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Determination of synthetic opioids in oral fluid samples using fabric phase sorptive extraction and gas chromatography-mass spectrometry

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ABSTRACT

New psychoactive substances (NPS) continue to emerge in the drug market every year, becoming a global threat to public health and safety. These compounds are mostly synthetic cannabinoids and designer cathinones. However, synthetic opioids have appeared on the recreational drug markets in recent years, particularly fentanyl and its derivatives ("fentanyls"). Fentanyl and its analogs are related to harmful intoxications and an increase in opioid-related mortality in many countries, such as in the United States and Europe in the last years. Taking the drug related global crisis into consideration, this work developed and validated an effective and sensitive method based on fabric phase sorptive extraction (FPSE) followed by gas chromatography-mass spectrometry (GC-MS) for the simultaneous determination of 11 fentanyl analogs in oral fluid samples. The extraction was carried out using a sol-gel Carbowax 20 M sorbent immobilized on 100% cellulose fabric substrate and using ethyl acetate as the desorption solvent. The limits of detection (LODs) and quantification (LOQs) ranged from 1 to 15 ng mL^{-1} and 5 to 50 ng mL^{-1}, respectively. Intra-day and inter-day precision were found within 8.2% and 8.6%, respectively, while accuracy ranged from -5.5 to 9.1%, in accordance with the established criteria. The absolute recovery values were in the range of 94.5%–109.1%. The validated method demonstrated its great potential to detect and quantify fentanyl analogs in possible forensic work and off-site analysis in road traffic cases.

1. Introduction

New psychoactive substances (NPS) comprise a complex and diverse group of compounds that have emerged on the global illicit drug market in recent years. The European Monitoring centre for Drugs and Drug Addiction (EMCDDA) has monitored more than 830 NPS by the end of 2020 [1]. Synthetic opioids have recently emerged on the illicit NPS market with a progressive increase in their consumption, such as fentanyl and its analogues, which have created epidemic-level harms in some countries [2,3]. Between 2009 and 2020, 67 new synthetic opioids were detected including 10 first reported in 2020 [1].

Fentanyl is a potent synthetic opioid synthesized and introduced on the market since the 1960s. It has a potency about 50–100 times greater than morphine and is structurally based on the main chain of phenylethyl piperidine (**Table S1**). It has multiple sites for the addition or substitution of several chemical functional groups producing analogous compounds (known as "fentanyls" or fentanyl analogues), with similar or even higher analgesic/toxic effects than fentanyl [4]. A small number of pharmaceutical fentanyls have been widely used in human medicine (such as fentanyl, alfentanil, sufentanil, and remifentanil) for pain control and anesthesia, and in veterinary medicine to immobilize large animals (as in the case of carfentanil and thiafentanil). Nevertheless, fentanyl has a

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long history of illicit abuse as a substitute for heroin and other controlled natural or semi-synthetic opioids or mixed with stimulants, such as cocaine or methamphetamine [5]. Additionally, fentanyl analogues have been involved in more than 250 deaths in recent years, many of which were directly attributed to these substances, probably due to their high potency [2].

The increased consumption and the emergence of new synthetic opioids make it urgent to develop sensitive and selective analytical methods for the detection of these compounds in biological matrices. The most popular analytical technique in the field of bioanalysis is liquid chromatography coupled to tandem mass spectrometry (LC-MS / MS) [5–13]. However, this technique requires large volumes of organic solvents and the acquisition, operational and maintenance cost of LC-MS(MS) are quite high. Gas chromatography coupled to mass spectrometry (GC-MS) offers a viable alternative to LC-MS and is commonly used in forensic toxicology laboratories for the detection and quantification of this type of drugs of abuse and other compounds of toxicological interest in different matrices [4,13–17].

The sample preparation is the most important step in the development of an analytical method. The fundamental objective is to selectively extract the analyte of interest from the matrix containing it, prior to its determination using the selected analytical technique. The most commonly used extraction techniques for fentanyl and its derivatives in complex matrices include liquid-liquid extraction (LLE) [6,10,11] and solid-phase extraction (SPE) [7–9,16,18,19]. However, these two conventional techniques are time consuming and require large volumes of organic solvents and test sample, which is limited in forensic toxicology. Recently, new trends have been implemented in the development of analytical methods, leading to the miniaturization and simplification of these traditional sample preparation techniques. Methodologies have been developed that made use of miniaturized techniques for the extraction of fentanyls in different biological matrices. Da Cunha et al. [12] developed a method based on microextraction by packed sorbent (MEPS), a miniaturized format of SPE, for the determination of nine fentanyls (fentanyl, sufentanil, alfentanil, acrylfentanil, thiofentanyl, valerylfentanyl, furanylfentanyl, acetylfentanyl and carfentanil) in urine samples. Saraji et al. [20] compared dispersive liquid-liquid microextraction (DLLME) and hollow fiber liquid-liquid microextraction (HF-LLME) techniques for the determination of fentanyl, alfentanil, and sufentanil in water, plasma, and urine samples. Gardner et al. [21] also used DLLME for the analysis of fentanyl in postmortem urine samples. These microextraction methodologies used small sample volumes as well as elution or extraction solvents. However, they have several drawbacks such as the complexity of the samples (need of additional clean-up step), instability of the solvent drops in microextraction, formation of air bubbles or sorbent obstruction. Additionally, biological samples required pre-extraction preparation, such as filtration, dilution and/or protein precipitation [12,20,21], increasing the total time of the method.

In 2014, Kabir and Samanidou used sol-gel coating technology to develop a new extraction technique (fabric phase sorptive extraction, FPSE), where an ultra-thin uniform sorbent coating is used on a small fragment of natural or synthetic fabric substrate (cellulose, polyester and fiberglass) of different nature (hydrophilic, hydrophobic, or both), as an extraction sorbent [22]. The chemical bonding between the substrate and the polymer ensures very high thermal, solvent and chemical stability of the FPSE membrane. Sol-gel coating technology allows the use of typical functional ligands commonly used in SPE (such as C8 or C18), as well as polymers used in solid-phase microextraction (SPME), such as polydimethylsiloxane (PDMS). Furthermore, FPSE combines the extraction mechanisms of SPE (exhaustive extraction) and SPME (equilibrium extraction) into a single technology platform. On the one hand, a continuous flow system is created between the porous network of the sol-gel sorbent coating and the permeability of the substrate (exhaustive extraction mode). On the other hand, the FPSE medium is in contact with the sample, whose mass is transferred by the sorbent until a balance is established between the sorbent and the sample matrix (equilibrium extraction mode). Unlike other microextraction techniques, FPSE does not require any sample pretreatment process to minimize matrix interference (such as filtration, protein precipitation, or centrifugation), and the FPSE membrane can be inserted directly into the sample, regardless of the complexity of the sample [22]. Despite being a relatively new technique, it has already been successfully applied in the extraction of different compounds of toxicological interest in biological samples, such as estrogens, benzodiazepines, androgens and progestogens, penicillin, anti-inflammatory drugs or parabens [23] (see Refs. 1–17 in Supplementary material).

The proposed method employed a sol-gel Carbowax 20M sorbent coated FPSE membrane for determining fentanyl and ten synthetic derivatives (4-aminophenyl-1-phenethylpiperidine, 4-fluoroisobutyrylfentanyl, p-fluorofentanyl, acryl fentanyl, cyclopropyl fentanyl, methoxyacetylfentanyl, carfentanil, valeryl fentanyl, furanyl fentanyl, and tetrahydrofuranyl fentanyl) and gas chromatography-mass spectrometry in oral fluid samples. Fabric phase sorptive extraction factors such as stirring mode, desorption and sample volumes, extraction/desorption times and pH of the sample matrix were evaluated using a screening experimental design. Based on the limited published scientific literature on miniaturized extraction techniques for this type of psychoactive compounds, no studies using FPSE to extract fentanyls in forensic samples combined with chromatography techniques (GC or LC) have been published.

2. Experimental

2.1. Chemicals and materials

Sol-gel sorbent-coated FPSE membranes were prepared in Florida International University, Miami, Florida, USA. Methyltrimethoxysilane (MTMOS), trifluoroacetic acid (TFA), acetone, polytetrahydrofuran (PTHF) and dichloromethane were purchased from Sigma-Aldrich (St. Louis, MO, USA). Polydimethyldiphenylsiloxane (PDMDPS) and octadecyl trimethoxysilane (ODS-TMS) were purchased from Gelest Inc. (Morrisville, PA, USA). Sodium hydroxide, hydrochloric acid and CW20M were purchased from Fisher Scientific (Pittsburg, PA, USA). The HPLC-grade solvents water and acetonitrile were obtained from the Fisher Scientific (Pittsburg, PA, USA). Muslin cotton fabric (100% cellulose) was purchased from Jo-Ann Fabric (Miami, FL, USA). An Eppendorf Centrifuge 5415 R (Eppendorf North America Inc., Hauppauge, NY, USA) was used to remove unwanted and interfering micro particles from the sol solutions prior to sol-gel coating on the fabric substrate used to create fabric phase sorptive extraction membrane. A Philips XL30 Scanning Electron Microscope equipped with an EDAX detector was used to obtain SEM images. A Barnstead NANOPure Diamond (Model D11911) deionized water system (Barnstead Inc., Dubuque, IA, USA) was used to obtain deionized water (18.0 MΩ).

FPSE method development and validation were carried out in Santiago de Compostela, Spain. 4-aminophenyl-1-phenethylpiperidine (4-ANPP), 4-fluoroisobutyryl fentanyl (4-FIBF), p-fluorofentanyl (p-FF), acryl fentanyl (AF), cyclopropyl fentanyl (CPF), methoxyacetylfentanyl (MAF), carfentanil, furanyl fentanyl (FF) and tetrahydrofuranyl fentanyl (THFF), all at 1 mg mL^{-1}, valeryl fentanyl (VF) and furanyl fentanyl-d5 (FF-d5), at 100 µg mL^{-1}, were supplied by Chiron AS (Stiklestadveien, Trondheim, Norway). Fentanyl, at 1 mg mL^{-1}, and the internal standards

(IS) 4-ANPP-d5, fentanyl-d5, and VF-d5, all at 100 µg mL^{-1} were obtained from Cerillant (Round Rock, TX, USA) (**Table S1** in Supplementary material). Acetonitrile (ACN), methanol (MeOH), and ethyl acetate (EtOAc) in LC-grade, and disodium hydrogen phosphate anhydrous were purchased from Merck (Darmstadt, Germany). A Milli-Q system from Millipore (Bedford, MA, USA) was used to purified water. Single-use Omnifix™-F syringes (1 mL) were provided by Braun (Barcelona, Spain) and PTFE syringe filters (0.22 µm pore size) were purchased from Thermo Scientific (Waltham, MA, USA).

2.2. Creation of sol-gel coated fabric phase sorptive extraction membrane

Inherently hydrophilic 100% cellulose Muslin cotton fabric was used as the substrate for the sol-gel sorbent coating. Commercial cotton fabric contains different surface finishing chemicals as well as other additives that are applied to improve the general appearance of the fabric surface. However, these chemicals mask a significant portion of the surface hydroxyl functional group and needs to be treated prior to sol-gel sorbent coating to maximize the surface hydroxyl group. During the sol-gel sorbent coating, the growing network of sol-gel sorbent gets chemically bonded to the fabric substrate via surface hydroxyl group. As such, it is important to maximize the number of accessible surface hydroxyl group that ensures higher sorbent loading during the sol-gel sorbent coating process. The detailed surface treatment process of cellulose fabric is presented elsewhere [24,25].

Since the target analytes of current study are of medium and low polarity (logP 2.57–5.28), four different sol-gel sorbents designed for medium and low polarity compounds were synthesized and evaluated. The sol-gel sorbents include sol-gel octadecylsilane (sol-gel C18), sol-gel polydimethyldiphenylsiloxane (sol-gel PDMDPS), sol-gel polytetrahydrofuran (sol-gel PTHF) and sol-gel Carbowax® 20M (sol-gel CW20M). The formulations of sol-gel sorbents are described elsewhere [25,26]. Briefly, the sol solution was prepared by the sequential addition of 5 g organic/inorganic polymer, 10 mL acetone: methylene chloride (1:1 v/v), 5 mL methyltrimethoxysilane and 2 mL trifluoroacetic acid (containing 5% v/v water). The sol solution was vortexed vigorously after adding each of the ingredients to ensure that the resulting solution becomes homogeneous and particle free. The sol solution was then subjected to sonication to remove any trapped air bubbles. Finally, the sol solution was transferred into a 30 mL amber reaction vessel and a 10 cm x 5 cm piece of clean and treated cotton fabric was gently immersed into the sol solution. The sol solution was allowed to create the sol-gel sorbent coating on the fabric substrate for 4 h at room temperature.

At the end of the sol-gel sorbent coating process, the sorbent coated fabric was removed from the reaction vessel and was stored in a desiccator overnight. Subsequently, the sol-gel sorbent coated fabric was rinsed with acetone: methylene chloride (1:1 v/v) under sonication for 30 min. The sol-gel sorbent coated membrane was then air dried for 1 h and was cut into 1 cm x 1 cm pieces. The membranes were cut with scissors and tweezers, to avoid manipulating the membrane with hands. Both the scissors and tweezers were previously washed with acetone to avoid possible contamination. The FPSE membranes were then stored in an air-tight container until their application in sample preparation.

2.3. Standard stock solutions, calibration, and QC samples

Individual stock solutions for each fentanyl at a concentration of 1 mg mL^{-1} or 100 µg mL^{-1}, supplied in methanol or acetonitrile, were stored at -20 °C. Working solutions with all analytes, used for optimization and further experiments, were prepared daily by appropriate dilution of stock solutions in methanol or ethyl acetate. Standard addition calibration curves were constructed in the concentration range (5–1500 ng mL^{-1}) by spiking blank oral fluid (OF) samples with aliquots from working solutions. The internal standards were prepared (250 ng mL^{-1}) by dilution of the stock solutions. The quality control samples (QC) were freshly prepared with additions at three concentration levels (50 or 75, 250, and 1000 ng mL^{-1}) on the blank OF sample.

2.4. Oral fluid samples collection

Drug-free human oral fluid (OF) samples were obtained from healthy volunteers using Salivette® devices (Sarstedt, Germany). The device is available with a simple cotton swab, which is inserted on the mouth. The swab should be chewed gently for about 1 min to stimulate salivation. Finally, the swab is transferred to the tube for subsequent centrifugation. Collected OF samples were stored at -20 °C until analysis, without the aid of external salivary stimulants, any type of pretreatment or dilution. All studies were conducted according to the "Ethical Principles for Human Medical Research" of the World Medical Association [27] and in accordance with the evaluation criteria and subsequent authorization established by the Research Ethics Committee of Galicia (Spain) [28].

2.5. Fabric phase sorptive extraction procedure

Prior to the extraction step, the FPSE membrane coated with sol-gel CW 20M/sol-gel C18/sol-gel PTHF/sol-gel PDMDPS (1 cm x 1 cm) were immersed in 2 mL of acetonitrile: methanol (50:50, v/v) solution for 5 min to remove any undesirable residue. Subsequently, the membrane was rinsed with 2 mL of ultrapure water for 5 min to remove residual organic solvents, and then air-dried before use. Under optimized conditions, the clean FPSE membrane was transferred to a 5 mL screw-capped glass tube vial containing 0.5 mL of spiked OF at the desired concentration, adjusted at pH 7 with 200 µL of phosphate buffer 0.05 M. The vial was agitated in an ultrasound bath (FB15055 Fisherbrand™, Madrid, Spain with ultrasonic frequency of 37 kHz and a power total consumed of 280 W) equipped with an immersion basket with handle at 25 °C by temperature control with a thermometer, for 30 min. Then, the FPSE membrane was removed from the vial, washed with ultrapure water, and dried over a lint-free-tissue. Afterward, it was put in contact with 0.25 mL of ethyl acetate, stirred in an ultrasound bath at 25 °C, for 10 min. Finally, the FPSE membrane was removed, and the extract was filtered (0.22 µm) and analyzed by GC-MS. The FPSE membranes can be reused again after a final wash with 2 mL ACN: MeOH (1:1, v/v) for 10 min.

2.6. Gas chromatography-mass spectrometry

The GC-MS analysis was performed using an Agilent 7890B gas chromatograph combined with an Agilent 7650A automatic liquid sampler and an Agilent 5977B quadrupole mass spectrometer (Agilent Technologies, Palo Alto, CA, USA). The separation was performed using a HP-5MS capillary column (30 m x 0.25 mm ID x 0.25 µm film thickness) from Agilent Technologies (Palo Alto, CA, USA). Helium (99.9999% purity, Nippon gases, Spain) was used as carrier gas at a constant flow of 1.2 mL min^{-1}. The initial GC oven temperature was set at 100 °C (held 1 min) and increased in a ramp of 42 °C min^{-1} until 200 °C, and up to 280 °C at 15 °C min^{-1} (held 12 min). The total run time was finally 20.71 min. The injected volume was set to 1 µL, in splitless mode. The quadrupole mass spectrometer was operated in positive electron impact (EI) ionization mode (70 eV). Transfer line, MS source, and MS quadrupole temperatures were set at 280 °C, 230 °C, and 150 °C, respectively. Initially, a full scan mode (mass range 50–550

Table 1
Retention times and *m/z* values for each analyte.

Compound	Retention time (min)	Quantifier ion (Q) (*m/z*)	Qualifier ions (q_1, q_2) (*m/z*)	% Ion ratios (q/Q) q_1	q_2
4-ANPP	08.4 ± 0.2	146	189, 091	77.4	27.7
4-FIBF	09.5 ± 0.2	277	164, 207	45.9	39.3
***p*-FF**	09.7 ± 0.2	263	164, 207	53.0	25.2
Fentanyl	09.9 ± 0.2	245	146, 189	57.1	32.1
AF	10.1 ± 0.2	243	146, 189	56.0	24.5
CPF	10.8 ± 0.2	257	146, 189	50.1	43.7
MAF	10.9 ± 0.2	261	158, 218	24.8	21.1
Carfentanil	10.9 ± 0.2	303	187, 154	14.8	12.5
VF	11.1 ± 0.2	273	146, 189	80.0	47.7
FF	12.9 ± 0.2	283	240, 158	58.3	17.8
THFF	13.2 ± 0.2	287	146, 189	50.4	32.7

amu) was used to select the highest-intensity ions for each compound. The identification and determination of fentanyl analogs were carried out through retention times and *m/z* ratios of characteristic ions. The most intense ion was selected for quantification, and the following two were selected as qualifiers [29] using the selected ion monitoring mode (SIM) to determine the target analytes. The qualifier-to-target percentage was determined by dividing the abundance of the selected qualifier ions by that of the target ion (quantifier) multiplied by 100% (**Table 1**).

2.7. Analytical performance

The method was validated in terms of linearity, limits of detection and quantification, precision, and accuracy (bias), recovery, matrix effects, and stability, in accordance with the international guidelines of Scientific Working Group for Forensic Toxicology (SWGTOX), Food and Drug Administration (FDA), and European Medicines Agency (EMA) [30–32]. The calibration model was established using at least six-point calibration curves, between the LOQ of each analyte up to 1500 ng mL^{-1}, in five replicates per concentration level. The limit of detection (LOD) was defined as the lowest concentration with a signal-to-noise ≥ 3. The limit of quantification (LOQ) criteria was established as a signal-to-noise of at least 10 and was quantified within ± 20% precision of each concentration. LOD and LOQ were evaluated with decreasing analyte concentrations in pooled OF samples from six different sources, spiking with analytes of interest. Precision and accuracy (bias) were evaluated at three concentrations levels: low QC (50 or 75 ng mL^{-1}, depending on the compound), medium QC (250 ng mL^{-1}), and high QC (1000 ng mL^{-1}) in triplicate over five different days ($n = 15$). One-way analysis of variance (ANOVA) was performed at each QC concentration level to assess the potentially significant variability of within-run and between-run precision (95% confidence level, $p < 0.05$). The precision was evaluated by measuring the coefficient of variation (%CV) between samples, and the established acceptance criteria was that the %CV should not exceed 20% at each concentration level. The bias was determined by calculating the error percentage of the QC samples for each concentration level considered. The highest average acceptable bias from nominal concentration was ± 20%. The matrix effect (ME) was evaluated by calculating the relationship between the slope of the calibration curve for each fentanyl after a post-extraction addition (blank OF sample subjected to the FPSE procedure, with subsequent addition of compounds and IS) and the slope of the external standard calibration curve, using the following equation:

$$ME\ (\%) = \left(\frac{slope\ postextraction\ addition}{slope\ external\ standard\ calibration} \right) 100$$

It was considered that there is no matrix effect if the value is close to 100%. Recovery was studied using the previous QC samples, with five replicates for each of the three concentration levels. Recovery values (%) were calculated as:

$$Recovery\ (\%) = \left(\frac{concentration\ obtained}{added\ concentration} \right) x\ 100$$

where *concentration obtained* was the concentration of the compound measured in QC samples and *added concentration* is the concentration level of the compounds added to QC samples. Recoveries close to 100% and within the range 80–120% were considered as acceptance criteria. Freeze-thaw stability was performed on low QC and high QC samples ($n = 3$). The OF was fortified with QC solutions on day zero. Three aliquots at each concentration were quantified and the remaining OF sample was stored at -20 °C and applied during 5 freeze-thaw cycles (three cycle/24 h and two cycles/7 days). Autosampler stability was evaluated at low QC and high QC ($n = 3$). QC samples were analyzed on day zero, stored in the autosampler at 20 °C ± 2 and re-injected every 3 h until 12 h, and then reinjected at 24 h, 48 h, and 72 h. In the two stability studies described above, analytes were considered stable within ± 20% and were required to be within 80–120% recovery.

3. Results and discussion

3.1. Characterization of the FPSE membrane

3.1.1. Scanning electron microscopy (SEM) of FPSE membranes

Fig. 1 presents the scanning electron microscopy images of sol-gel CW20M sorbent coated membranes (a) at 100x magnification and (b) at 1000x magnification. The SEM images revealed the distinct homogeneous coating on the surface of individual cellulose microfibril. Fabric phase sorptive extraction, by design, simultaneously exploits the exhaustive extraction principle generally accepted as the key extraction mechanism exploited in solid phase extraction (SPE) and equilibrium driven extraction principle generally accepted as the extraction mechanism used in solid phase microextraction (SPME). One major criterion for exhaustive extraction (as in SPE) is that the SPE cartridge or the SPE disk must be permeable for the sample containing the target analytes. Same criterion is also applicable to FPSE membrane if it is to exploit the exhaustive extraction principle. As shown by the arrows in **Fig. 1 (a,b)**,

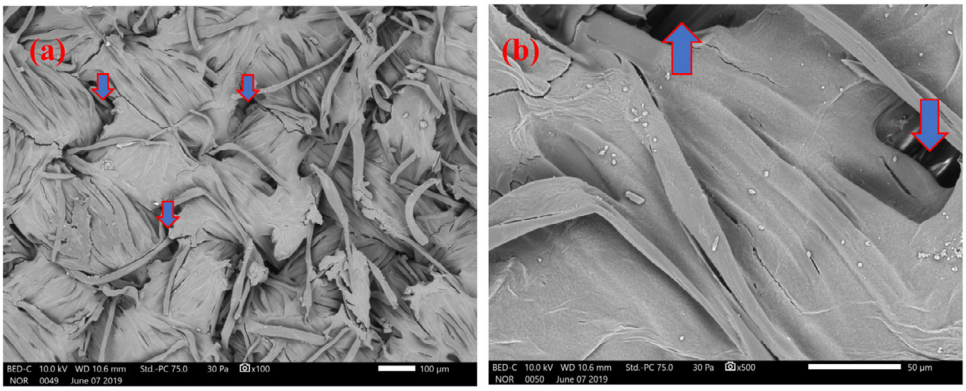


Fig. 1. Scanning electron microscopy images of sol-gel CW20M coated FPSE membrane at (a) 100x magnification; (b) 1000x magnification.

the through pores are conserved even after an apparently thick sol-gel CW20M sorbent coating on the fabric substrate. These through pores allow rapid permeation of the sample matrix through the FPSE membrane during extraction and ensures fast extraction kinetic. The incorporation of SPME and SPE extraction principles into FPSE is one of its major advantages that sets it apart from other contemporary solid sorbent based sorptive extraction and microextraction techniques.

3.1.2. FT-IR spectroscopy

Fig. S1 in Supplementary material presents the FT-IR spectra of the different building blocks of the sol-gel CW20M sorbent coated FPSE membrane. FT-IR spectra shed light on the functional makeup of the building blocks as well as the successful integration of these building blocks into the final product, sol-gel CW20M coated FPSE membrane.

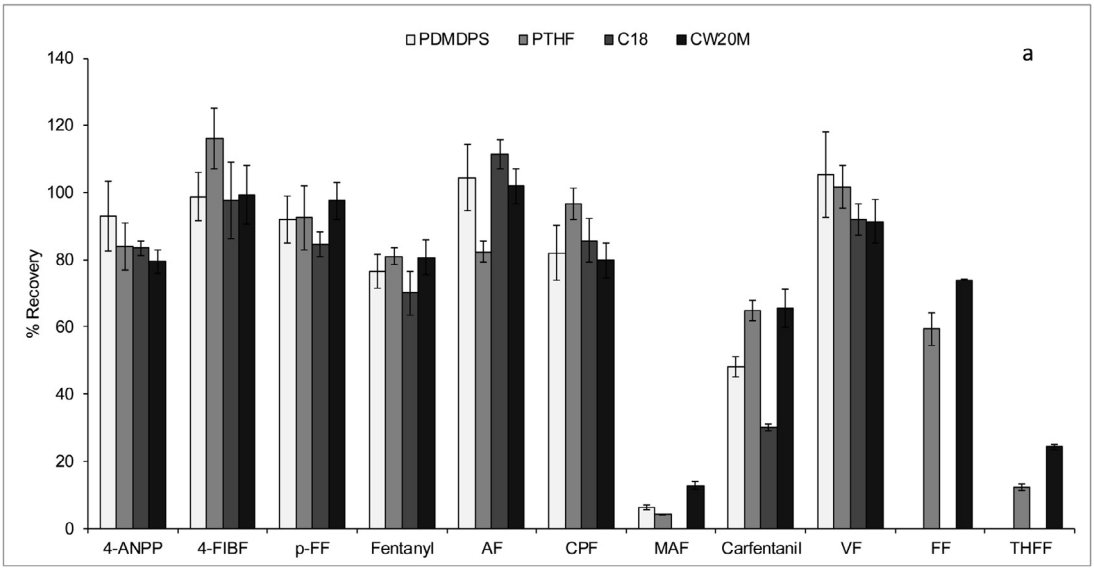
The characteristic peaks of CW20M polymer (**Fig. S1a**) include 2876 cm^{-1}, and 1359 cm^{-1} that represent different vibration modes of C-H bonds. The peaks at 1359 cm^{-1} and 1278 cm^{-1} can be attributed to C-C bonds [33]. Major peaks in FT-IR spectra of methyltrimethoxysilane include 1267 cm^{-1} and 788 cm^{-1} (**Fig. S1b**) that may be attributed to the vibration of CH_3 groups of the precursor. Several bands such as antisymmetric stretching vibration of Si-O-Si at ~ 1035 cm^{-1} and symmetrical deformation vibration of Si-CH_3 at ~1273 cm^{-1} (**Fig. S1c**) appear simultaneously in sol-gel CW20M coated FPSE membrane and CW20M polymer and/or methyltrimethoxysilane precursor are indicative of the successful integration to the fabric substrate via covalent bonding.

3.2. Optimization of FPSE protocol

In order to maximize the extraction efficiency of the FPSE method, the type of FPSE sorbent coating and the type of desorption solvent were first investigated. A number of factors that may affect the extraction and desorption performance were then evaluated simultaneously by an experimental design, such as: sample volume, extraction time, stirring mode, back-extraction solvent volume, and sample pH.

3.2.1. Selection of FPSE sorbent coating

Fentanyl and its derivatives have broad polarity values with log *P* ranging from 2.57, for MAF, to 5.28, for FF (**Table S1**). Therefore, the type of sorbent coating of the FPSE membrane with different sol-gel sorbents of different polarities was evaluated in order to select the most suitable FPSE phase for the extraction of these compounds. Four different polarities of sol-gel sorbent coatings were investigated: three biocompatible sorbents, such as sol-gel poly(dimethyldiphenylsiloxane) (PDMDPS, non-polar), sol-gel poly(tetrahydrofuran) (PTHF, medium polar), and sol-gel poly(ethylene glycol) (Carbowax 20M, highly polar), and a non-biocompatible sorbent, sol-gel C18 (non-polar). It is worth mentioning that although C18 sol-gel is not biocompatible, it is frequently used in SPE and MEPS as a common sorbent for extracting polar and non-polar compounds [12,34,35]. All sol-gel coatings were created on 100% cotton (cellulose) as substrate, providing a strong hydrophilic property to the FPSE membrane. The initial experiments were carried out with 1 mL of ultrapure water containing a concentration of 1 µg mL^{-1} of each analyte. The FPSE membrane was completely immersed inside the sample contained in a vial with a magnetic stirrer bar. The extractions were performed for 30 min with magnetic stirring. The back-extraction was carried out using 0.5 mL of ethyl acetate, which is compatible with GC-MS, under magnetic stirring for 15 min. All experiments were performed in triplicate for each FPSE phase. Finally, the extracts were filtered and injected into the GC-MS system. Experimental results (**Fig. 2a**) show that sol-gel C18 coating was not suitable for the extraction of MAF, FF, and THFF. The non-biocompatibility of membrane C18 may have resulted in lower sorbent availability for analyte-sorbent interactions and subsequently lower extraction values due to its inability to repel cellular materials such as proteins, peptides, lipids, phospholipids, and other macromolecules on its surface. Furthermore, the other non-polar coating (PDMPDPS) was also not suitable for THFF and FF extraction. This could be attributed to the fact that MAF and THFF are the most polar compounds of the target analytes, so they are hardly retained in the non-polar coating of the membrane. FF is the least polar compound, so it is possibly retained on the non-polar FPSE membrane. However, ethyl acetate is not strong enough to desorb the analytes from the phase, while in the sol-gel Carbowax 20M the remaining compound passes to the organic solvent, being less polar than the phase. In general, the most polar phase (Carbowax 20M) was the most suitable for five of the compounds, the intermediate phase (PTHF) was suitable for three of the fentanyls, the biocompatible non-polar phase (PDMDPS) was suitable for two of them, and phase C18 was only for AF. Therefore, the polar phase (Carbowax 20M) was chosen for subsequent extractions. It is important to note that the analyte extraction in FPSE is primarily governed by intermolecular interactions between the sol-gel sorbent and the analytes, not by the polarity of the analyte. Although, Carbowax 20M (CW20M) is a highly polar polymer, sol-gel CW was created using methyl trimethoxysilane and CW 20M that made the composite sorbent an ideal for both nonpolar and polar analytes. The presence of London dispersion, hydrogen bonding capability as well as dipole-dipole interactions in the same sorbent (sol-gel CW20M) ensures the maximum intermolecular interactions between the FPSE membrane and the target analytes, leading to exhaustive/near exhaustive extraction in a relatively short period compared to other classical microextraction techniques.



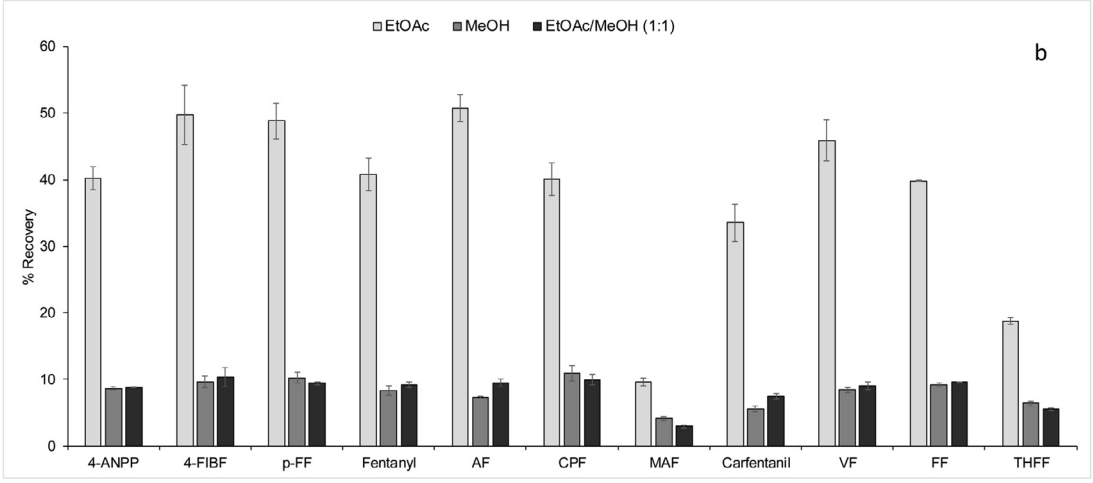


Fig. 2. Influence of different sol-gel coatings in the FPSE extraction of 11 fentanyls ($n = 3$) (a); desorption solvent study ($n = 3$) (b).

Extraction recovery calculation model: Kabir and Samanidou [22] developed a calculation algorithm (Absolute Recovery Calculator, ARC) for each of the FPSE sorbents in order to simplify the sorbent selection process, an important step in the method development exercise [24,27]. This ARC is based on a second-order mathematical model that correlates log K_{ow} values of a wide range of target analytes with their extraction efficiencies for each of the FPSE sorbents. This can be used as a predictive tool to select the appropriate FPSE phase. Considering the fact that the analytes may need to be extracted from a large variety of sample matrices e.g., environmental, food, biological, the models were created using a large number of compounds possessing log K_{ow} values ranging from 0.3 to 5.07 (highly polar to nonpolar) dissolved in deionized water (in absence of any matrix interferents). As such, during the method development using complex sample matrices, especially biological samples, the absolute recovery value may deviate from the model calculated value. In addition, due to the difference in functional makeup of the analytes, two compounds with same log K_{ow} value may demonstrate different extraction recovery values under identical extraction conditions. The expected extraction efficiency values (20.27–104.47%) for all fentanyls and their actual absolute recoveries (0–115.93%) are shown in **Table S2** for the different sorbents studied. Different values were obtained for the actual recoveries (0–111.45% for sol-gel C18 sorbent; 0–105.30% for sol-gel PDMDPS sorbent; 4.13–115.93% for sol-gel PTHF sorbent, and 12.61–101.87% for sol-gel CW20M sorbent), concerning the expected recoveries (51.15–80.78% for sol-gel C18 sorbent; 20.27–51.57% for sol-gel PDMDPS sorbent; 62.75–74.76% for sol-gel PTHF sorbent, and 62.90–104.47% for sol-gel CW20M sorbent). Dipole-dipole interactions, London dispersion, and π-π interactions between sorbent and analytes predominate in the FPSE technique for efficient extraction [22]. Furthermore, the FPSE phases coated with sol-gel Carbowax 20M solutions stimulate hydrogen bonding interactions with the analyte if they contain hydrogen bond donors and acceptors. The greater the total number of hydrogen bond donors and acceptors in an analyte, the greater the probability of a satisfactory interaction between the analyte and the sorbent, resulting in greater extraction efficiency. However, the ab-

sence of donors and acceptors causes a loss in extraction recovery (recoveries ranged between 12.61 and 101.87%) compared to the expected value (values ranged between 62.90 and 104.47%) at its log K_{ow} value. In this case, fentanyls have a low number of hydrogen bond donors and acceptors (between 2 and 4), which may explain the low recoveries found to those predicted by the mathematical model. In addition, the high percentages of functionally rich matrix interferents including protein, lipid, phospholipid, peptides may have also interacted with the target analytes, resulting in low absolute recoveries for some analytes. Still, sol-gel Carbowax 20M has been predicted to be the best sorbent for the studied fentanyl analogs, according to ARC and experimental results.

3.2.2. Desorption solvent

The proper selection of the desorption solvent is of great importance to obtain the highest extraction efficiency for all target analytes in FPSE, as in SPE or MEPS techniques. Due to the strong chemical bond between the membrane substrate and the coating, FPSE allows any solvent for the elution of the analyte after extraction [22]. Ethyl acetate, methanol, and a mixture of ethyl acetate: methanol (1:1, v/v), suitable for subsequent injection in GC-MS, were studied as desorption solvents. The protocol carried out is the same as that described for the selection of the sorbent in the previous section, selecting the Carbowax 20M phase as the FPSE membrane. As can be seen in **Fig. 2b**, the highest extraction efficiency for all analytes was obtained with ethyl acetate. The target compounds have an intermediate-low polarity (log K_{ow} 2.57–5.28) and ethyl acetate is less polar than methanol, which makes it more suitable for the release of the compounds (retained in polar coating, CW20M) towards a less polar solvent. Therefore, ethyl acetate was selected for the desorption of the fentanyls.

3.2.3. Screening design

An asymmetric screening design $2^3 3, 3^4 1 // 16$ was used to examine the influence of factors affecting FPSE, such as sample volume, extraction, and desorption time, stirring mode, the volume of desorption solvent, and the pH sample. The objective is to identify those factors that influence the extraction with the fewest possible experiments. For this, seven variables (three factors at two levels, three factors at three levels, and one factor at four levels) were studied in only 16 experiments (**Table S3**). The variables to study were:

- b_1: stirring mode during extraction (magnetic stirring and ultrasounds).
- b_2: stirring mode during desorption (magnetic stirring and ultrasounds).
- b_3: volume of desorption solvent (250 and 500 µL).
- b_4: sample volume (300, 500, and 800 µL).
- b_5: extraction time (10, 20, 30 min).
- b_6: desorption time (5, 10, 15 min).
- b_7: pH of the sample (6, 7, 8, and 9).

The design responses obtained (chromatographic peak areas) were related to the seven factors studied, using the following mathematical model:

$$y = {}_0 + \sum_{i=1}^{3} {}_{i/B-A} x_{BA} + \sum_{j=4}^{6} {}_{j/B-A} x_{BA} + {}_{j/C-A} x_{CA} + {}_{j/C-B} x_{CB} + {}_{7/B-A} x_{BA} + {}_{7/C-A} x_{CA} + {}_{7/D-A} x_{DA} + {}_{7/C-B} x_{CB} + {}_{7/D-C} x_{DC}$$

where $A = 1$, $B = 2$, and C = 3, and represent the factor levels, y is the chromatographic response, and the constant term b_0 is the mean response for all experiments. The model describes the effects on the FPSE of replacing the level of one factor for another. The results were generated and evaluated using the statistical software *NemrodW®* [36]. The ANOVA results show that none of the factors studied has been statistically significant ($p < 0.05$) for the extraction with FPSE, although their trends were. This can be seen reflected in the Delta Weights graphs (**Fig. 2S**), which represent the relative effects of each compound on the response to a variable change. If the effect is positive, the bar moves to the right and if the effect is negative, the bar moves to the left. The dashed vertical lines represent the limits of statistical significance at a 95% confidence level. Another way to represent the data is through Total Effects graphs (**Fig. 2S**), in which the length of the bar is proportional to the effect of each factor level on the response. **Fig. 3** represents the results obtained by the design for the compound carfentanil, by way of example, using the Delta Weights (**Fig. 3A**) and Total Effects (**Fig. 3B**) graphs.

It has been possible to set the optimal conditions for the simultaneous extraction of the target fentanyls, analyzing the results of the screening design for all compounds. The behavior of the family of compounds studied has been the same in relation to extraction and the influence of the factors considered has been similar in all cases. For both extraction and desorption steps, sonication stirring has a positive effect and is more favorable than magnetic stirring. This is because sonication generates high frequency and amplitude ultrasounds leading to cavitation (growth and collapse of gas bubbles) to agitate particles in the liquid medium, thus providing uniform dispersion between each other and favoring mass transfer (passage of analytes) from the aqueous phase (sample) to the FPSE membrane coating, or from the FPSE coating to the desorption solvent, respectively. Magnetic agitation, compared to sonication, is much more "mild" method. Regarding the volume of the desorption solvent, it is observed that varying from a volume of 0.25 mL, which is the minimum volume to cover the FPSE membrane (1 × 1 cm), to a volume of 0.5 mL has a negative effect on the extraction (**Fig. 3A**), indicating that a volume of 250 µL is sufficiently capable of concentrating the target analytes. Therefore, a volume of 250 µL has been set for desorption during FPSE. On the other hand, the ideal goal is to use the smallest sample volume possible. However, it has been observed that varying from a sample volume of 0.3 mL to 0.5 mL has a greater positive effect on the extraction of fentanyls while increasing the volume to 0.8 mL the effect is already negative. At higher sample volume, the total mass of the matrix interferents increases proportionately but not the contact surface area of the FPSE membrane. These macromolecular interferents may retain some of the analytes via intermolecular interactions, resulting in reducing the freely available analytes for extraction on FPSE membrane. Higher mass of the matrix interferents may also block the active interaction sites of the FPSE membrane for the effective extraction. Thus, an intermediate sample volume (500 µL) has been chosen. The extraction time for all the compounds has been set at 30 min, while for the desorption time it has been observed that a time between 10 and 15 min was optimal for the compounds. To shorten the analysis time, 10 min was sufficient to carry out this desorption. It may be related to the fact that excessive time can cause active membrane sites to become saturated. In addition, the FPSE technique combines the SPME mechanism, indicating that equilibrium has been reached at these time intervals, respectively. All compounds have a pKa ranging from 7.76 (for carfentanil) to 9.03 (for 4-ANPP) (**Table S1**). Therefore, the sample pH has been studied between 6 and 9. The results show that the optimum pH obtained for all compounds was 7.

Finally, FPSE membrane reusability was investigated by repeating extraction process for several times. The results showed that the FPSE membrane could be used more than 33 times without considerable decrease in extraction efficiency (**Table 2**) and without appreciating frayed edges of the fabric after ultrasonication cycles. The results confirmed the good durability and high stability of the FPSE membranes. These advantages could make it suitable for

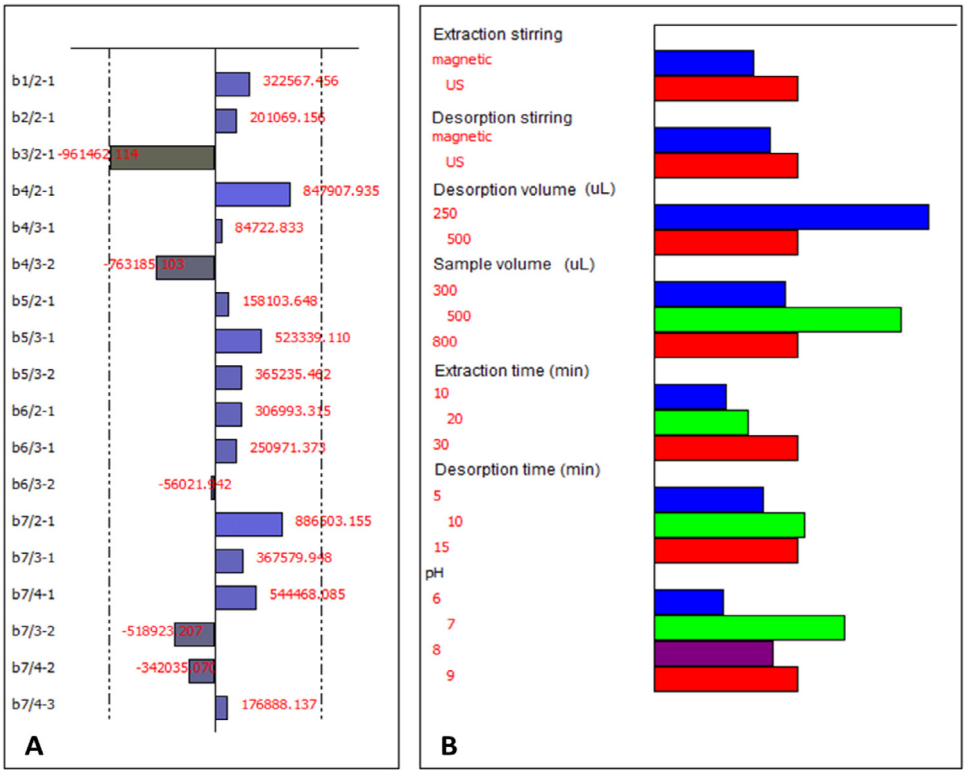


Fig. 3. Delta Weights (A) and Total Effects (B) graphs for carfentanil.

Table 2
Analytical validation of the proposed method.

Compound	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	*QC Level* (ng mL^{-1})	Precision (n = 15) Intra-day (% CV)	Inter-day (% CV)	Accuracy (n = 15) (% bias)	Recovery (n = 5) (%)	± SD	Matrix effect (%)
4-ANPP	15	50	50	7.9	7.5	0.06	100.1	± 6.8	91.4
			250	0.5	1.0	0.5	100.5	± 0.6	
			1000	1.2	1.7	2.1	102.1	± 1.3	
4-FIBF	15	50	50	4.7	4.7	5.9	105.9	± 4.4	**138.9***
			250	0.7	0.7	0.6	100.6	± 0.6	
			1000	1.6	2.1	2.5	102.5	± 1.6	
***p*-FF**	5	15	50	4.0	4.4	4.4	104.4	± 3.8	98.4
			250	0.7	1.0	0.4	100.4	± 0.7	
			1000	1.1	1.4	1.6	101.6	± 1.1	
Fentanyl	10	25	50	5.3	5.9	−3.2	96.8	± 4.7	105.7
			250	0.4	0.6	0.6	100.6	± 0.4	
			1000	1.2	1.8	2.1	102.1	± 1.3	
AF	15	50	50	5.0	4.7	9.1	109.1	± 4.7	**152.6***
			250	0.4	0.6	0.8	100.8	± 0.4	
			1000	1.0	2.0	2.4	102.4	± 1.3	
CPF	5	15	50	4.0	7.0	3.0	103.0	± 4.8	**77.8***
			250	0.4	0.7	0.7	100.7	± 0.4	
			1000	2.4	2.8	2.9	102.9	± 2.3	
MAF	15	50	50	3.4	4.3	−1.0	99.0	± 3.3	**152.9***
			250	0.6	0.6	0.5	100.5	± 0.6	
			1000	1.4	2.1	2.1	102.1	± 1.5	
Carfentanil	1	5	50	8.2	8.6	−5.5	94.5	± 6.7	**174.7***
			250	0.6	0.6	0.7	100.7	± 0.6	
			1000	1.4	1.6	2.0	102.0	± 1.3	
VF	5	15	50	2.5	5.5	2.2	102.2	± 3.5	108.3
			250	0.5	0.6	0.4	100.4	± 0.5	
			1000	2.0	2.1	2.6	102.6	± 1.9	
FF	25	75	75	1.6	2.2	1.7	101.7	± 1.7	**153.8***
			250	0.5	0.7	0.3	100.4	± 0.5	
			1000	1.5	1.8	2.5	102.5	± 1.4	
THFF	15	50	50	5.1	6.2	1.6	101.6	± 4.9	100
			250	0.5	0.7	0.7	100.7	± 0.5	
			1000	1.4	2.1	1.8	101.8	± 1.5	

SD: Standard deviation.
*: compounds that present matrix effect.

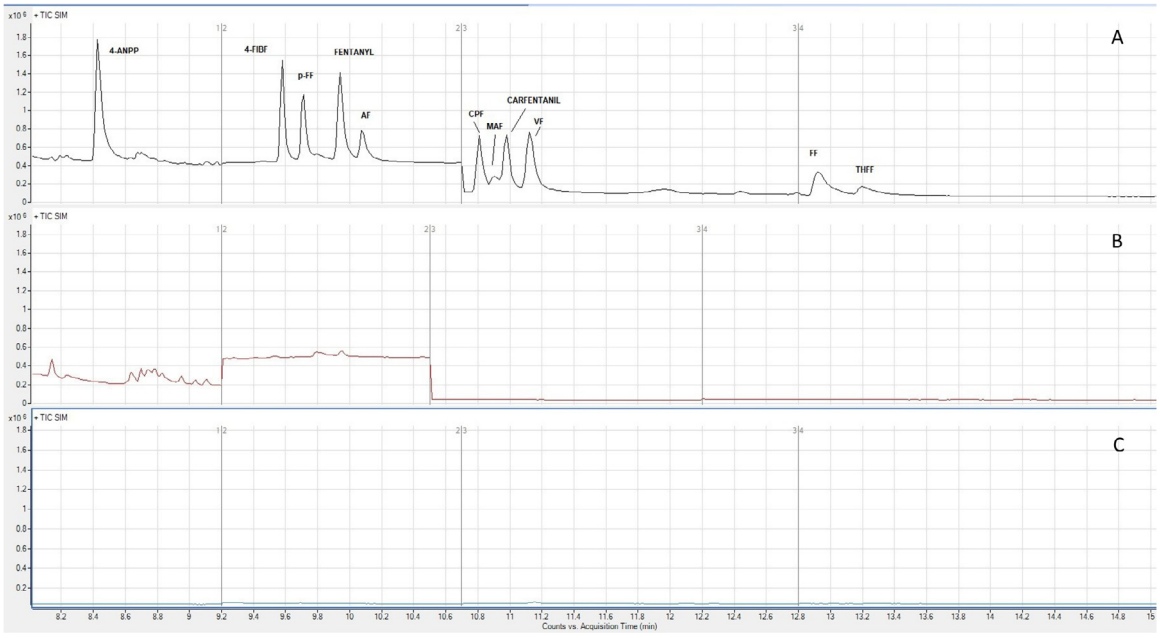


Fig. 4. Total-Ion-Chromatograms of a saliva sample spiked at 1 μg mL^{-1} (a), a blank saliva sample (b) and a blank response of sorbent (blank phase) (c).

routine chemical analysis. However, it is not intended to be reused when it comes to real samples to avoid false positive results.

3.3. Method validation

A validation protocol of the optimized FPSE-GC-MS method was carried out, following the bioanalytical guidelines [30–32] (Section 2.7). The selectivity of the method was studied evaluating possible endogenous interferences in the retention time of the analytes. For this, ten samples of blank saliva from healthy volunteers were subjected to the FPSE-GC-MS procedure. The results did not show the existence of possible interferences in the retention time of the compounds comparing it with a spiked OF sample at 1 μg mL^{-1} (**Fig. 4**).

The calibration curves were constructed in the LOQ-1500 ng mL^{-1} range, with five replicates for each concentration level. Linearity was evaluated using the least-squares regression method. The coefficients of determination (R^2) ranged between 99.2630 and 99.9914%, which means that the adjusted models explain the variability in a percentage very close to 100%. Also, the correlation coefficients (r) obtained in the range 0.9963–0.9999 indicate a relatively strong relationship between the variables (**Table S4**). Therefore, the coefficients of determination and correlation indicated a good correlation between the variables. On the other hand, the slope of the calibration curve is a measure of the sensitivity of the analytical method. In this case, the highest sensitivity corresponded to THFF (0.003566), while the lowest sensitivity corresponds to AF (0.01530).

The LODs ranged from 1 to 15 ng mL^{-1}, except for FF (25 ng mL^{-1}). The LOQs ranged between 5 and 50 ng mL^{-1} (75 ng mL^{-1} for FF) (**Table 2**). There are fewer published methods for the determination of fentanyl derivatives by GC-MS. Unlike other published studies, this study has proposed an extraction method compatible with the principles of Green Chemistry, since it consumes low volume, both sample and regents. Dybowski and Dawidowicz [15] quantified furanyl fentanyl in blood samples using QuEChERS and GC-MS/MS, which provides greater sensitivity than the GC-MS technique used in this work. Although the limits obtained are lower than those obtained in this work, the amount of sample and reagents used are greater than in the optimized FPSE technique. Kahl et al. [18] determined furanyl fentanyl and ocfentanil, beside acetyl fentanyl and butyrfentanyl, in whole blood samples by SPE, using larger sample volumes and solvents. Gardner et al. [21] compared LLE, SPE, and DLLME techniques for the extraction of fentanyl in urine samples. Unlike the work of these authors, in the work present here, it has been possible to determine, in addition to fentanyl, ten fentanyl analogs, among which is the precursor 4-ANPP. Strano-Rossi et al. [37] developed an LLE-based method for the determination of fentanyl, norfentanil, alfentanil, and sulfentanil in urine samples. In addition, the limits obtained in this work encompass the toxic/lethal range found in the scientific literature for fentanyl derivatives [38,39]. On the other hand, the limits obtained are lower than those found by Gilbert et al. [14], who developed a confirmatory GC-MS method for the identification of 18 fentanyl derivatives; Buchalter et al. [17], who developed a GC method interfaced with cold electron ionization mass spectrometry, and ultraviolet vacuum detection using a flow divider for the determination of 24 fentanyl analogs. Likewise, the limits found are also lower than those reported by Shaks and Behonick [40] for carfentanil. These authors determined this compound in blood samples, using a more sensitive analytical technique, such as LC-MS/MS.

The intra- and inter-day precision did not exceed 8.2% and 8.6%, respectively, meeting the established criteria (%CV < 20%). The bias results for accuracy ranged from -5.5 to 9.1, being within ± 20% of the target concentration. The recovery values obtained at the three concentration levels were close to 100% (94.5–109.1%) (**Table 2**). These recoveries were higher than those obtained by da Cunha et al. [11], who determined 104 NPS, including fentanyl analogs, in OF samples using LLE-LC-MS/MS. Recovery is higher that published by da Cunha et al. [12] for fentanyl derivatives using MEPS-LC-MS/MS in urine samples, or Platosz et al. [19], which quantified synthetic opioids (including 4-ANPP, AF, FF, p-FF, fentanyl, and carfentanil) in hair samples by SPE-LC-MS/MS. A marked matrix effect has been observed for six of the fentanyl studied, such as 4-FIBF, AF, CPF, MAF, carfentanil, and FF, therefore the standard addition method was used to mitigate the matrix effect and for the quantification of fentanyls in OF samples (**Table 2**).

Freeze-thaw stability was studied in QC samples at low concentration (50 ng mL^{-1}, except for FF, which was established at 75 ng

mL^{-1}) (**Fig. S3a**) and high concentration (1000 ng mL^{-1}) (**Fig. S3b**) in three replicates each level. The QC samples were stored at -20 °C and studied in three freeze-thaw cycles every 24 h and, subsequently, in two freeze-thaw cycles every 7 days. The results for the long stability collected in show that none of the analytes studied were stable after two weeks. 4-ANPP, 4-FIBF, and fentanyl were stable for one week, while *p*-FF was stable for 3 days (72 h), for the two-concentration level studied. For the rest of the compounds, differences in the recoveries were observed between the low and high concentration level QC samples. For the low QC concentration level, AF and VF were stable for 48 h, while carfentanil and FF were stable up to 72 h. However, CPF, MAF, and THFF stability decreased considerably during the first 24 h, with recoveries of 76.8%, 79.3%, and 66.4%, respectively. For the high QC sample level, the compounds were stable within 3 to 7 days, except for FF, which was stable up to the first 24 h (79.6%).

Autosampler stability was also evaluated with low-level QC samples (**Fig. S3c**) and high-level QC samples (**Fig. S3d**) ($n = 3$). The QC samples were stored in the autosampler at 20 ± 2 °C and analyzed every 3 h until 12 h, and were re-injected at 24 h, 48 h, and 72 h. The results show that 4-FIBF and VF were the compounds more stable (48 h) at both concentration levels. For the rest of the analytes, certain differences were observed in the two concentration levels. For low concentration QC samples, CPF was the most stable together with 4-FIBF and VF (48 h), followed by fentanyl (24 h), carfentanil (12 h), 4-ANPP and MAF (9 h), *p*-FF, and AF (6 h), and finally FF (3 h). THFF was stable less than 3 h in the autosampler. The high concentration level of the QC, AF, carfentanil, and VF samples, were the most stable compounds together with 4-ANPP and 4-FIBF during the 48 h. The least stable compound turned out to be MAF, which was stable until 3 h.

The fully optimized and validated FPSE-GC-MS method was applied to the analysis of real oral fluid samples. A total of 15 OF samples from volunteer individuals and 8 OF samples from patients with drug addiction and undergoing detoxification treatment were analyzed. Due to the limited volume collected in the Salivette® devices, the samples were analyzed in duplicate. The results were negative for the fentanyl studied. However, the proposed methodology provides an effective analytical tool for its application in clinical and forensic toxicology to identify and confirm the presence of these compounds that may go unnoticed due to their structural similarity in screening tests, based on immunoassay or colorimetric techniques.

4. Conclusions

In this work, a robust method based on the FPSE technique combined with GC-MS has been proposed for the determination of fentanyl and ten derivatives in oral fluid samples. The set of desirable conditions required in the sample preparation process has been effectively determined using an asymmetric screening experimental design. This design allows to obtain the optimal parameters with a minimum number of experiments, such as sample volume and pH (500 µL, pH 7), the volume of back-extraction solvent (250 µL of ethyl acetate), shaking mode (sonication), as well as the extraction and desorption times (30 min and 10 min, respectively). The proposed method provides good linearity, as well as precision and accuracy (CV <20%), and good recoveries (approximately 100%). On the other hand, the values obtained in the present study attest to the good performance of the proposed method and attest to the importance of having validated methods to detect and quantifying fentanyl, which can be difficult to detect with other methods. Furthermore, to our knowledge, this study constitutes the first application of FPSE to the analysis of fentanyls in oral fluid samples and their subsequent determination by GC-MS.

Declaration of Competing Interest

All authors declare that they have no conflict of interest.

CRediT authorship contribution statement

Ana M. Ares-Fuentes: Investigation, Validation, Formal analysis, Visualization, Writing – original draft. **Rosa A. Lorenzo:** Conceptualization, Methodology, Writing – review & editing, Supervision. **Purificación Fernández:** Conceptualization, Methodology, Writing – review & editing, Supervision. **Ana M. Fernández:** Resources. **Kenneth G. Furton:** Resources. **Abuzar Kabir:** Resources, Writing – review & editing. **Antonia M. Carro:** Conceptualization, Methodology, Formal analysis, Data curation, Writing – review & editing.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2021.462768.

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