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Determination of synthetic cannabinoids in oral fluids by liquid chromatography with fluorescence detection after solid-phase extraction ☆

P. García-Atienza, H. Martínez-Pérez-Cejuela, E.F. Simó-Alfonso, J.M. Herrero-Martínez, S. Armenta*

Analytical Chemistry Department, Universitat de València, 50th Dr. Moliner St., Burjassot 46100, Spain

A R T I C L E I N F O

Method name:
Determination of synthetic cannabinoids in oral fluids by liquid chromatography with fluorescence detection after solid-phase extraction

Keywords:
Synthetic cannabinoids
Liquid chromatography-fluorescence detection
Oral fluid samples

A B S T R A C T

Synthetic cannabinoids are one of the most consumed new psychoactive substances, being absolutely necessary the development of analytical methodologies for the determination of these substances in biological fluids. In this study, a liquid chromatography with fluorescence detection (LC-FD) method has been developed for the analysis of 8 synthetic cannabinoids in oral fluids. The method has been validated in terms of linearity, precision and extraction recoveries, giving limits of detection as low as 0.7 μg L^{-1}, and limits of quantification of 2.6 μg L^{-1}. Different silica and polymeric commercial solid sorbents such as C18, Supel-Select HLB, EB2 Extrabond® and SampliQ-OPT were tested, concluding that Supel-Select HLB provided quantitative recoveries for the extraction of synthetic cannabinoids in oral fluids.

- Analysis of synthetic cannabinoids in oral fluids.
- Analytical procedure based on liquid chromatography with fluorescence detection.
- Sample treatment based on solid phase extraction with HLB cartridges.

Specifications table

Subject area:	Chemistry
More specific subject area:	Analytical Chemistry, Forensic Analysis
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☆ **Related research article** P. García-Atienza, F.A. Esteve-Turrillas, S. Armenta, Evaluation of the in-vitro inhalation uptake of MDMB-4en-PINACA using a smoking simulation chamber, Microchem. J. 181 (2022) 107,737. https://doi.org/10.1016/j.microc.2022.107737
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* Corresponding author.
E-mail address: Sergio.armenta@uv.es (S. Armenta).

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Method details

Background

According to the United Nations Office on Drugs and Crime (UNODC) Early Warning Advisory (EWA) on new psychoactive substances (NPS), synthetic cannabinoid receptor agonists are the second largest group of substances, only preceded by stimulants [1]. Synthetic cannabinoids receptor agonists are functionally like Δ^9-tetrahydrocannabinol (THC), the main psychoactive compound in marijuana, both interact directly with the endocannabinoid system, although synthetic cannabinoids have greater potential [2]. They appeared in the 70′s, being initially developed as therapeutic agents in pain treatment. Despite this fact, at the beginning of this century, they began to appear in the illicit drug market [3], and in 2008 synthetic cannabinoid receptor agonists were detected for the first time as cannabis substitutes in herbal preparations [4].

Nowadays, according to the European Monitoring centre for Drugs and Drug Addiction (EMCDDA), synthetic cannabinoids receptor agonists are one of the most seized NPS in Europe, being monitored more than 220 different synthetic cannabinoids since their appearance in 2008. Furthermore, around 6000 deaths related to these substances were reported in 2020 [5]. Currently, they are sold as herbal mixtures for smoking. These adulterated herbals appeared around 2004, being its main route of consumption inhalation through conventional or/and electronic cigarettes [6].

The selection of a biological fluid for drug analysis depends on the specific purpose of the testing [7]. Among the different available biological fluids for drug analysis, UNODC recommends urine as the preferred sample matrix [8], since it allows easy accessibility, and all drug metabolites are practically excreted in the urine, being the detection window of those metabolites higher than in blood. However, urine has also remarkable drawbacks including partial/complete analyte metabolization, being impossible the detection of the parent compound, and sampling procedures could invade individual privacy.

On the other hand, the metabolism of synthetic cannabinoids is often unknown and it is usual to find the same metabolite from different parent compounds [9]. For this reason, blood is the preferred fluid to analyze drugs in the original version taking into account the “under-the-effects” window [10]. However, the analysis of blood requires a really invasive sampling procedure (venepuncture) executed by trained personal. In this context, alternative biological fluids, such as sweat and oral fluid (OF), have been gaining popularity as forensic specimens overcoming the above-mentioned drawbacks. OF presents the advantages of easy sample collection by non-invasive procedures, reduced risk of adulteration due to the direct observation of sampling and presence of the parent compounds instead of metabolites. In this sense, it can be used for rapid on-site screening, which is really interesting in forensic and clinical applications [11]. However, the main limitation is the high heterogeneity of OFs, therefore, sampling procedure may influence on the final concentration of the analyzed substances [12].

OF matrix often contains numerous compounds such as proteins or enzymes that can hinder the analyte determination. As a result, a sample clean-up is commonly necessary to remove the interferents present in this type of matrices. Furthermore, extraction approaches typically increase the sensitivity of the method, providing significant enrichment factors, which allow the analysis of NPS at trace levels. Among the available techniques, solid-phase extraction (SPE) and liquid phase microextraction (LPME) are the most commonly employed pretreatment procedures. Concretely, SPE presents some intrinsic advantages such as simplicity and quantitative recoveries, which are highly desirable. In addition, it allows pre-concentration of the sample in cases where the drug content in the sample is very low. Indeed, it has been previously used for the on-line extraction of synthetic cannabinoids and metabolites in OFs by using polystyrene-divinyl benzene sorbents [13] and the off-line version by using cyclodextrin-silica porous sorbents [14].

Liquid chromatography tandem mass spectrometry (LC-MS/MS) [15,16] and gas chromatography tandem mass spectrometry (GC–MS) [17,18] are often used for the analysis of synthetic cannabinoids in OFs. However, this instrumentation is highly-sophisticated and very expensive. The need of analytical procedures based on high performance instrumentation, which provides appropriate analytical features but, at the same time, assumable acquisition costs has led us to develop an analytical procedure based on the combination of SPE, using commercially available sorbents, and liquid chromatography with fluorescence detector (LC-FD).

Oral fluid sampling

Synthetic cannabinoids-free OF samples were collected from males and females ranging from 25 to 40 years old after freely-given informed consent. Spitting has been the method of choice for oral fluid sampling. This method provides several advantages, such as easy collection of sample, low cost and minimization of the evaporation of saliva in case of long-time samplings, among others. Due to the low homogeneity of the sample (emulsion (foam), mucin aggregates and aqueous phase), spitting should be followed by a homogenization step. OF samples were collected by direct expectoration or spitting into Eppendorf tubes. OF samples contaminated with food and/or other debris from the mouth were centrifuged, the supernatant was separated, homogenized using a vortex stirrer and 1.0 mL of oral fluid was loaded into the SPE cartridge. Synthetic cannabinoids-free OF samples were pooled and immediately analyzed or stored at −20 °C until their analysis by the recommended procedure to assess the absence of synthetic cannabinoids. Then, 1.0 mL of blank OFs were spiked with synthetic cannabinoids to obtain final concentrations ranging from 5 to 50 μg L^{-1}.

It should be highlighted that under no circumstances have the authors trafficked or provided illegal substances, aimed, promoted, facilitated, stimulated, or forced in any way the consuming of illegal substances.

Preparation of standard solutions

Synthetic cannabinoids receptor agonists standards, including 5F-NPB 22, ADB-CHMICA, ADB-CHMINACA, 5F-ADB, MDMB-FUBINACA, MMB-CHMICA, MDMB-4en-PINACA and MDMB-CHMCZCA were acquired from LGC standards (Barcelona, Spain), Sigma-Aldrich (St. Louis, EE. UU.) and "Laboratorio de control de Drogas de la Delegación de gobierno de la Comunidad Valenciana" (Valencia, Spain). Table 1 shows different physico-chemical properties of evaluated compounds, including their molecular weights and chemical structures. Concentrated standard solutions were prepared at 1000 mg L^{-1} concentration level in methanol and stored in amber glass vials at −15 °C. From these stock solutions, a standard solution of 100 mg L^{-1} containing all the evaluated synthetic cannabinoids was prepared in methanol and stored in amber glass vials at −15 °C. Finally, this solution was further diluted with methanol to obtain daily standard solutions with different concentration levels ranging from 5 to 1000 μg L^{-1}.

Extraction procedure

Commercial SPE extraction cartridges, including a C18 silica-based sorbent and three different polymeric materials such as HLB, EB2 and OPT, were connected to the Visiprep™ SPE vacuum manifold (Sigma-Aldrich, Bellefonte, PA, USA). In this study, Extrabond C18 200 mg/3 mL from Scharlau (Barcelona, Spain); Supel-Select HLB 60 mg/3 mL, acquired from Sigma-Aldrich; EB2 Extrabond 60 mg/3 mL, commercially available from Scharlau; and SampliQ-OPT 60 mg/3 mL from Agilent (Santa Clara, CA, USA) were used for analyte extraction. Extraction procedure implies cartridge conditioning with 1.0 mL of methanol followed by equilibration with 1.0 mL ultrapure water to remove the excess of organic solvent, at a flow rate lower than 3.0 mL min^{-1}. Next, 1.0 mL sample was loaded into the cartridge at 1.0 mL min^{-1}, before the ultrapure water from the equilibration step was completely run through.The washing step was performed with 1.0 mL deionized water at 1.0 mL min^{-1} flow rate and cartridges were dried in the vacuum manifold for 5 min. Finally, 1.0 mL methanol was used for the elution of synthetic cannabinoids and this extract was injected into the LC system after its filtration through 0.45-μm polytetrafluoroethylene filter.

Chromatographic conditions

Agilent 1100 Series equipped with G1322A degasser, a G1311A quaternary pump and a G1328A fluorescence detector was used for chromatographic method development. A Scharlau Kromaphase 100 C18 column (150 × 4.6 mm, 5 μm) was employed for analyte separation. The column was kept at room temperature during the separation. The mobile phase composition was (A) water and (B) acetonitrile. The gradient was adjusted to a linear gradient from 75% B to a 100% B in 8 min; and finally, a 100% B isocratic elution was maintained during 4 min. Flow rate of mobile phase was 1.0 mL min^{-1}, the injection volume was set at 20 μL. The most appropriate excitation and emission wavelengths of synthetic cannabinoids were established using a FP-750 spectrofluorometer from Jasco (Madrid, Spain). The excitation wavelength was set at 297 nm, and the most appropriate emission wavelength were selected for each evaluated synthetic cannabinoid.

Method validation

The effect of four commercial cartridges based on different sorbent materials (C18 silica-based and polymeric Supel-Select HLB, EB2 Extrabond® and SampliQ-OPT) were evaluated during the validation of the analytical method using OF samples spiked with synthetic cannabinoids at 400 μg L^{-1} concentration level. EB2 Extrabond® is based on pyrrolidone-modified spherical polystyrene divinylbenzene polymer. SampliQ-OPT is an amide modified divinyl benzene polymer. Supel-Select HLB is comprised of a hydrophobic (divinylbenzene) and a hydrophilic component (N-vinylpyrrolidone). The last sorbent employed is the traditional reversed-phase material C18 modified silica particles.

Fig. 1 shows the recoveries obtained for the extraction of spiked OF samples using the aforementioned SPE cartridges.

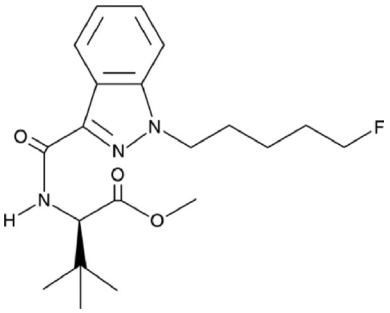
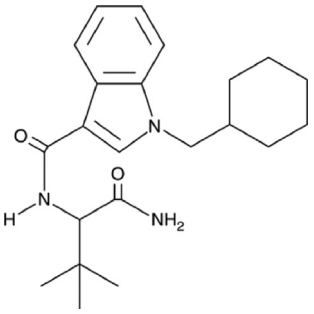
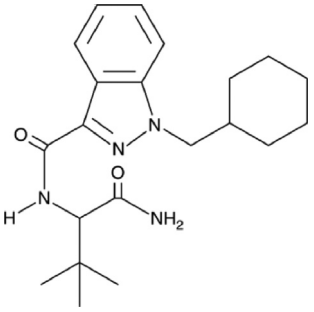
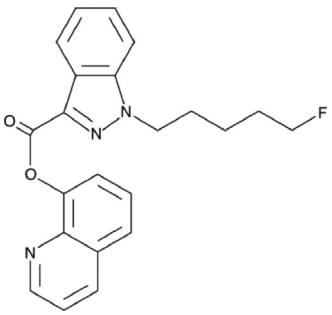
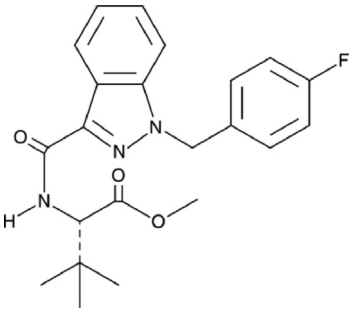
As it can be seen in Fig. 1, synthetic cannabinoids recoveries provided by HLB are slightly higher than those obtained by C18, EB2 and OPT cartridges. In addition, recovery values for most of the evaluated synthetic cannabinoids are higher than 80%, with relative standard deviation values lower than 8%.

Different elution volumes, from 250 to 1000 μL, were tested for synthetic cannabinoid elution in order to increase the preconcentration factor. However, recoveries of most of the analytes substantially decreased, especially those obtained for MDMB-CHMCZCA, which has the highest log *P* value (6.6). Thus, 1.0 mL methanol was proposed as elution volume.

On the other hand, different organic solvents, such as methanol, acetonitrile and mixtures of them (50:50 methanol:acetonitrile) were evaluated as elution solvent. In all the cases, recoveries obtained were statistically comparable and, thus, methanol was selected for further analysis.

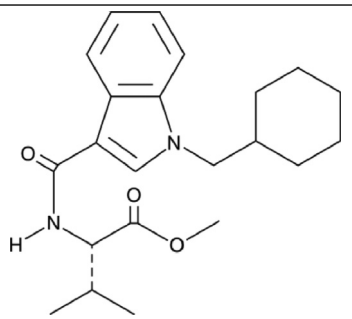
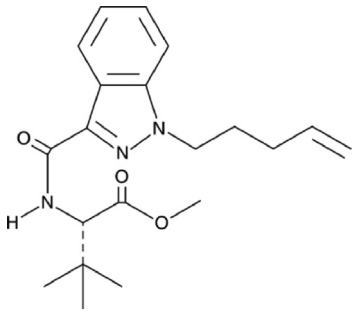
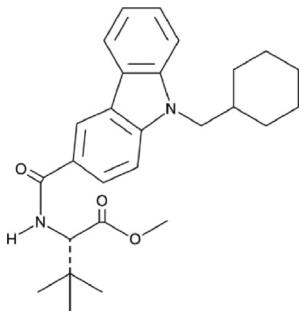
To assess a complete elution of the evaluated synthetic cannabinoids from the SPE column, 1.0 mL OF was spiked at 100 μg L^{-1} concentration level and was loaded into the Supel-Select HLB SPE column. After the washing step, three consecutive 1.0 mL methanol elution volumes were percolated. Recoveries obtained from the first fraction ranged from 73 to 96%, whereas recoveries from the second fraction varied from 2 to 6%, and those of the third fraction were below the LOQ of the procedure (see Fig. 2). Thus, considering both elution fractions, recoveries from the evaluated synthetic cannabinoids ranged from 79 to 99%, being MDMB-CHMCZCA the most affected by the second elution fraction varying from 73 ± 3 to 80 ± 3%. In order to avoid extra efforts in terms of time and reagents, only in those OFs where MDMB-CHMCZCA provides a positive result is recommended the use of a second methanol elution.

Table 1
Name, common abbreviation, molecular weight and chemical structure of the different analyzed synthetic cannabinoids.

Abbreviation	Name	M.W. (g mol^{-1})	Chemical Structure
5F-ADB	N-[[1-(5-fluoropentyl)−1H-indazol-3-yl]carbonyl]−3-methyl-D-valine, methyl ester	377.5	
ADB–CHMICA	N-[1-(aminocarbonyl)−2,2-dimethylpropyl]−1-(cyclohexylmethyl)−1H-indole-3-carboxamide	369.5	
ADB–CHMINACA	N-[1-(aminocarbonyl)−2,2-dimethylpropyl]−1-(cyclohexylmethyl)−1H-indazole-3-carboxamide	370.5	
5F–NPB-22	1-(5-fluoropentyl)−1H-Indazole-3-carboxylic acid, 8-quinolinyl ester	377.4	
MDMB-FUBINACA	N-[[1-[(4-fluorophenyl)methyl]−1H-indazol-3-yl]carbonyl]−3-methyl-L-valine, methyl ester	397.4	

(*continued on next page*)

Table 1 (*continued*)

Abbreviation	Name	M.W. (g mol^{-1})	Chemical Structure
MMB–CHMICA	N-[[1-(cyclohexylmethyl)−1H-indol-3-yl]carbonyl]-L-valine, methyl ester	370.5	
MDMB-4en-PINACA	3-methyl-N-[[1-(4-penten-1-yl)−1H-indazol-3-yl]carbonyl]-L-valine, methyl ester	357.5	
MDMB-CHMCZCA	N-[[9-(cyclohexylmethyl)−9H-carbazol-3-yl]carbonyl]−3-methyl-L-valine, methyl ester	436.6	

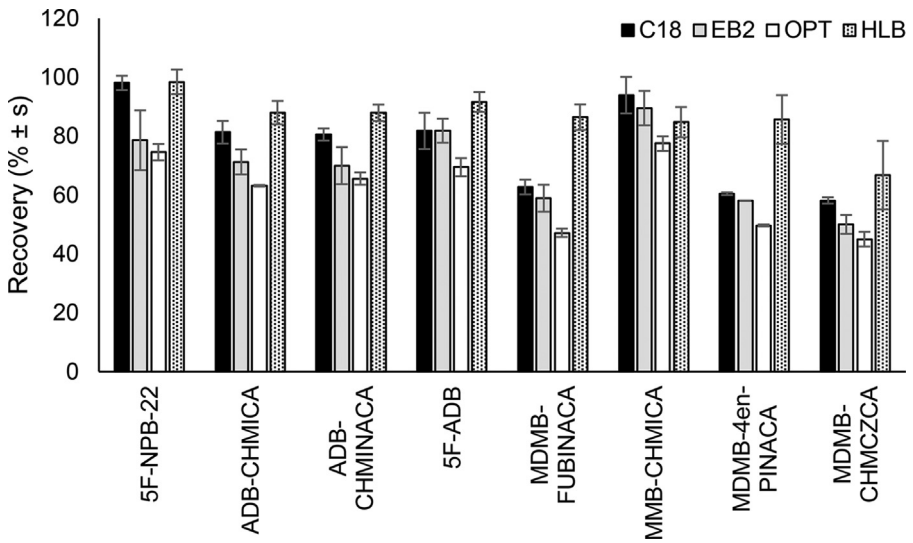


Fig. 1. Recoveries obtained for the analysis of OF samples spiked with synthetic cannabinoids at 400 μg L^{-1} concentration level using different sorbents for SPE extraction.

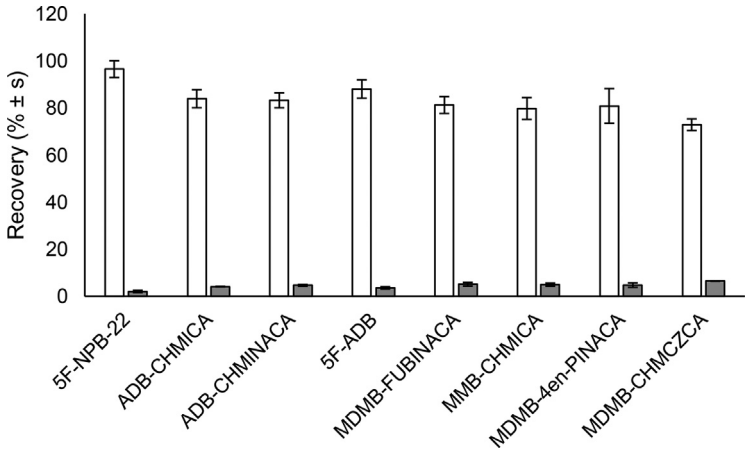


Fig. 2. Effect of different elution fractions on the total recovery of synthetic cannabinoids from OFs.

Table 2
Analytical features of the developed LC-FD procedure for synthetic cannabinoid determination.

Synthetic cannabinoid	Retention time (min)	Asymmetry factor	Peak resolution	Linear range (μg L^{-1})	R^2	LOD (μg L^{-1})	LOQ (μg L^{-1})	RSD (%)
5F-NPB-22	6.3	0.88	2.0	2.5–1000	0.999	0.7	2.5	4.8
ADB-CHMICA	6.9	0.92	2.5	2.3–1000	0.994	0.7	2.3	4.5
ADB-CHMINACA	7.9	0.91	1.1	2.2–1000	0.994	0.7	2.2	4.6
5F-ADB	8.2	0.94	0.9	2.6–1000	0.990	0.8	2.6	5.0
MDMB-FUBINACA	8.4	0.93	1.5	2.2–1000	0.996	0.7	2.2	4.3
MMB-CHMICA	8.8	0.91	2.0	2.3–1000	0.997	0.7	2.3	4.6
MDMB-4en-PINACA	9.4	0.89	7.3	2.3–1000	0.994	0.7	2.3	4.7
MDMB-CHMCZCA	11.6	0.92	–	2.1–1000	0.999	0.6	2.0	4.1

[a]LOD and LOQ calculated as three and ten times the standard deviation of the signal of 5 μg L^{-1} (n = 10) synthetic cannabinoid standard divided by the slope of the respective calibration curve.
[b]RSD established from ten independent measurements of a 5 μg L^{-1} spiked OF sample analyzed by the recommended procedure in different days.

Additionally, emission spectra of the synthetic cannabinoids, for a fixed excitation wavelength, set at 297 nm, were obtained. Fig. 3 shows the fluorescence spectra, which indicate the most appropriate emission wavelength for each analyzed synthetic cannabinoid, being selected 353 nm as the maximum emission wavelength for most of the synthetic cannabinoids. Others, such as ADB-CHMICA, MMB-CHMICA and MDMB-CHMCZCA, presented maximum emission wavelengths at 330, 340 and 375 nm, respectively. Thus, those values were selected for the analytical determination of synthetic cannabinoids.

Fig. 4 shows several LC-FD chromatograms corresponding to a standard solution of 50 μg L^{-1} of synthetic cannabinoids (Fig. 4a), a synthetic cannabinoid-free OF (Fig. 4b) and an OF sample spiked with 5 μg L^{-1} of synthetic cannabinoids (Fig. 4c).

As shown in Fig. 4, an adequate separation of the chromatographic peaks was obtained with the proposed experimental conditions. Peak symmetry and resolution values were provided in Table 2. In all the cases, asymmetry factor values close to the unity were obtained, indicating an appropriate peak shape without tailing nor fronting. On the other hand, resolution values higher than 1 were also obtained, being the most critical pairs those composed by ADB-CHMINACA – 5F-ADB and 5F-ADB – MDMB-FUBINACA. Furthermore, Fig. 4 emphasizes that the extraction procedure was adequate, being most of the components of the sample matrix eliminated or drastically reduced.

The proposed LC-FD procedure was validated in terms of linearity, precision, limits of detection (LOD) and quantification (LOQ) (see Table 2). Calibration curves were obtained, by ordinary least squares regression. Concentrations of seven standard solutions (evenly spaced) ranged from 5 to 1000 μg L^{-1} were prepared. The linearity of the method was established by analyzing the calibration curves of standard solutions from 2.2 to 1000 μg L^{-1}, obtaining a good linearity with correlation coefficient values (R^2) between 0.990 and 0.999. In all the cases, it was observed homoscedasticity of the variance, a random pattern and normal distribution of residues with standardized residue values lower than ± 2, indicating a good fit for the linear models. LOD and LOQ were calculated as three and ten times the standard deviation of the measurement for ten times the 5 μg L^{-1} standard (Signal to noise ca. 3) and divided by the slope of the calibration curves. LOD and LOQ values ranged from 0.6 to 0.8 μg L^{-1} and 2.0 to 2.6 μg L^{-1} for MDMB-CHMCZCA and 5F-ADB, respectively. Intra-day precision was established as the relative standard deviation (RSD%) of ten independent measurements of a spiked sample at 5 μg L^{-1} synthetic cannabinoids concentration level on different days, providing values lower than 5%.

Stability of the analytes in the OF matrix as well as in the resulting extracts stored at −18 °C (freezer) was also evaluated. OF matrix was spiked with synthetic cannabinoids at 10 μg L^{-1} concentration level and was analyzed by the recommended procedure during 21 days. From the results, it was concluded that OF samples can be stored in the freezer for 15 days, whereas the extracts

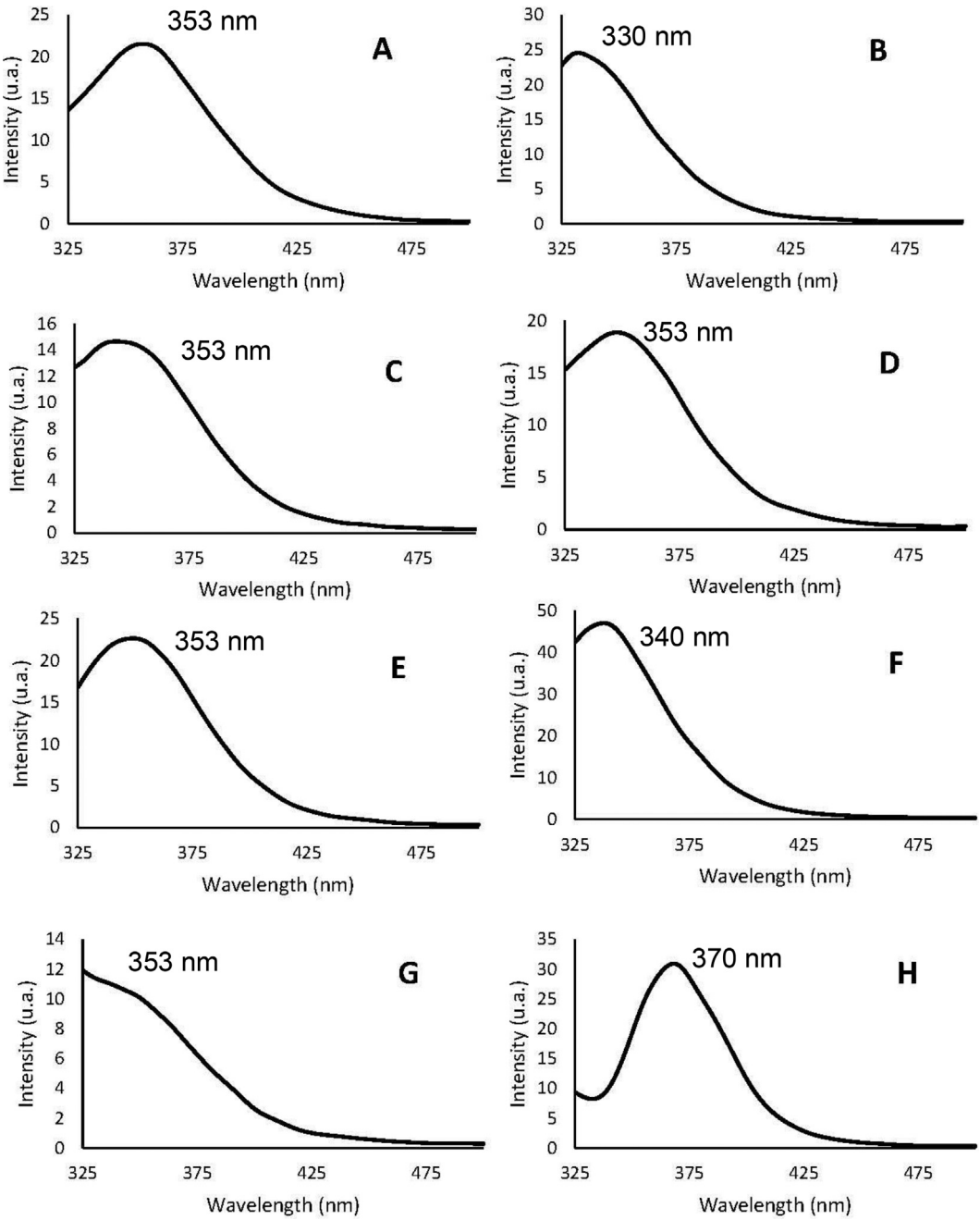


Fig. 3. Fluorescence spectra of the evaluated synthetic cannabinoids using 297 nm as excitation wavelength. A: 5F-NPB-22, B: ADB-CHMICA, C: ADB-CHMINACA, D: 5F-ADB, E: MDMB-FUBINACA, F: MMB-CHMICA, G: MDMB-4en-PINACA, H: MDMB-CHMCZCA.

were stable over the 21-day period analyzed. Despite these good results, it is recommended to perform the SPE extraction of synthetic cannabinoids from OF as soon as possible and the obtained extract could be stored for longer periods of time.

Method trueness was estimated as the recoveries obtained for the analysis of OF samples spiked with synthetic cannabinoids at concentration levels ranging from 5 to 50 μg L^{-1}. In previously published studies of synthetic cannabinoid determination in OFs, different concentration levels were reported ranging from 0.8 to 40 μg L^{-1} [19]. The obtained recoveries are shown in Table 3, with values ranging from 76 to 98%. As it can be seen, for most of the evaluated synthetic cannabinoids, recoveries were quantitative in the studied concentration range.

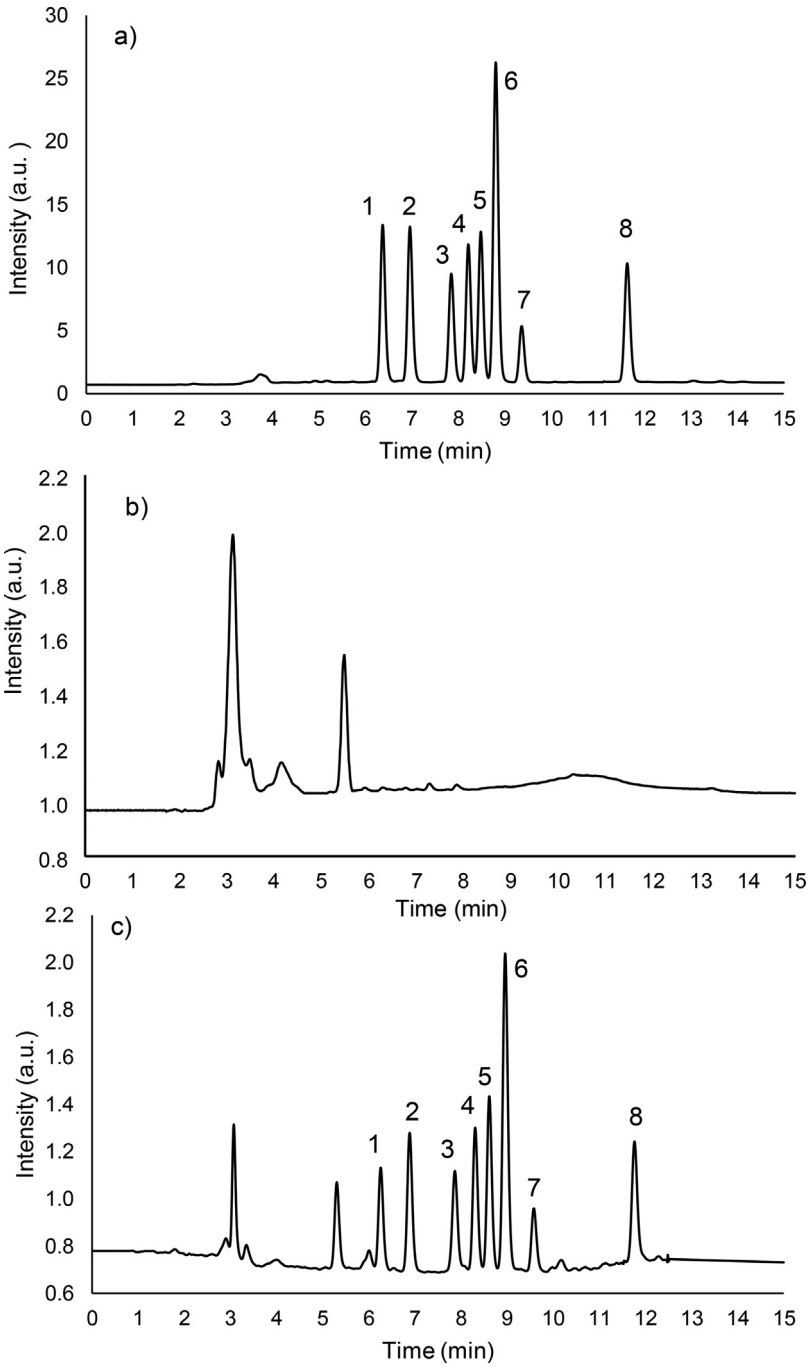


Fig. 4. Chromatograms of (a) synthetic cannabinoids standard solution of 50 μg L^{-1}, (b) a synthetic cannabinoid-free OF and (c) an OF sample spiked with synthetic cannabinoids at 5 μg L^{-1} concentration level. Peak identification: (1) 5F-NPB-22, (2) ADB-CHMICA, (3) ADB-CHMINACA, (4) 5F-ADB, (5) MDMB-FUBINACA, (6) MMB-CHMICA, (7) MDMB-4en-PINACA, (8) MDMB-CHMCZCA.

Table 3
Recovery assessment of synthetic cannabinoids in spiked OF samples after the SPE protocol using HLB columns. Recovery (%) ± sd (n = 3).

Synthetic cannabinoid	Recoveries (%) ± sd		
	Spiked concentration level (μg L^{-1})		
	5	10	50
5F NPB22	85 ± 2	91 ± 5	94 ± 7
ADB-CHMICA	88 ± 3	91 ± 12	92 ± 9
ADB CHMINACA	96 ± 1	94 ± 5	95 ± 6
5F ADB	99 ± 4	94 ± 5	98 ± 5
MDMB FUBINACA	90 ± 16	89 ± 3	92 ± 11
MMB CHMICA	85 ± 14	86 ± 8	82 ± 6
MDMB-4en-PINACA	84 ± 7	91 ± 8	89 ± 7
MDMB-CHMCZCA	76 ± 3	73 ± 5	76 ± 9

Conclusions

In this work, a simple method based on the SPE extraction using commercial HLB cartridges has been established for the determination of synthetic cannabinoids in OFs by LC-FD. The validated method shows a linear relationship between peak area and synthetic cannabinoid concentration from 2.2 to 1000 μg L^{-1}, with satisfactory LOD (0.6–0.7 μg L^{-1}) and LOQ values (2.2 and 2.6 μg L^{-1}). In the extraction procedure, C18 silica-based cartridges can be used, as potential alternative to HLB sorbents for synthetic cannabinoids extraction from OFs.

Ethics statements

This article employed human oral fluid samples obtained from males and females ranging from 25 to 40 years old after freely-given informed consent. Moreover, it should be highlighted that under no circumstances have the authors trafficked or provided illegal substances, aimed, promoted, facilitated, stimulated, or forced in any way the consuming of illegal substances.

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.

CRediT authorship contribution statement

P. García-Atienza: Validation, Data curation, Writing – original draft. **H. Martínez-Pérez-Cejuela:** Validation, Data curation, Writing – review & editing. **E.F. Simó-Alfonso:** Writing – review & editing, Funding acquisition, Resources. **J.M. Herrero-Martínez:** Conceptualization, Supervision, Writing – review & editing, Funding acquisition, Resources. **S. Armenta:** Conceptualization, Supervision, Writing – review & editing, Funding acquisition, Resources.

Data availability

Data will be made available on request.

Acknowledgments

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