquid microextraction (DLLME) [7, 16], or protein precipitation [8, 12, 13] to the analysis of SCRA on biological specimen have been reported. Nevertheless, although such alternatives are an improvement, they still have shortcomings, such as using hazardous organic solvents (chloroform, toluene, etc.), large sample volumes (> 500 µL), and requiring expensive reagents or devices. These practices have been discouraged by the growing trend of finding more environmentally friendly alternatives during method development, such as it is postulated by Green Analytical Toxicology (GAT) [21].

The use of switchable hydrophilicity solvents (SHS) has been proposed in 2010 for the extraction of soybean oil [22]. These SHS are molecules with poor polarity but can become completely water-miscible by simply switching the pH of the system. This change in polarity occurs due to the protonation or deprotonation of specific functional groups in their chemical structures, e.g. amidines and amines. The switchable hydrophilicity solvent-based homogenous liquid–liquid microextraction (SHS-HLLME) makes use of that chemical feature to extract non-polar substances from aqueous samples, such as drugs of abuse and pharmaceuticals from biological specimens [23-27]. Briefly, the SHS is mixed with a concentrated acid solution so the excess of protons in the system protonates the amino group of the SHS making it positively charged, thus allowing polar interactions, such as hydrogen bonds. In this state, the SHS is hydrophilic and forms a miscible solution with aqueous samples, such as urine or plasma. Thereafter, to separate an organic layer and extract substances with low polarity, a base is then added in excess enough for the hydroxyl groups to neutralize the protons, increase the pH, and consequently deprotonate the SHS. In its neutral state, non-polar interactions are favored instead of hydrogen bonds, thus the SHS has a hydrophobic behavior and is immiscible in the aqueous sample, forming a two-layered system (Fig. 2). Although the SHS-HLLME technique is somewhat recent, few applications have been reported and it is already considered a promising green alternative for sample preparation [28]. Therefore, considering SHS-HLLME merits further studies, we applied this technique to the analysis of SCRA for the first time and we describe in this article the full optimization and validation process covering 31 compounds.

2. Materials and methods

2.1 Chemicals and reagents

All reagents were of analytical or HPLC grade. Methanol, acetonitrile, chloroform, toluene, hexane, ethyl acetate, dipropylamine (DPA), N,N-dimethylcyclohexylamine (DMCHA), sodium chloride (NaCl), hydrochloric acid (HCl), sodium hydroxide (NaOH), ammonium formate, and formic acid were

purchased from Merck (Darmstadt, Germany). Ultrapure water was produced using a Milli-Q system (Millipore, Billerica, Massachusetts).

All analytes and internal standards (IS) were acquired from Cerilliant Corporation (Round Rock, Texas, EUA). The stock solutions were available at 1.0 mg/mL, while for JWH-210-OH-*d*5 was at 100 µg/mL. For validation, the standard solutions were diluted and a pool containing all analytes at 1 µg/mL was prepared. Nine different concentrations, 0.6, 1.2, 1.5, 3, 4.8, 6, 30, 48, and 60 ng/mL, were then prepared from this pool and used to fortify blank plasma samples to obtain the calibration curve and quality controls (QC). All solutions were diluted in methanol and stored at −20 °C.

2.2 Liquid chromatography-tandem mass spectrometry

A Waters ultra-performance liquid chromatography (UPLC) Acquity System equipped with an Acquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 µm) was used for all analyses. This system was coupled to a Quattro Premier XE mass spectrometer (MS) (Waters Corporation, Milford, MA). Mobile phase A was ammonium formate (pH 3.1, 1 mM)-formic acid (999:1, v/v) and mobile phase B was methanol-formic acid (999:1, v/v). The method was kept at a constant flow rate of 0.3 mL/min with column oven temperature at 40 °C. The chromatographic gradient was as follows: initially, 10% of B for 0.5 min, ramp to 70% within 3.5 min, which was kept for 2.5 min; second ramp to 100% of B within 2 min, which was kept for another 2 min. Initial conditions were restored in 1 min and kept for 1.5 min to re-equilibrate the system. The method's run time was 13 min and 7.5 µL of the extracts were injected.

The multiple reaction monitoring (MRM) approach was used for all analytes with three transitions for each compound and the MS was operated in positive ionization mode. The electrospray capillary voltage was set to 2.5 kV, desolvation gas flow rate to 1,100 L/h, cone gas flow rate to 200 L/h, desolvation temperature to 450 °C, and source temperature to 120 °C. The capillary voltage, collision energy, and *m/z* transitions used as quantifiers and qualifiers for each analyte, and retention times are displayed in Table S1.

2.3 Sample preparation

The extraction procedure consisted of two steps: i) protein precipitation and ii) SHS-HLLME technique.

2.3.1 Protein precipitation

Three hundred microliters of acetonitrile were added to a 300 µL aliquot of human plasma to obtain protein precipitation. In addition, 40 µL of the pool of analytes and 20 µL of the IS were added to each vial to obtain the intended concentrations. Then, vials were vortexed for 10 s, centrifuged (5 min at 6000 rpm), and the supernatant was transferred to a thin glass vial in which the acetonitrile was evaporated under a gentle N_2 stream at room temperature.

2.3.2 SHS-HLLME technique

Once the acetonitrile used for protein precipitation was evaporated, 500 µL of a DPA-HCl (6 M) (1:1, v/v) mixture were added to the samples and briefly vortexed until complete miscibility of the SHS (≈ 2 s). Next, 500 µL of NaOH (10 M) were added, vortex for 5 s, and 2 min later centrifuged for 5 min at 6000 rpm. Finally, the organic layer was transferred to autosampler vials, evaporated to dryness under N_2 stream at room temperature, and resuspended with 50 µL of mobile phase A.

2.4 Method optimization

Two-way Analysis of Variance (ANOVA) was used for univariate analysis and a factorial screening 2^k followed by a central composite design (CCD) were used for multivariate statistical analyses. In both statistical approaches, significance was considered when $p < 0.05$, as for multivariate analyses, the adjusted coefficient of determination (r^2) obtained by ANOVA served to determine the percent of data variance explained by the model. All data were processed using GraphPad Prism® 8 and Statistica® 10 software.

2.5 Method validation

The method was validated for the quantitation of 31 SCRA in plasma samples according to the ANSI/ASB standard 036, 1st Edition 2019 guide for method validation in Forensic Toxicology, and supporting literature [29, 30]. Therefore, linearity, limit of detection (LOD), limit of quantitation (LOQ), bias, precision, selectivity, carryover, matrix effect (ME), recovery (RE), and process efficiency (PE) were the parameters evaluated.

2.5.1 Linearity

The linear range encompasses the plasmatic concentrations in which SCRA are usually found [9, 31]. To determine the linearity, five calibration curves each composed of six concentrations were freshly prepared and analyzed on the same day. Each concentration was prepared using fortified blank plasma samples and the linear regression was accepted when $r^2 \geq 0.99$. In addition, heteroscedasticity weighting was applied whenever necessary.

2.5.2 Limit of detection and limit of quantitation

To establish the LOD values, fortified blank samples in decreasing concentrations were experimentally tested. Plasma from three different donors was employed to ensure reproducibility. The LOD for each analyte was then determined as the concentration capable of providing a signal-to-noise ratio of, at least, 3:1 with variations in the retention times not exceeding ± 0.05 min. In addition, the ratio of daughter ions for each analyte should be consistent with higher concentrations.

The LOQ established for all analytes was the first concentration of the calibration curve. To establish this value as LOQ, three samples from different donors were analyzed on three different days with freshly prepared calibration curves. The identification and quantitation evaluated were: bias (± 20%), precision (≥ 80%), signal-to-noise ratio (≥ 3:1), and retention times (± 0.05 min).

2.5.3 Bias and precision

The evaluation of bias and precision was performed by preparing three QC levels using blank plasma samples. The specimens were fortified to achieve the intended concentrations and analyzed on five different days with a freshly prepared calibration curve for each run. Bias should not exceed ± 20% and precision should be ≥ 80%. Both within-run and between-run precisions were assessed and are expressed as coefficient of variation (CV).

2.5.4 Matrix effect, recovery, and process efficiency

The ME, RE, and PE were examined according to Matuszewski et al. [29]. To that end, the three QC were used in three different experiments. In set 1, neat standards prepared in mobile phase A were analyzed. In set 2, blank plasma samples were extracted and the analytes were added to the organic supernatant, evaporated under N_2 stream, and injected into the instrument. In set 3, blank plasma samples were fortified before the extraction procedure. For all sets, five replicates of each QC level were used, while each blank samples used for sets 2 and 3 were from different donors. The ME was calculated by dividing set 2 by set 1, RE by dividing set 3 by set 2, and PE by set 3 by set 1.

2.5.5 Selectivity

The selectivity of the method was evaluated with three different approaches. First, ten blank plasma samples from different subjects were extracted and analyzed by the method to assess the presence of common endogenous interferents. Next, neat standards of exogenous substances were prepared in mobile phase A at a 100 ng/mL concentration and injected into the instrument. The common drugs analyzed were amphetamine-type stimulants (amphetamine, methamphetamine, 3,4-methylenedioxymethamphetamine, 3,4-methylenedioxyamphetamine, cathinone, methcathinone, methylone, dimethylone, methedrone, butylone, mephedrone, eutylone, 4-chloroethcathinone, pentedrone, dibutylone, N-ethylpentedrone, dipentylone, N-ethylpentylone, and N-ethylheptedrone), cannabinoids (THC, cannabidiol, and cannabinol), cocainics (cocaine, benzoylecgonine, cocaethylene, and anhydroecgonine methyl ester), opioids (morphine, 6-acetylmorphine, and codeine), antidepressants (paroxetine, fluoxetine, norfluoxetine, sertraline, amitriptyline, nortriptyline, imipramine, and desipramine), benzodiazepines (midazolam, nitrazepam, nordazepam, oxazepam, flunitrazepam, bromazepam, alprazolam, clonazepam, and diazepam), barbiturates (phenobarbital, secobarbital, and pentobarbital), and others (caffeine, nicotine, and cotinine).

Finally, as JWH-210-OH-d5 was used as IS, the presence of non-labeled molecules (i.e. JWH-210-OH) as impurity or product of degradation was assessed. To that end, a blank plasma sample was fortified only with the labeled IS (JWH-210-OH-d5) and another only with the SCRA aimed by the method. Samples were then extracted and analyzed by the method. For the three interference studies described above, no interfering peaks should be visualized in the analyte's detection window.

2.5.6 Carryover

The carryover effect was investigated by analyzing three consecutive blank injections after a 10-fold higher concentration (100 ng/mL) was analyzed. There should not be any analyte signal in the blank injections.

3. Results and discussion

3.1 Preliminary experiments

The first experiment performed in this study sought to find a microextraction technique suited to analyze SCRA in plasma that would also fit GAT guidelines [21]. With that in mind, DLLME seemed a good candidate, for it is simple, fast, and efficient. Thus, a screening of common organic solvents used for cannabinoid extraction was performed and as expected, the mixture of chloroform-methanol (1:3, v/v) yielded the best recovery for most analytes (Fig. 3). In the same experiment, however, the SHS-HLLME was also included in the comparison. The technique was performed using two SHS with basic properties (DPA and DMCHA) and simultaneously compared with DLLME in terms of analyte recovery. The only DLLME mixture that provided better recoveries than that of chloroform-methanol was toluene-acetonitrile (1:3, v/v) for APINACA (24%) and ACHMINACA (49%). In contrast, when SHS-HLLME was employed with DPA, extraction of JWH-210, APINACA, ACHMINACA, and JWH-175 was 20%, 226%, 423%, and 175% better than DLLME, respectively. Better recoveries were also observed with DMCHA for APINACA (45%) and ACHMINACA (100%) (Fig. 3). This preliminary finding suggested that SHS-HLLME could be a good candidate for the aimed purpose. Interestingly, data published by Xu and colleagues agree with these findings as they compared DLLME with SHS-HLLME, and showed that both yielded similar analyte recovery [25]. However, although these miniaturized techniques are fast, cheap, and simple to perform, DLLME has more disadvantages, e.g. more steps are involved and higher volumes of more toxic solvents are commonly used. It is also worth pointing out that chloroform is highly hazardous and its use is strongly discouraged by eco-friendly guidelines [21]. With that in mind, we decided to investigate the use of SHS-HLLME as a sample preparation for analyzing SCRA in plasma samples.

A following experiment was carried out to confirm which SHS would work better for the target analytes. As shown in Fig. 4a, the extraction using DMCHA

yielded statistically significant higher recoveries for 5-fluoro EDMB-PICA, PB-22, MDMB-4en-PINACA, APINACA, and ACHMINACA, while DPA was only better for 4-cyano MMB-BUTINACA. This could be explained by the higher hydrophobicity of DMCHA compared to DPA (LogP = 1.9 and 1.6, respectively), which represents it has a higher affinity for non-polar substances, such as the synthetic cannabinoids herein studied. However, except for the aforementioned SCRA, both SHS yielded similar recoveries for most of the aimed analytes ($p > 0.05$). Hence, DPA was chosen to favor analytes with lower intensities, such as 4-cyano MMB-BUTINACA (Fig. 4a). As a result, this experiment confirms that SHS-HLLME can efficiently extract the aimed analytes from plasma samples at low concentrations (1 ng/mL), thus the technique was further optimized to improve its efficiency.

3.2 SHS-HLLME optimization

For the optimization study, four conditions that could significantly impair analyte recovery were identified and screened in a full factorial screening 2^4. As the type of SHS was studied in previous experiments, it was not included in the present screening (Fig. 4). Thus, the volumes of the DPA-HCl (6 M) (1:1, v/v) mixture and NaOH (10 M) (200-500 µL for both solutions), addition of NaCl (60 mg), and extraction time (0-5 min) were examined. The only statistically significant variables were the volumes of DPA-HCl and NaOH (Fig. 4b and c). Both these factors were then studied in a CCD to find the optimum volumes that would yield the best response, i.e. analyte recovery. For all SCRA analyzed, the most important factor was the volume of NaOH and optimum responses were achieved with 400 to 600 µL (Fig. 4d and e). In addition, for SCRA that the volume of DPA-HCl was also relevant, the highest analyte recovery was achieved with volumes ranging from 400 to 600 µL as well (Fig. 4d and e).

The addition of salts, such as NaCl, also known as salting-out effect, can improve the extraction of drugs from biological samples by reducing their solubility in the aqueous phase. However, previously published works have studied the salting-out effect in SHS-HLLME and showed no positive impact on analyte recovery [25-27, 32]. This was also observed in the present work, as adding NaCl did not improve the extraction of SCRA from plasma samples (Fig. 4b and c). After NaOH is added to trigger SHS deprotonation, HCl is neutralized forming H_2O and NaCl. It is plausible to assume that the NaCl formed during this step is enough to produce the salting-out effect or that it indeed has no impact on SHS-HLLME. Another factor that was not relevant for SCRA recovery was the extraction time (Fig. 4b and c). After NaOH is added, different times have been considered for analyte extraction and phase separation (0-10 min) [23-25, 27, 33]. Based on such reports, we evaluated up to 5 min of extraction time but no statistical difference was observed in overall SCRA recovery (Fig. 4b and c). This could be explained by the infinitely large surface area between SHS and sample, making the transfer of analytes to the organic layer very fast. Altogether, both

these parameters make the technique simpler and faster, as no addition of salt is required and analyte extraction happens quickly.

In contrast to the previous parameters, the volumes of DPA-HCl and NaOH solutions were statistically significant for the technique, of which higher volumes improved analyte recovery (Fig. 4d and e). As more volumes of DPA-HCl and NaOH were added to the samples, more organic supernatant was formed and capable of being recovered. That could explain the higher analyte recovery achieved when a combination of 400-600 µL of both DPA-HCl and NaOH was employed. On the other hand, high volumes of DPA-HCl (> 300 µL) combined with low volumes of NaOH (< 300 µL) do not suffice for triggering the pH switch and SHS deprotonation, therefore was incompatible with the technique. Thus, 500 µL of each solution was chosen. It is worth mentioning that such volume is not an inconvenience as it corresponds to only 250 µL of DPA that is considerably less hazardous than other solvents commonly used in microextraction techniques, e.g. chloroform and toluene [28]. In addition, the concentrated acid and base solutions neutralize each other producing H_2O and NaCl, also contributing to the greenness of the technique.

3.3 Method validation

Linearity, LOD, and LOQ were established according to relevant plasmatic concentrations of SCRA [9, 31]. Therefore, the linear range and LOQ were defined as 0.1 to 10 ng/mL and 0.1 ng/mL, respectively (Table 1). In addition, a statistical analysis showed a lack of homogeneity in the calibration model, thus the 1/x weighting was applied for all analytes providing $r^2 \geq 0.99$. As for LOD, the lowest concentration was aimed, thus the values achieved varied among analytes (0.01 to 0.08 ng/mL) (Table 1). Based on available literature, these values should suffice for detecting SCRA in plasma when not possible to quantitate [9, 31].

The accuracy and imprecision for all SCRA were assessed on five different days by analyzing the three QC levels. The within-run precision was higher than 85% for all analytes, which is represented by CV < 15% (Table 1). As for between-run precision, there were only 3 analytes with CV values > 15%, 4-cyano MMB-BUTINACA, JWH-081, and JWH-175 (18.8%, 17.4%, and 11%, respectively). In addition, the accuracy of the method was also high for all analytes (> 90%), with the exceptions being JWH-122, JWH-210, and APINACA with biases of 11.8%, 10.6%, and 12.4%, respectively (Table 1). Thus, even though the between-run imprecision and bias were > 15% and ± 10%, respectively, for three of the analytes covered by the method, these are still within acceptable limits, indicating the technique has satisfactory accuracy and precision for the intended purpose.

The ME study showed that most SCRA were affected by intense ion enhancement (> 50%), especially ADB-FUBIATA, 5-fluoro EMB-PINACA, JWH-073, and ACHMINACA with values > 100% (Table 2). Curiously, analytes with high chemical similarity and eluting very closely were affected with considerably

different ME, such as 5-fluoro MDMB-PINACA (≈ 0.4%), 5-fluoro EMB-PINACA (≈ 129.4%), and 5-fluoro EDMB-PICA (≈ 78.1%) or also APINACA (≈ -50.5%) and ACHMINACA (≈ 121.0) (Tables S1 and 2). On the other hand, RE was between 36 and 56.7%, and PE ranged from 18.5 to 148.4% (Table 2). Although the ME observed in the present study was intense for some of the aimed SCRA, it did not impact bias and precision, hence is in agreement with validation guidelines [30]. In addition, intense ME for SCRA has been reported elsewhere with other techniques [9]. In the last step of interferents assessment, no relevant signal in the analytes' detection windows and carryover effect was observed. Thus, the method proved to be selective for the aimed SCRA suffering no relevant interference from common endogenous and exogenous substances.

As proof of applicability, the method was employed to analyze one authentic sample from a known user of marijuana. Analysis showed the presence of MDMB-4en-PINACA at 0.81 ng/mL, although the subject reported having no intention of using synthetic cannabinoids. A representative chromatogram from the authentic sample is presented in Fig. S1 and this confirms that the method can be used for real sample analyses.

We presented a full optimization followed by validation of the proposed SHS-HLLME resulting in a technique suited to efficiently detect and quantitate 31 synthetic cannabinoids at low concentrations. The present method requires only 300 µL of plasma samples, which can be considered a low volume if compare with other works. For example, Hwang and co-workers described a micro-solid-phase extraction to analyze SCRA using only 180 µL of plasma [10]. However, the method can be complex and time-consuming for it has more steps than the SHS-HLLME reported herein and requires over 7 min of only vortexing and sonication. In addition, 300 µL of toluene were used to extract each sample and only LODs above 2 ng/mL were achieved. Although the work focuses on 13 SCRA and other drugs of abuse, such drawbacks as using highly toxic extraction solvents, expensive devices, and having a complex analytical pipeline are not in agreement with GAT guidelines [10]. In contrast, Kneisel and Auwärter proposed a highly sensitive analytical method in which an LLE was developed and validated to analyze 27 SCRA on serum samples [9]. Briefly, 1 mL of samples were extracted with 1.5 mL of a hexane-ethyl acetate (99:1, v/v) mixture, mixed for 5 min, and centrifuged for another 20 min. The reported LODs ranged from 0.01 to 2 ng/mL and were similar to the values achieved with the SHS-HLLME reported in the present work, especially for the SCRA included in both techniques: JWH-210 (0.07 ng/mL); JWH-203 (0.05 ng/mL); JWH-019 and RCS-8 (0.03 ng/mL); JWH-073, JWH-081, JWH-122, RCS-4, AM-2201, and AM-2233 (0.02 ng/mL); and JWH-015 (0.01 ng/mL) (Table 1) [9]. Adamowicz and Tokarczyk have published a method for the screening of 143 drugs of abuse, which included 4 SCRA. The sample preparation consisted of precipitation of 200 µL of blood with 600 µL of acetonitrile added 50 µL at a time with a 10 s-vortexing after each addition. Although the approach of using only protein precipitation is simple, the

procedure described could be time-consuming when analyzing multiple samples simultaneously. In comparison, the SHS-HLLME proposed herein takes similar time and involves fewer pipetting steps. In addition, the LODs reported for the SCRA in their work ranged from 0.07 to 0.46 ng/mL, of which AM-2201 was the only analyte included in both works (0.09 ng/mL against 0.03 ng/mL achieved by the SHS-HLLME) (Table 1) [8].

As for DLLME in SCRA analysis, we have found two works. In the first one, Odoardi et al. used 500 µL of whole blood and 350 µL of chloroform-methanol (1:2.5, v/v) as extraction and dispersive solvents, respectively [7]. In this screening method, 70 NPS from different groups were included, but the LODs achieved for synthetic cannabinoids were inferior (≥ 0.2 ng/mL) to those obtained by the SHS-HLLME herein described (Table 1). On the other hand, Tomai et al. reported a DLLME for the analysis of 6 SCRA in oral fluid using 500 µL of sample followed by protein precipitation with 1 mL of acetonitrile [16]. Although the technique was highly sensitive yielding LODs of 0.002-0.021 ng/mL, chloroform was used as extraction solvent. In addition, when taking the analytes included in both methods into comparison, the LODs achieved in the present work were over 2-fold higher than the DLLME reported by Tomai et al., JWH-073 (0.021 ng/mL) and JWH-019 (0.018 ng/mL) (Table 1), indicating that the SHS-HLLME could also be a valuable technique to analyze oral fluid [16]. In terms of simplicity of handling, DLLME and SHS-HLLME can be somewhat equivalent. For example, previously published articles show the use of dry ice in SHS-HLLME, which can be inconvenient and prone to analytical variability [27, 32, 33]. This can be easily overcome by using basic solutions, such as NaOH, to change the pH of the system, thus improving reproducibility [23-26, 34]. In contrast, DLLME by default requires more steps, e.g. the addition of the extraction and dispersive solvents, buffer solutions, NaCl, and even water and protein precipitation in some cases [7, 16]. Hence, these techniques could be used as alternatives to each other allowing the simplest and most effective to be chosen.

To our knowledge, SHS-HLLME was mostly used in the analysis of food and urine, while only one other work has applied it to plasma samples [23]. As a result, the use of a protein precipitation step was necessary and this was achieved by the addition of an organic solvent. Although efficient, acetonitrile needed to be removed through evaporation prior to the extraction itself (Section 2.3) and this additional step is laborious and time-consuming. In that regard, Scheid et al. have reported an interesting alternative by using zinc sulfate for protein precipitation instead of organic solvents [23]. This approach dismisses this evaporation step and the zinc sulfate is not incompatible with the extraction as organic solvents are. This should be taken into consideration for future applications of SHS-HLLME, especially for samples that require protein precipitation.

The number of published methods for SCRA analysis in blood-related matrices are scarce. These specimens, such as plasma, are particularly relevant in cases of acute toxicity or pharmacokinetic studies, for example. In addition, synthetic cannabinoids have been shown to be extensively metabolized producing a great number of metabolites that have to be included in urine screenings [12]. However, new substances are constantly introduced into the drug market and the process to identify such biotransformation products is complex and time-consuming. As a result, many SCRA remain undetected until comprehensively studied, hence analyzing the parent compounds in blood or plasma can circumvent that limitation until more information on metabolites is available. Overall, the SHS-HLLME described in the present work can be a valuable tool in that regard, especially if considered that a compound belonging to the new emerging SCRA group called OXIZIDs was included in the method [5, 35] (Tables S1, 1, and 2). This shows that the technique reported herein can be used for synthetic cannabinoids in general and has a potential application for those that appear in the illicit drug market in the future.

4. Conclusions

The SHS-HLLME technique is somewhat recent and only few applications have been reported. This work described the first application of this SHS-based extraction for the analysis of synthetic cannabinoids on plasma samples. Method optimization showed that only 250 µL of DPA were required for 300 µL of plasma to achieve satisfactory sensitivity. Moreover, the salting-out effect is dismissed and analyte extraction is achieved in extremely short times, making SHS-HLLME simpler and faster than DLLME. Although some limitations still need to be addressed, such as solvent evaporation, this SHS-based technique is a promising eco-friendly alternative for drug analysis, including the major NPS group of synthetic cannabinoids.

Author contributions

André Luis Fabris: Conceptualization, Methodology, Validation, Investigation, Writing – original draft. Aline Franco Martins: Writing – review. Jose Luiz Costa: Resources, Authentic samples, Writing – review, Funding acquisition. Mauricio Yonamine: Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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.

Acknowledgements

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo [grant numbers 2021/09857-8, 2020/10809-5], Conselho Nacional de

Desenvolvimento Científico e Tecnológico [grant numbers 142056/2020-0, 303275/2017-0, 315640/2021-9] and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES/PROCAD [INSPEQT Project, grant number 16/2020].

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FIGURE/TABLES CAPTIONS

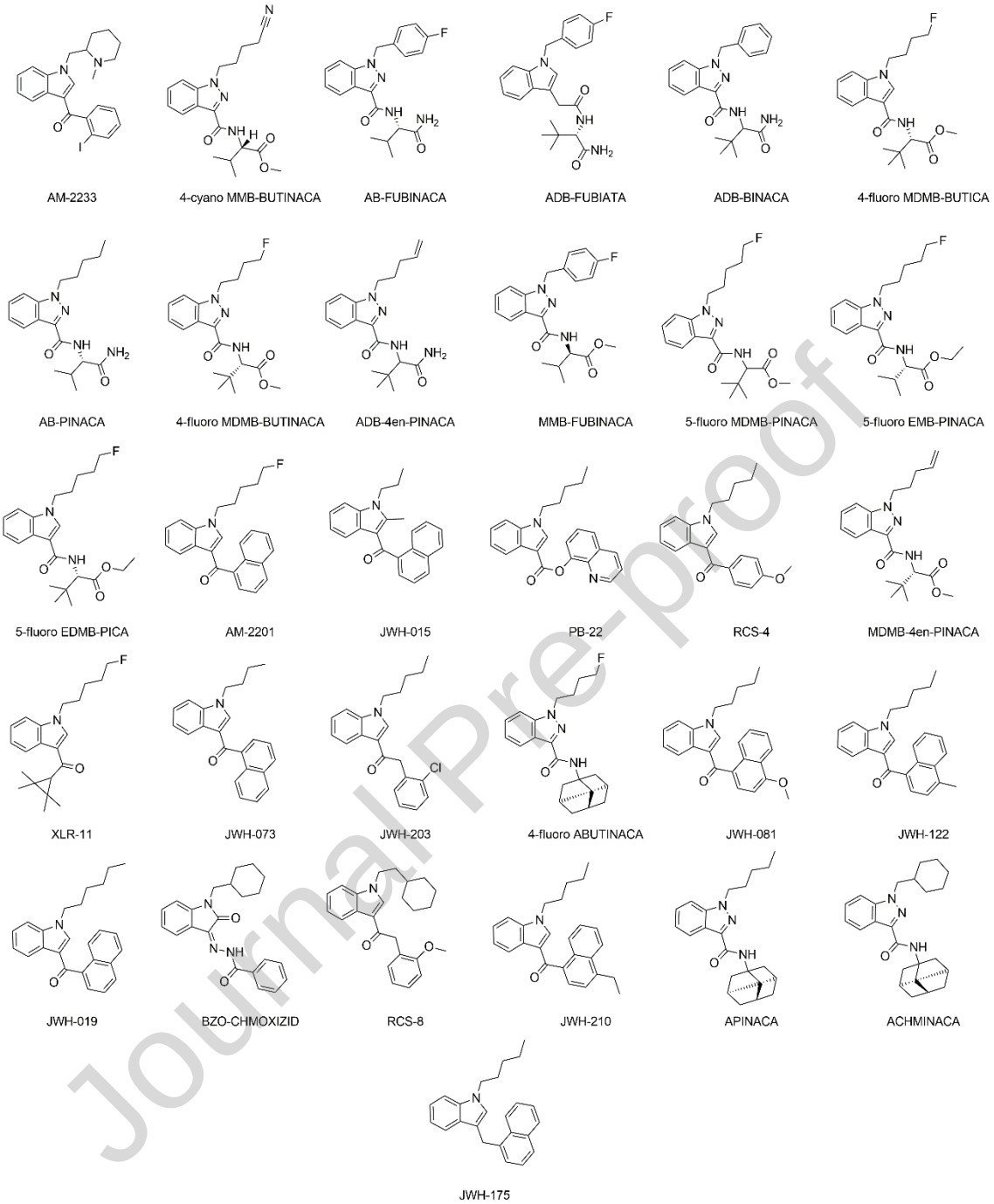

Figure 1. Chemical structures of all synthetic cannabinoids covered by the method.

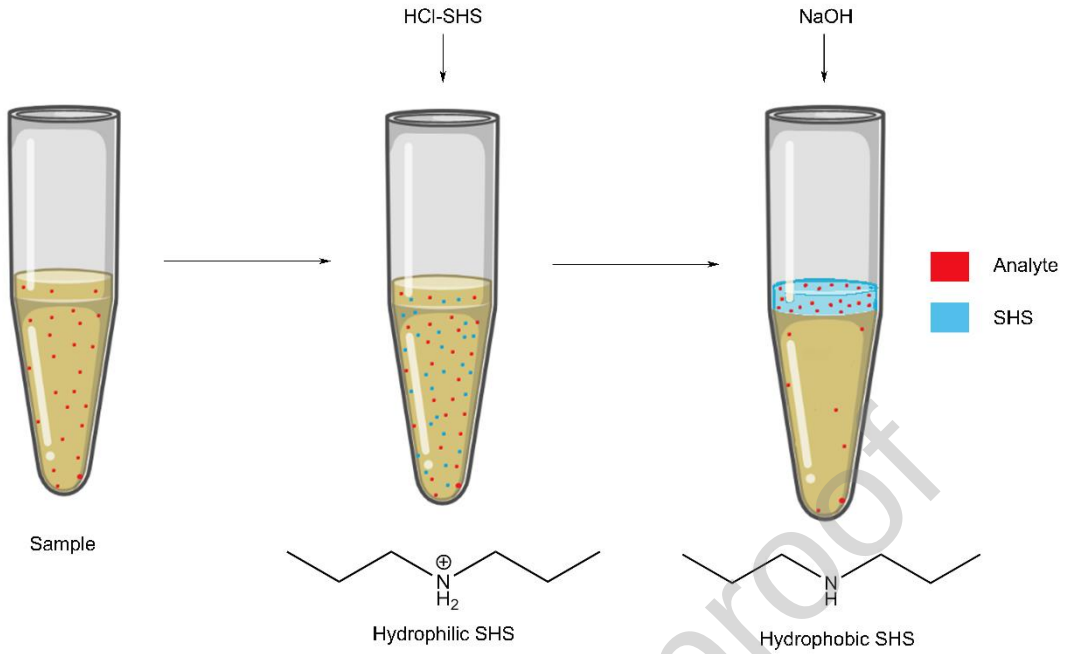

Figure 2. Principle of SHS-HLLME. The SHS is mixed with a proton donor substance (HCl) and then added to an aqueous sample (human plasma). The protonation of the SHS increases its hydrophilicity, making it miscible with the plasma sample. Finally, addition of a base (NaOH) deprotonates the SHS making it once again hydrophobic and immiscible to the sample; as a consequence, it is possible to collect the organic layer from the vial. SHS-HCl: mixture of the switchable hydrophilicity solvent with HCl (i.e. protonated SHS).

Analyte	DLLME							SHS-HLLME	
	Toluene-acetonitrile (1:1.5)	Toluene-acetonitrile (1:3)	Toluene-acetonitrile (1:5)	Hexane-methanol (1:1.5)	Hexane-methanol (1:3)	Ethyl acetate-acetonitrile (1:1.5)	Ethyl acetate-acetonitrile (1:3)	DPA	DMCHA
AM-2233	-74%	-79%	-78%	-63%	-63%	-96%	-95%	-47%	-85%
4-cyano MMB-BUTINACA	-59%	-76%	-74%	-93%	-96%	-92%	-93%	-35%	-78%
AB-FUBINACA	-77%	-78%	-76%	-99%	-99%	-94%	-94%	-31%	-78%
ADB-BINACA	-81%	-78%	-77%	-97%	-98%	-94%	-95%	-39%	-68%
4-fluoro MDMB-BUTICA	-72%	-75%	-72%	-84%	-83%	-93%	-95%	-26%	-63%
AB-PINACA	-79%	-81%	-81%	-97%	-97%	-95%	-96%	-56%	-76%
4-fluoro MDMB-BUTINACA	-76%	-77%	-75%	-89%	-82%	-95%	-97%	-31%	-65%
5-fluoro EDMB-PICA	-90%	-80%	-78%	-96%	-91%	-97%	-98%	-35%	-64%
AM-2201	-98%	-84%	-79%	-100%	-98%	-98%	-98%	-38%	-67%
JWH-015	-98%	-82%	-78%	-100%	-97%	-98%	-100%	-30%	-67%
PB-22	-97%	-81%	-78%	-100%	-96%	-96%	-98%	-24%	-61%
RCS-4	-98%	-83%	-78%	-100%	-97%	-98%	-98%	-31%	-66%
MDMB-4en-PINACA	-93%	-81%	-78%	-98%	-95%	-97%	-98%	-28%	-64%
XLR-11	-97%	-82%	-78%	-100%	-97%	-98%	-98%	-25%	-63%
JWH-073	-98%	-81%	-76%	-100%	-97%	-97%	-98%	-24%	-63%
JWH-203	-97%	-80%	-76%	-100%	-97%	-97%	-98%	-24%	-64%
4-fluoro ABUTINACA	-96%	-81%	-76%	-100%	-96%	-97%	-98%	-25%	-64%
JWH-081	-95%	-74%	-69%	-100%	-96%	-96%	-96%	-10%	-56%
JWH-122	-94%	-71%	-65%	-100%	-96%	-96%	-96%	-3%	-53%
JWH-019	-94%	-70%	-65%	-100%	-95%	-96%	-95%	-3%	-52%
RCS-8	-94%	-68%	-62%	-100%	-95%	-96%	-95%	-38%	-49%
JWH-210	-81%	-36%	-32%	-100%	-85%	-88%	-86%	20%	-11%
APINACA	-68%	24%	-18%	-100%	-70%	-85%	-77%	226%	45%
ACHMINACA	-49%	49%	-13%	-100%	-64%	-77%	-72%	423%	100%
JWH-175	-80%	-8%	-36%	-100%	-71%	-94%	-90%	175%	-26%

Figure 3. Heat map showing the recoveries achieved with DLLME and SHS-HLLME. Each value represents the relative recovery of each mixture compared to chloroform-methanol (1:3, v/v) in DLLME. Negative values indicate lower recoveries, while positive values indicate higher recoveries than those of chloroform-methanol were achieved. SHS-HLLME for was performed with a SHS-HCl (6 M) (1:1, v/v) solution for both SHS. DPA: dipropylamine; DMCHA: N,N-dimethylciclohexylamine.

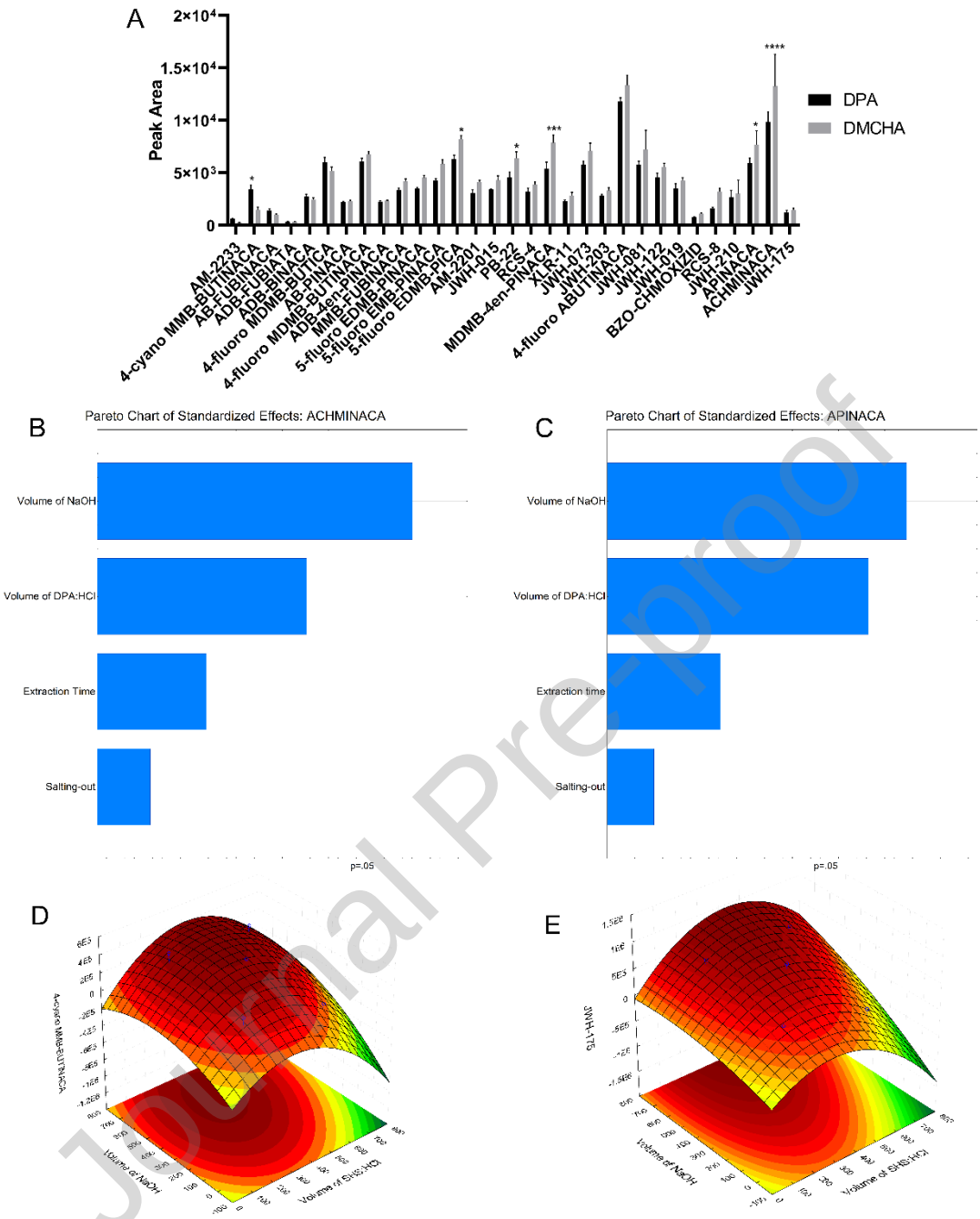

Figure 4. Optimization of the SHS-HLLME. Comparison between analyte recoveries yielded by two different SHS (A). Pareto charts showing the significant variables among those screened in the Factorial Screening (B and C). Surface response graphs of the significant variables that were previously identified, volumes of DPA-HCl and NaOH, indicating the optimum conditions for SCRA extraction (D and E). Two-way ANOVA followed by post-hoc Tukey Test was used for graph A (* $p < 0.05$; *** $p < 0.001$; **** $p < 0.001$), while bars crossing the red line indicate $p < 0.05$ in graphs B and C. SHS: switchable hydrophilicity solvent; DPA: dipropylamine; DMCHA: dimethylhexylamine

Table 1. Validation results for all analytes covered by the proposed SHS-HLLME.

Analyte	LOD (ng/mL)	LOQ (ng/mL)	Linear range (ng/mL)	QC (ng/mL)	Bias (%)	Imprecision (%)	
						Within-run	Between-run
AM-2233	0.08	0.1	0.1-10	0.2 0.8 8.0	2.4 3.6 3.9	13.0 8.6 10.4	14.7 11.8 10.5
4-cyano MMB-BUTINACA	0.03	0.1	0.1-10	0.2 0.8 8.0	4.1 1.6 3.4	14.2 12.9 10.2	18.8 17.4 10.8
AB-FUBINACA	0.03	0.1	0.1-10	0.2 0.8 8.0	2.1 2.7 4.5	5.1 5.9 10.6	9.9 7.0 11.5
ADB-FUBIATA	0.08	0.1	0.1-10	0.2 0.8 8.0	0.8 2.3 4.4	8.6 5.2 9.4	11.4 7.3 9.8
ADB-BINACA	0.03	0.1	0.1-10	0.2 0.8 8.0	1.4 2.1 3.9	9.3 3.2 5.7	11.4 5.8 7.3
4-fluoro MDMB-BUTICA	0.03	0.1	0.1-10	0.2 0.8 8.0	4.3 6.7 4.2	10.7 10.5 8.2	11.2 11.6 9.8
AB-PINACA	0.05	0.1	0.1-10	0.2 0.8 8.0	7.7 5.6 5.6	11.9 10.2 6.8	14.9 11.1 7.8
4-fluoro MDMB-BUTINACA	0.03	0.1	0.1-10	0.2 0.8 8.0	4.0 5.0 4.5	8.4 8.6 6.6	10.0 9.2 6.5
ADB-4en-PINACA	0.03	0.1	0.1-10	0.2 0.8	-0.6 9.1	2.4 8.9	5.9 9.7

				8.0	6.5	9.5	10.2
MMB-FUBINACA	0.05	0.1	0.1-10	0.28 0.28 8.0	-2.73 .4 .0	10.71 .1 .6	12.08 0 7
5-fluoro MDMB-PINACA	0.05	0.1	0.1-10	0.28 0.28 8.0	6.53 .4 .5	6.03 .0 .3	7.06 5.34 5.3
5-fluoro EMB-PINACA	0.03	0.1	0.1-10	0.28 0.28 8.0	-1.12 .7 .7	8.82 .5 .9	11.54 4.75 5.5
5-fluoro EDMB-PICA	0.03	0.1	0.1-10	0.28 0.28 8.0	6.63 .9 .3	8.06 .2 .7	8.61 7.14 7.4
AM-2201	0.03	0.1	0.1-10	0.28 0.28 8.0	1.41 .9 .4	4.96 .8 .1	7.27 7.75 5.1
JWH-015	0.01	0.1	0.1-10	0.28 0.28 8.0	3.54 .6 .3	6.59 .8 13.5	13.4 10.9 14.5
PB-22	0.01	0.1	0.1-10	0.28 0.28 8.0	5.36 .4 .5	4.09 .3 .5	8.611 .0 .8	10
RCS-4	0.01	0.1	0.1-10	0.28 0.28 8.0	0.75 .3 .8	5.45 .1 2.3	7.65 5.12 .6
MDMB-4en-PINACA	0.01	0.1	0.1-10	0.28 0.28 8.0	2.23 .5 .3	6.08 .2 .7	8.310 .3 9.2

XLR-11	0.03	0.1	0.1-10	0.28	0.05.78.0	9.81.74.8	12.013.27.7
JWH-073	0.01	0.1	0.1-10	0.28	7.71.01.3	12.19.06.5	14.312.39.5
JWH-203	0.03	0.1	0.1-10	0.28	-0.71.0-0.6	11.27.99.2	13.311.311.4
4-fluoro ABUTINACA	0.01	0.1	0.1-10	0.28	-0.74.3-1.4	8.71.80.29	9.411.912.1
JWH-081	0.03	0.1	0.1-10	0.28	5.06.48.9	14.810.911.43	17.412.415.3
JWH-122	0.01	0.1	0.1-10	0.28	8.74.61.81	12.55.59.2	13.78.510.9
JWH-019	0.01	0.1	0.1-10	0.28	3.44.7-0.5	7.51.90.45	10.112.012.6
BZO-CHMOXIZID	0.03	0.1	0.1-10	0.28	-0.64.54.5	13.02.77.2	14.43.79.1
RCS-8	0.08	0.1	0.1-10	0.28	2.11.34.8	13.511.45.8	14.414.17.8
JWH-210	0.05	0.1	0.1-10	0.28	10.63.2	11.91.59	13.17.0

				8.0	4.0	3.7	5.6
APINACA	0.08	0.1	0.1-10	0.2 0.8 8.0	12.4 4.3 5.0	7.8 2.5 4.3	10.9 3.5 6.0
ACHMINACA	0.08	0.1	0.1-10	0.2 0.8 8.0	6.3 -1.3 3.9	9.9 7.8 6.7	12.1 10.1 9.7
JWH-175	0.08	0.1	0.1-10	0.2 0.8 8.0	7.0 7.7 5.7	1.2 8.1 6.1	14.1 10.5 11.0

LOD: limit of detection; LOQ: limit of quantitation; QC: quality controls.

Table 2. ME, RE, and PE values for the synthetic cannabinoids at each QC level. ME: matrix effect; RE: recovery; PE: process efficiency.

Analyte	ME (%)			RE (%)			PE (%)		
	low	medium	high	low	medium	high	low	medium	high
AM-2233	3.9	1.2	6.2	6.4	7.7	6.9	6.7	2.5	3.9
4-cyano MMB-BUTINACA	6.2	12.5	3.8	2.9	0.8	1.3	0.2	5.7	9.8
AB-FUBINACA	3.9	2.9	3.2	3.3	3.9	1.3	3.6	9.6	6.7
ADB-FUBIATA	43.3	42.1	53.7	9.7	7.2	9.3	20.9	14.3	9.7
ADB-BINACA	4.8	3.9	9.1	6.0	4.6	9.3	1.2	4.2	8.6
4-fluoro MDMB-BUTICA	4.3	7.9	3.4	5.3	7.1	1.7	9.9	9.6	3.9
AB-PINACA	1.6	3.6	1.8	4.2	1.5	0.9	1.4	7.9	6.2
4-fluoro MDMB-BUTINACA	7.1	7.5	2.3	2.3	1.9	1.4	5.0	0.1	7.3
ADB-4en-PINACA	2.5	2.5	4.9	3.6	5.8	2.5	0.8	9.9	5.8

MMB-FUBINACA	9.5	7.0	4.4		0.1	8.0	6.3		1.9	8.2	5.2
5-fluoro MDMB-PINACA	.0	.4	2.1		4.4	0.7	0.1		7.1	0.9	9.3
5-fluoro EMB-PINACA	41.3	29.4	26.5		1.3	2.4	0.5		9.7	7.2	1.8
5-fluoro EDMB-PICA	4.0	8.1	2.4		7.3	3.7	2.0		7.0	7.8	2.3
AM-2201	9.1	1.3	6.0		7.6	4.9	1.2		5.6	7.9	0.2
JWH-015	5.7	3.5	6.1		3.9	0.9	0.1		1.4	0.9	6.7
PB-22	1.2	8.9	8.5		6.2	1.7	1.4		9.0	0.4	1.5
RCS-4	7.5	.1	.5		1.0	4.0	3.8		9.9	8.0	4.0
MDMB-4en-PINACA	4.5	2.7	.9		0.5	1.0	2.6		7.8	6.2	3.0
XLR-11	1.1	.6	.9		5.3	3.6	1.0		0.4	5.6	3.0
JWH-073	09.9	01.5	07.3		4.4	0.5	0.6		3.3	1.7	4.2
JWH-203	2.8	7.6	6.2		4.0	3.2	1.4		7.3	8.0	4.6
4-fluoro ABUTINACA	0.9	2.2	8.1		1.0	5.8	1.6		2.0	4.2	5.7
JWH-081	0.6	5.8	8.6		3.7	0.9	9.3		3.3	7.3	6.3
JWH-122	5.6	5.8	9.4		3.9	1.8	0.9		2.6	5.1	1.1
JWH-019	2.6	8.7	4.0		6.8	3.1	2.1		0.8	2.8	0.7
BZO-CHMOXIZID	7.0	6.2	1.7		6.7	3.1	6.5		3.4	1.6	9.0
RCS-8	5.4	16.8	14.1		1.2	9.7	0.7		5.9	3.1	5.0
JWH-210	45.5	55.6	51.9		7.9	1.5	9.6		6.1	8.5	9.1
APINACA	46.2	50.5	49.3		8.9	0.9	0.9		0.9	0.3	0.8
ACHMINACA	85.9	21.0	11.0		1.9	2.6	7.5		48.4	4.2	9.2
JWH-175	2.3	1.0	2.3		6.0	8.6	7.9		2.0	8.3	7.6

The concentrations for the low, medium, and high QC levels are: 0.2, 0.8, and 8 ng/mL, respectively. ME: matrix effect; RE: recovery; PE: process efficiency.

Table S1. MRM parameters for the SCRA covered by the method.

Figure S1. Chromatogram of the authentic sample analyzed by the proposed technique. The chromatogram on the top represents the synthetic cannabinoid found in the sample, MDMB-4en-PINACA, and on the bottom the IS used for all analyses, JWH-210-OH-*d*5.

CRediT authorship contribution statement

André Luis Fabris: Conceptualization, Methodology, Validation, Investigation, Writing – original draft. Aline Franco Martins: Writing – review. Jose Luiz Costa: Resources, Authentic samples, Writing – review, Funding acquisition. Mauricio Yonamine: Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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.

Conflict of interest

The authors declare no conflict of interest.

Highlights

- A new technique using switchable hydrophilicity solvents was proposed.
- Green Analytical Toxicology guidelines were aimed during method development.
- Simultaneous analysis of 31 synthetic cannabinoids on plasma samples.
- SHS-HLLME is a promising eco-friendly technique for toxicological analyses.