Journal of Chromatography B, 878 (2010) 2175–2183

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Hollow fiber-based liquid phase microextraction with factorial design optimization and gas chromatography–tandem mass spectrometry for determination of cannabinoids in human hair

Elissandro Soares Emídio[a], Vanessa de Menezes Prata[a], Fernando José Malagueño de Santana[b], Haroldo Silveira Dórea[a,*]

[a] Laboratório de Análise de Compostos Orgânicos Poluentes, Departamento de Química, Universidade Federal de Sergipe, Av. Marechal Rondon, s/n., Jardim Rosa Elze, 49100-000 São Cristóvão, SE, Brazil
[b] Departamento de Fisiologia, Universidade Federal de Sergipe, Av. Marechal Rondon, s/n., Jardim Rosa Elze, 49100-000 São Cristóvão, SE, Brazil

ARTICLE INFO

Article history:
Received 26 March 2010
Accepted 2 June 2010
Available online 9 June 2010

Keywords:
Cannabinoids
Hollow fiber liquid-phase microextraction
Fractional factorial design
GC–MS
Hair

ABSTRACT

A new method, based on hollow fiber liquid-phase microextraction (HF-LPME) and gas chromatography–tandem mass spectrometry (GC–MSMS), was developed for determination of Δ^9-tetrahydrocannabinol (THC), cannabidiol (CBD) and cannabinol (CBN) in samples of human hair. Since hair is a solid matrix, the samples were subjected to alkaline digestion using NaOH. The aqueous solutions obtained were extracted using a 6 cm polypropylene fiber (600 μm i.d., 200 μm wall thickness, 0.2 μm pore size) for each extraction. A 2^{5-1} fractional factorial design for screening, and a central composite design for optimization of significant variables, was applied during development of the extraction method. The variables evaluated were the type of extraction solvent, pH, stirring speed, extraction time, and acceptor phase volume. The optimized conditions for the proposed extraction procedure were 10 mg of hair sample; 20 μL of butyl acetate; aqueous (pH 14) donor phase containing 6.8% NaCl; 600 rpm stirring speed; 20 min extraction time. A linear response was obtained in the ranges 1–500 pg mg^{-1} (CBD and CBN) and 20–500 pg mg^{-1} (THC), with regression coefficients >0.99. Precision, determined as the relative standard deviation, was 3.3–8.9% (intra-day) and 4.4–13.7% (inter-day). Absolute recoveries varied in the ranges 4.4–4.8% (CBD), 7.6–8.9% (THC) and 7.7–8.2% (CBN). Limits of detection (LOD, S/N = 3) and quantification (LOQ, S/N = 10) were 0.5–15 pg mg^{-1} and 1–20 pg mg^{-1}, respectively. The method was successfully used to determine CBD, THC and CBN in hair samples from patients in a drug dependency rehabilitation center. Concentrations varied in the ranges 1–18 pg mg^{-1} (CBD), 20–232 pg mg^{-1} (THC) and 9–107 pg mg^{-1} (CBN), confirming the suitability of the method for monitoring studies.

1. Introduction

During the last decade, alternative or unconventional sample matrices have become increasingly important on the field of toxicology, due to their advantages compared to conventional matrices used in routine analyses. In general, the main advantages of the new procedures are non-invasive collection and simple application [1]. The most commonly used unconventional matrices used in toxicological analyses are saliva, sweat, fingernails and hair [2].

Hair analysis has been used forensically since the 1980s to investigate the chronic use of drugs of abuse, after pioneering work in opioid analysis by Baumgartner et al. [3]. Because of its solid and durable nature, hair differs from the other biological matrices normally used in toxicological analyses, such as blood and urine. Successful hair analyses can be performed even centuries after its growth [4].

Cannabinoids are terpene-phenolic compounds possessing 21 carbons, and comprise the principal class of the constituents of *Cannabis sativa* L. [5], with 95 cannabinoids having been isolated from the plant [6]. Of these, Δ^9-tetrahydrocannabinol (THC) is the main psychoactive component. The carboxylic acids of THC and cannabidiol (CBD) are quantitatively important in the plant, however under the influence of heat or light the carboxylic group is readily lost as CO_2, forming the neutral cannabinoids THC and CBD [7]. These same conditions favor the formation of cannabinol (CBN) from THC in the *Cannabis* plant, due to oxidative degradation [8].

Since the non-psychoactive cannabinoids (CBD and CBN) are normally found in hair samples together with the main psychoac-

* Corresponding author. Tel.: +55 79 2105 6654; fax: +55 79 2105 6651.
E-mail address: hdorea@ufs.br (H.S. Dórea).

1570-0232/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2010.06.005

tive constituent (THC), the identification of all three compounds is used to indicate that an individual has had contact with products derived from *Cannabis*. Determination of the main THC metabolite (11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid—THCCOOH) is recommended to distinguish between passive (cannabis smoke) and active (intentional consumption) exposures [9].

Various analytical methods employing gas chromatography coupled with mass spectrometry have been developed for the simultaneous determination of these three compounds, with sample preparation by liquid–liquid extraction (LLE) [10–14], solid-phase extraction (SPE) [15], supercritical fluid extraction (SFE) [16], solid-phase microextraction (SPME) [17–21] and solid-phase dynamic extraction (SPDE) [22]. The limitations of these extraction techniques include consumption of large quantities of solvents [23], high equipment costs [24] or variations in the pre-concentration factor between different batches of fibers [25].

In 1999, Pedersen-Bjergaard and Rasmussen introduced an extraction technique that reduced solvent consumption, termed hollow fiber liquid-phase microextraction (HF-LPME) [26]. Here, the extraction solvent (5–50 μL) is contained within the lumen of a porous hollow polypropylene fiber (1.5–10 cm long), with no direct contact with the sample [27], and the analytes are extracted by passive diffusion from the donor phase (matrix) to the acceptor phase (extraction solvent) [28]. HF-LPME can be performed in two-phase or three-phase modes. With two phases, the analytes are extracted from the aqueous matrix to a solvent immiscible with water present within the lumen of the fiber (acceptor phase), and immobilized in the pores of the fiber. In the three-phase mode, the acceptor phase comprises an acidic or alkaline aqueous solution, with an organic solvent immiscible with water immobilized in the pores of the membrane, providing a barrier between the (aqueous) sample and the aqueous acceptor phase [29]. Different to the single-drop microextraction (SDME) technique, which uses a drop of organic solvent (typically 1–3 μL) suspended from a microsyringe during the extraction process, HF-LPME allows use of high stirring rates to accelerate reaction kinetics, as well as a greater area of contact between the matrix and the extractor phase, resulting in enhanced mass transfer [25]. In HF-LPME the extractor phase is protected, so that the analysis of complex samples becomes viable since the membrane micropores provide efficient sample filtration, resulting in extremely clean extracts [30].

Here, an analytical method is proposed for the simultaneous determination of THC, CBN and CBD in hair, using HF-LPME, with separation and detection by GC–MS/MS. A fractional factorial design with subsequent central composite design was performed to optimize the main variables involved in the procedure. The method was validated and applied to samples of human hair obtained from users of *C. sativa* undergoing treatment in rehabilitation clinics.

2. Experimental

2.1. Reagents and standard solutions

The solvents 1-octanol (Fluka, Buchs, Switzerland), butyl acetate and n-octane (Sigma–Aldrich, St. Louis, MO, USA) were analytical grade. Cyclohexane (Tedia Company Inc., Fairfield, USA), isooctane (Mallinckrodt, Phillipsburg, NJ, USA) and toluene (Merck, Darmstadt, Germany) were all HPLC grade. Sodium hydroxide was obtained from Synth (São Paulo, Brazil), hydrochloric acid from Nuclear (São Paulo, Brazil) and anhydrous sodium carbonate from Vetec (Rio de Janeiro, Brazil). Ultrapure water was provided from a Milli-Q® system (Millipore, Mildford, MA, USA). Stock standard solutions of THC, CBN, CBD (1 mg mL^{-1}) and deuterated Δ^9-tetrahydrocannabinol (THC-D_3, 0.1 mg mL^{-1}) in methanol (Cerilliant, Round Rock, TX, USA). Mixed working standard solutions of the cannabinoids (1, 10, 100 and 1000 μg L^{-1}) were prepared by dilution of the stock solutions in methanol. The solutions were stored at 4 °C, protected from light. The stock solutions were stable throughout the period of the study (3 months).

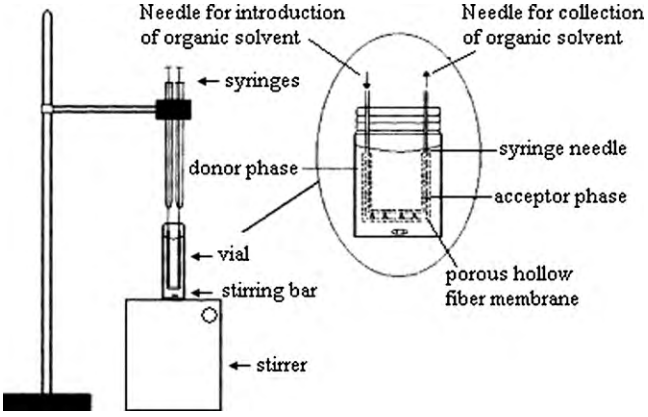

Fig. 1. Schematic representation of the hollow-fiber configuration. (Reproduced with permission from [31].)

2.2. Sample preparation

For method development and validation, hair samples were obtained from volunteers who did not use products containing cannabinoids. The hair was collected from the upper rear part of the head, cut as close as possible to the scalp, and stored at ambient temperature in paper envelopes. The length of the hair samples used in the analyses varied between 2 and 12 cm, with the entire length being analyzed. Prior to the decontamination procedure, the hair samples were carefully cut into small segments shorter than 2.0 mm. A 10 mg portion of hair was transferred to a 10 mL vial, and decontaminated using petroleum ether, deionized water and dichloromethane (2 mL of each solvent), in sequence, for 10 min in a sonicator (USC 1400, Unique, São Paulo, Brazil). After the decontamination process, the sample was dried at ambient temperature. Hair digestion used alkaline hydrolysis with 1 mL of NaOH (1 mol L^{-1}), at 85 °C for 15 min.

All of the participating patients were informed of the objectives of the study in a clear and detailed manner.

2.3. HF-LPME procedure

An Accurel Q3/2 polypropylene hollow fiber membrane (600 μm i.d., 200 μm wall thickness, 0.2 μm pore size), obtained from Membrana GmbH (Wuppertal, Germany) was used for extraction of analytes contained in the hair digestion solution. A 50 μL syringe (model 705SNR, Hamilton, Bonaduz, Switzerland) was used to introduce the acceptor phase, and another was used for its removal (U-shape configuration), as shown in Fig. 1. Before use, the hollow fiber was cleaned by sonication in acetone for 5 min, to remove any possible contaminants adhering to the fiber. After drying, the fiber was manually cut to a length of 6 cm. A new fiber was used for each extraction, hence avoiding any carryover between analyses. Following decontamination and alkaline digestion, 6.8% (w/v) of NaCl was added, after cooling the solution to ambient temperature. The fiber was immersed in the organic solvent for 10 s to saturate the pores, and then placed in ultrapure water for 10 s to remove any residual organic solvent present on the fiber surface. Before extraction, the microsyringe was washed 10 times with butyl acetate to avoid carryover or formation of air bubbles. For extraction, 20 μL of butyl acetate were injected into the fiber, which was then immersed in the aqueous matrix for 20 min at ambient

Table 1
Parameters for MS–MS detection of selected cannabinoids.

Cannabinoids	Parent ion (m/z)	Retention time (min)	Excitation storage level (m/z)	Excitation amplitude (eV[a])	Daughter ions (m/z)[b]
CBD	231	9.76	110	0.7	**174** (100%), 175 (16%)
THC-D_3	317	10.49	140	0.6	234 (33%), 243 (52%), 258 (26%), **302** (100%)
THC	299	10.52	132	1.9	193 (55%), **217** (100%), 243 (64%), 257 (78%)
CBN	295	11.10	130	1.4	**238** (100%), 239 (19%)

[a] eV: electron volt.
[b] Quantitation ions in bold letters.

temperature and stirring velocity of 600 rpm. After extraction, 5 μL of THC-D_3 surrogate standard (0.2 μg mL^{-1}) in butyl acetate was added to the extract, the mixture homogenized, and 1 μL portions injected into the GC–MS/MS.

2.4. Gas chromatography tandem mass spectrometry

Analyses were performed using a CP-3800 gas chromatograph, coupled with a Saturn 4000 MS/MS ion trap mass detector and equipped with a model 1079 split/splitless injector and a CP-8400 autosampler (Varian, Walnut Creek, CA, USA). The separation column was a Varian VF-5MS capillary (30 m × 0.25 mm i.d., 0.25 μm film thickness). Ultrapure helium (99.995%) was used as the carrier gas, at a flow rate of 1 mL min^{-1}. The oven temperature program was initial 60 °C, increased at 35 °C min^{-1} to 255 °C, maintained for 1 min, then a further ramp at 2 °C min^{-1} to 280 °C, maintained for 2 min. The injector, manifold, trap and transfer line temperatures were set at 260, 50, 190 and 280 °C, respectively. The solvent delay was 6 min. Splitless injection was employed, with a split valve off-time of 1 min. Data acquisition and processing were performed using Varian Star Workstation software (version 6.9). The total run time was 21 min. The MS was initially operated in full-scan mode, with 70 eV electron impact. The mass range was 50–400 m/z, with 0.79 s/scan and 0 threshold. The ionization filament emission current was 10 μA.

The mass spectrometer was operated in tandem MS/MS mode in order to improve the sensitivity and selectivity of the method. Mass spectrum analysis was performed in electron impact (EI) mode, with target TIC, emission current and average scans of 1000 counts, 10 μA and 1 microscan, respectively. The selected parent ions were m/z 231, 295, 299 and 317 for CBD, CBN, THC and THC-D_3, respectively. The daughter ion generated from fragmentation of the 302 m/z THC-D_3 parent ion presented a signal (peak area) smaller than that obtained by fragmentation of the 317 m/z parent ion, which was therefore selected for quantification. Excitation storage levels (storage RF) were m/z 110 (CBD), 132 (THC), 130 (CBN) and 140 (THC-D_3), and the isolation window for the compound spectra analyzed was m/z 3. A study of the collision-induced dissociation (CID) was undertaken to ensure adequate fragmentation of the cannabinoid parent ion, and maximize the detection limit of the technique. This was performed by varying the excitation energy from 0 to 90 eV in non-resonant mode, and from 0 to 2 eV in resonant mode, for direct injection of 5 μg mL^{-1} cannabinoid solutions.

2.5. Design of experiments

Univariate design was used to select the extraction solvent (acceptor phase). The solvents tested were toluene, 1-octanol, isooctane, butyl acetate, cyclohexane and n-octane. Extractions were performed in triplicate, using fortification with 100 pg mg^{-1} of the cannabinoids, addition of 12% (w/v) NaCl, 20 μL volumes of the solvents, an extraction time of 20 min and a stirring speed of 600 rpm.

After extraction solvent selection, the other parameters affecting the HF-LPME procedure were evaluated: extraction time, ionic strength, stirring speed, pH and volume of the acceptor phase. A fractional factorial design was used, with resolution V (2^{5-1}). Three replicates at the central point (total of 19 randomized experiments) were included in the design in order to estimate the experimental variance and check for loss of linearity between the levels chosen for each variable. Afterwards, a central composite design was performed to optimize the values of the significant variables obtained in the fractional factorial design, in order to improve the response. A 2^3 central composite design was performed, with six star points and three center points, totalling 17 experiments ($2^3 + (2 \times 3) + 3$). The value of α used to establish the condition of rotability was 1.682. The data were processed using Statistica® 6.0 software.

2.6. Method validation

Validation of the analytical method was according to the guidelines of the Society of Toxicological and Forensic Chemistry (GTFCh, Germany), which provide specific recommendations for hair analysis procedures [32]. Performance of the HF-LPME method was assessed in terms of selectivity, linearity, precision, recovery, and limits of detection and quantification. Selectivity was determined from analysis of six sample blank replicates (free of analytes), and also from analyses of the sample blank plus surrogate THC-D_3 standards at a concentration of 100 pg mg^{-1} (in duplicate). The linearity of the calibration curve was determined in triplicate in the ranges 1–500 pg mg^{-1} (CBD and CBN) and 20–500 pg mg^{-1} (THC), with five points. Precision (intra-day, $n = 5$) was determined on five different days (inter-day, $n = 10$), at concentrations corresponding to the upper and lower limits of the linear range of the calibration curve for each cannabinoid.

For determination of the absolute recovery, hair samples (10 mg) spiked with 10 and 5000 pg of CBD and CBN, and 200 and 5000 pg of THC, were analyzed in triplicate according to the HF-LPME procedure. The results were compared with direct injection of the standards in methanol (10, 200 and 5000 pg μL^{-1}), using 1 μL injection volumes. The limits of detection and quantification of the method were calculated as 3 and 10 times the signal to noise (S/N) ratio, respectively.

3. Results and discussion

3.1. Optimization of the GC–MS/MS conditions

The parent ions were initially selected from the mass spectra obtained in full-scan mode. Fragmentation of the parent ions was then achieved by collision-induced dissociation (CID) in resonant excitation mode, increasing the sensitivity compared to the non-resonant mode (with a threefold increase in the S/N ratio). The relative abundances of the parent ions after CID were around 5–15%. Confirmation of the presence of the analytes was based on their retention times and the presence of two higher intensity daughter ions. Table 1 provides the parameters for analysis of the cannabinoids using the tandem MS/MS mode.

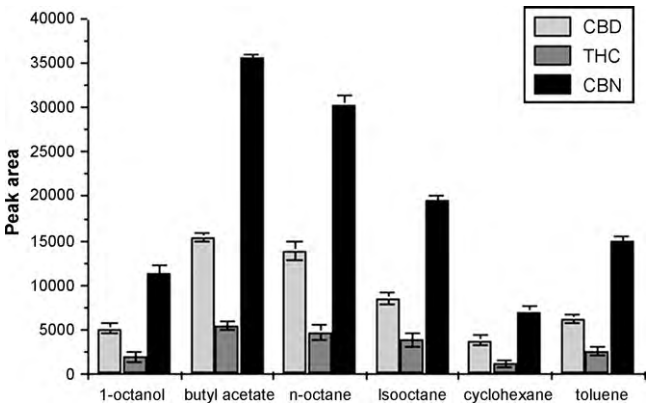

Fig. 2. Effect of extraction solvent on HF-LPME extraction efficiency ($n = 3$).

3.2. Optimization of the hollow-fiber LPME method

3.2.1. Organic solvent selection

The choice of solvent was based on affinity for the analytes, low solubility in water, low viscosity (to improve mass transfer), and (since the two phase mode was used) compatibility with direct injection onto the GC capillary column. Six organic solvents were investigated: toluene, 1-octanol, isooctane, butyl acetate, cyclohexane and octane. Butyl acetate was shown to provide the best results, based on consideration of the chromatographic peak areas and losses of the solvents on the fiber membranes (Fig. 2). When cyclohexane and toluene were used, bubbles were formed on the fiber surfaces, and solvent was lost during the extraction.

3.2.2. Fractional factorial design

The factors and levels employed in this design (Table 2) were selected during preliminary experiments taking into account the limitations of the experimental system. In this step, the response was evaluated from the THC peak area, since this was the analyte for which the sensitivity was lowest.

Table 2
Variables and levels investigated using the fractional factorial design experimental.

Variables	Levels		
	Low (−1)	Center (0)	High (+1)
(t) Extraction time (min)	10	20	30
(pH) Sample pH	4	7	10
(SS) Stirring speed (rpm)	300	600	900
(S) Ionic strength (NaCl, w/v%)	0	7.5	15
(O) Organic phase volume (μL)	8	14	20

Fig. 3 shows the Pareto diagram with results for the sorting of the variables. The value of Student's t-test parameter was 3.1824. The proposed model could explain around 98.4% of the variance (R^2), indicating a good fit of the experimental data. The effects of all of the variables and their interactions showed positive values. Similar results were obtained for CBD and CBN, with ionic strength (% NaCl) being the most significant factor.

Addition of salt (as NaCl, Na_2SO_4 or Na_2CO_3) reduces the solubility of the analytes in the matrix, and therefore increases their partitioning into the organic phase (the salting-out effect) [33]. Similar results have been previously described for THC, CBD and CBN [19–21]. The pH showed an important influence, with the analytes being extracted more efficiently under alkaline conditions (pH 10). At acid pH (pH 4) there was precipitation of hair proteins, which adhered to the fiber surface and possibly restricted diffusion of analytes to the lumen, ultimately reducing the analytical response. It is possible that under these conditions, the proteins (especially keratin) formed agglomerates that tended to precipitate. As expected, the yield of the extraction increased with increase of the volume of the acceptor (organic) phase present within the fiber lumen [34]. According to the Pareto diagram, extraction time and stirring speed were not statistically significant in the yield of the cannabinoid extraction, probably because the equilibrium condition had already

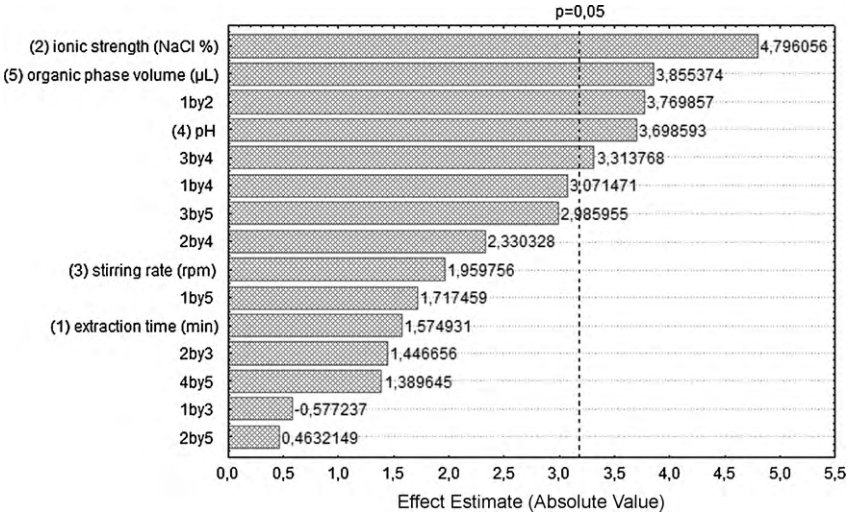

Fig. 3. Standardized main effect Pareto chart for the fractional factorial design of the screening experiment.

Table 3
Independent factors and levels used in the rotatable CCD.

Variables	Levels			Star points ($\alpha = 1.682$)	
	Low (−1)	Center (0)	High(+1)	$-\alpha$	$+\alpha$
(S) Ionic strength (NaCl; w/v%)	7	11	15	4	18
(pH) Sample pH	9	11	13	8	14
(O) Organic phase volume (μL)	15	20	25	12	28

Table 4
Estimated regression coefficients and analysis of variance of the predicted model.

Factor	Coefficient	*F*-value[c]	*p*-value
S[a]	−2532.690	6.770	0.035
pH	9557.399	96.413	0.000
O[b]	1.948	0.000	0.998
S^2	−1174.504	1.202	0.309
pH^2	5089.048	22.565	0.002
O^2	−433.986	0.164	0.698
$S \times pH$	−4675.375	13.515	0.008
$S \times O$	433.625	0.116	0.743
$pH \times O$	−281.375	0.049	0.831

[a] *S*: % NaCl.
[b] O: Organic phase volume.
[c] $F_{critical}(1;7;0.05) = 4.45$.

been reached at a lower level (extraction time) within the chosen domain. It was found that a high stirring speed led to the formation of air bubbles on the surface of the hollow fiber, and also to solvent evaporation.

3.2.3. Central composite design (CCD)

Based on the above results, the variables % NaCl (*S*), pH and organic phase volume (O) were included in the central composite design for optimization of the maximum responses to the cannabinoids. The extraction time and stirring speed were held constant at 20 min and 600 rpm, respectively. The central composite design consists of a 2^k factorial run, with $2k$ axial (α) and C_0 center point runs. The total number (N) of experiments required is given by: $N = 2^k + 2k + C_0$, where k is the number of variables. The levels of this design are provided in Table 3.

The regression coefficients of each model term are presented in Table 4, together with analysis of variance (ANOVA) of the effects. The R^2 value indicates that the model could explain 95.5% of the variability. The plots of experimental against predicted values (A), normal probability of residuals (B), and residuals against predicted response (C) are shown in Fig. 4. Fig. 4A and B reveals that the proposed model describes the experimental data well, since the points are located close to the straight line, hence assuring normality of the residuals. Fig. 4C shows points that are randomly distributed, characterizing a constant variance of errors. These features demonstrate the reliability of the data used in the design.

An ANOVA (Table 4) *p*-value less than 0.05 indicates that an effect is statistically significant at a 95% level of confidence. The data show that the effects of *S* and (especially) pH were significant. The organic phase volume showed no significant influence on the response (linear and quadratic terms), while the influences of *S* and pH were significant.

Analyses of the optimum CCD values were performed by the response surface method, using the quadratic model, with the responses (peak areas) for the three cannabinoids being transformed as a function of the desirability value (D). An overall D can be defined as the geometrical mean of all the individual desirabilities (d_i): $D = (d_1 * d_2 * d_n)^{1/n}$. The values of d_i vary between 0 and 1. The optimum CCD values were $S = 6.8\%$, pH = 14 and O = 28 μL, with $D = 1$ (Fig. 5).

Fig. 6 shows the desirability surface plot and the combinations of the three selected experimental variables. Fig. 6A confirms the existence of a significant negative interaction between pH and % NaCl, suggesting that a low salt level and a high pH increase the cannabinoid extraction efficiency. A reduction in response at high NaCl concentrations can also be seen. According to Psillakis and Kalogerakis [33], this can be explained by alteration of the physico-chemical properties of the sample (such as surface tension or viscosity), reducing the mass transfer rate of the analytes to the acceptor phase. High pH improves analyte response, as hasbeen

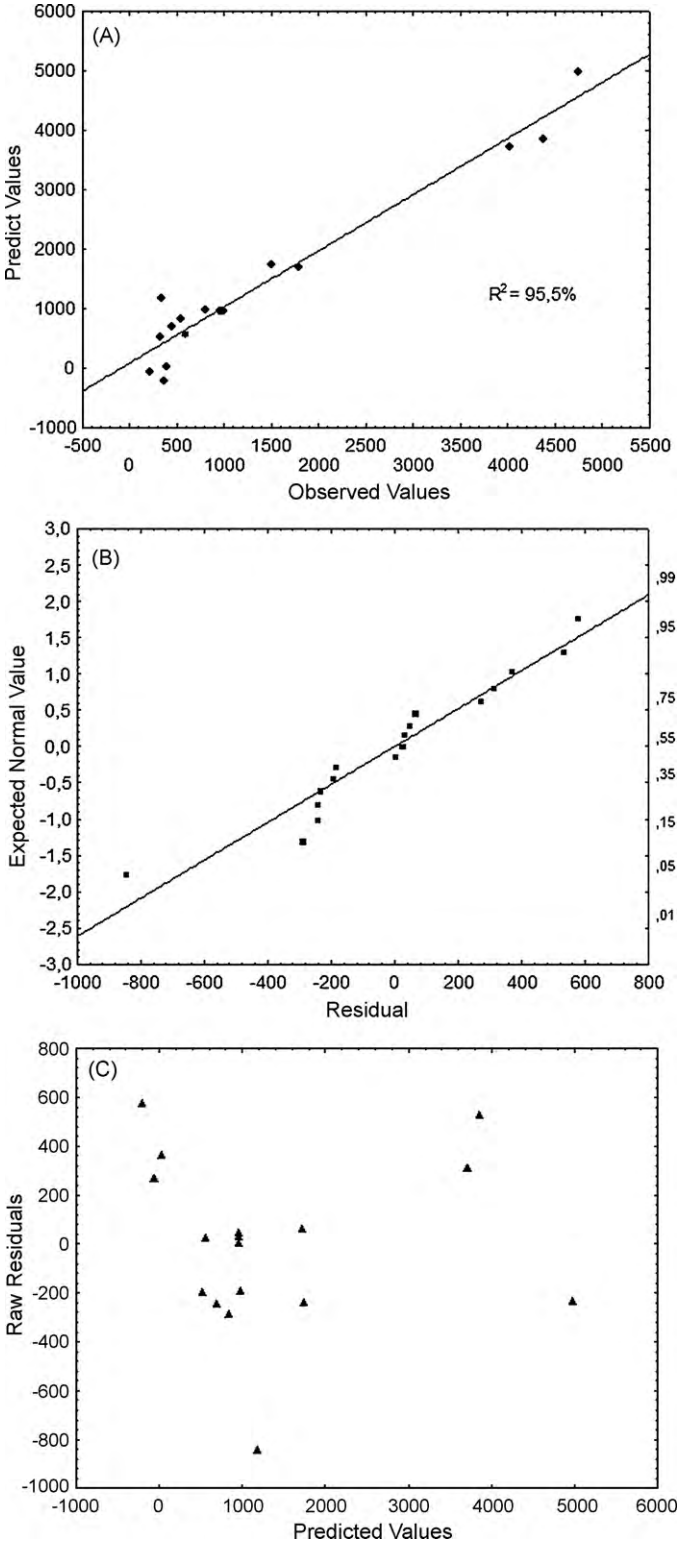

Fig. 4. Relation between experimental and predicted values (A). Normal probability plot of residuals (B). Plot of residuals vs. predicted response (C).

observed previously [17,19,22]. Fig. 6B and C shows the interactions of salt and pH with the organic phase volume, indicating that the latter did not influence the response (Table 4). Hence, an intermediate volume of solvent (20 μL) was used for method validation. Fig. 7B illustrates a chromatogram obtained under optimized conditions.

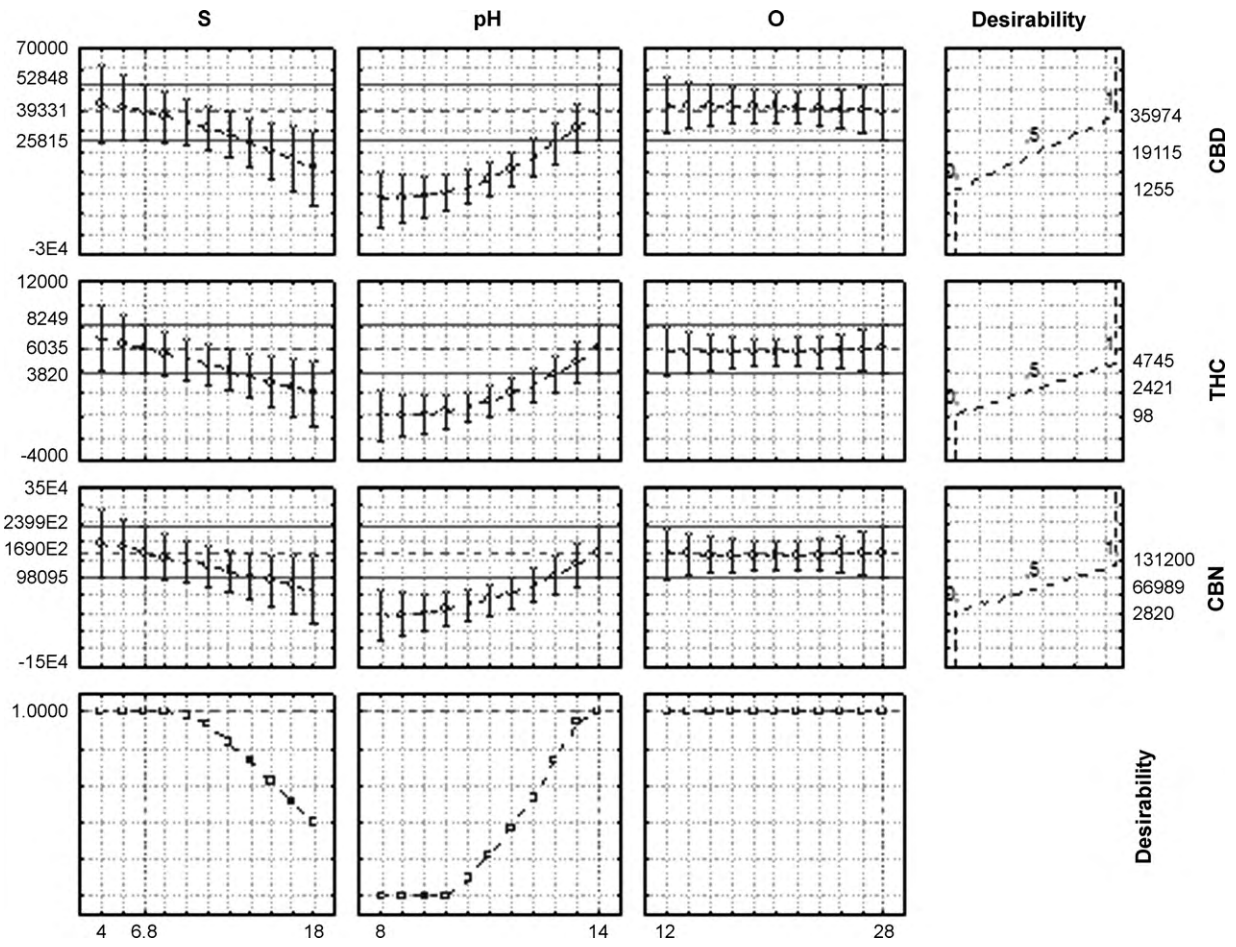

Fig. 5. Profiles for predicted values and desirability of the cannabinoids.

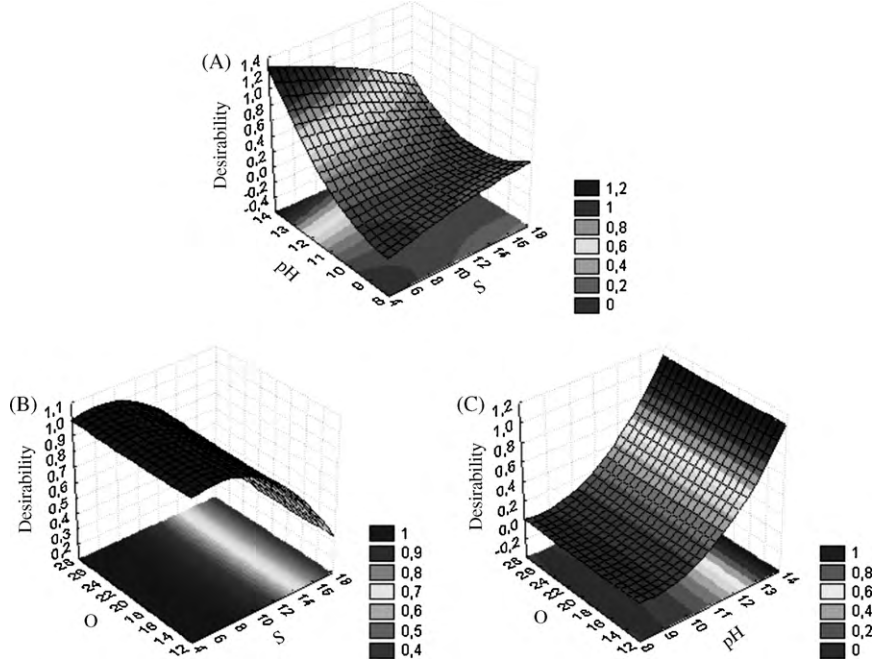

Fig. 6. Response surface plots of the overall desirability function (*D*) of the HF-LPME method.

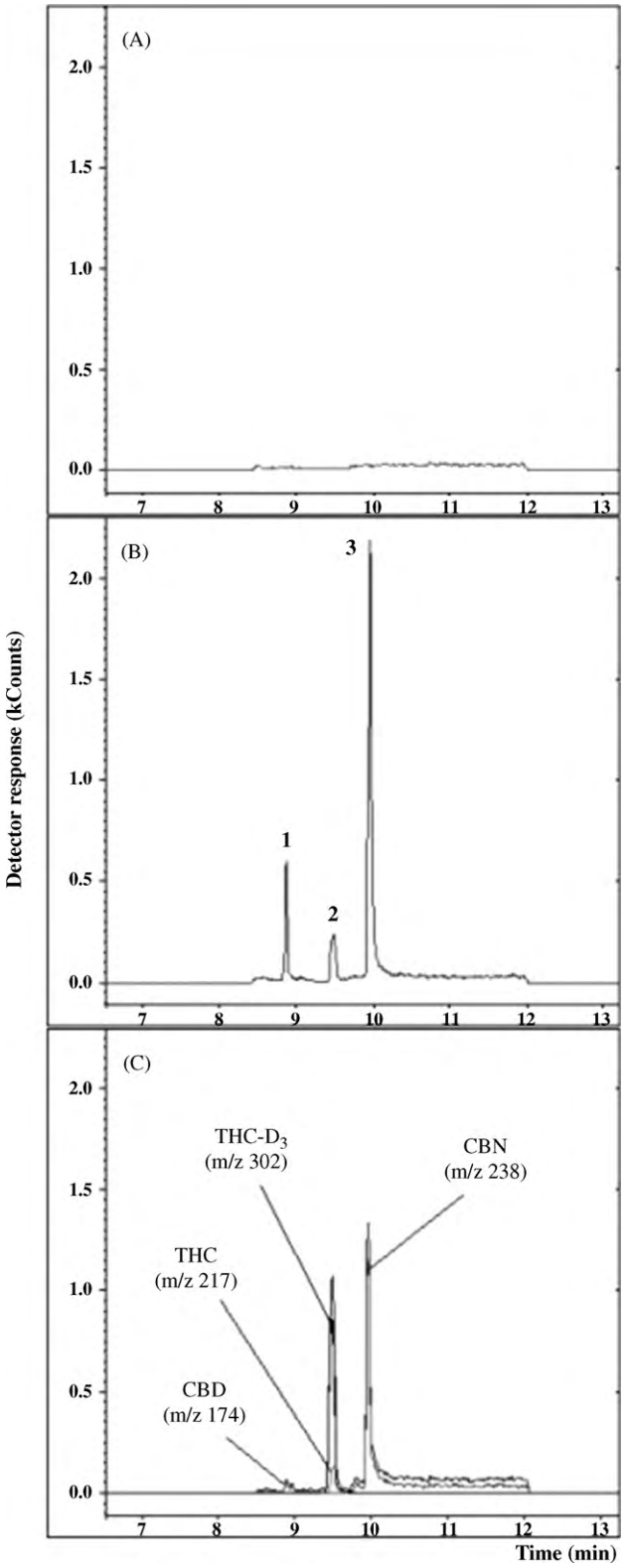

Fig. 7. Chromatograms obtained for analysis of hair using HF-LPME. (A) Sample blank; (B) hair sample spiked at 0.2 ng mg^{-1} of each analyte: 1-CBD; 2-THC; 3-CBN; (C) real hair sample containing 14 pg mg^{-1} of CBD, 232 pg mg^{-1} of THC and 107 pg mg^{-1} of CBN.

3.3. Method validation

From the results of the experiments investigating the effects of the different variables, the following conditions were selected for assessment of the efficiency of the method: butyl acetate acceptor phase, 600 rpm stirring speed, 6.8% NaCl, pH 14 aqueous donor phase, 20 μL of acceptor phase and 20 min extraction time. The performance results, in terms of selectivity, linearity, precision, recovery and limits of detection and quantification, are provided in Table 5. The method demonstrated good selectivity, with absence of interferences in determination of the cannabinoids (Fig. 7A). The calibration curve was linear within the range studied, with correlation coefficients better than 0.99. The RSD values varied in the range 3.3–8.9% (intra-day) and 4.4–13.7% (inter-day). The absolute recoveries were satisfactory, with values of 4.4–4.8% (CBD), 7.6–8.9% (THC) and 7.7–8.2% (CBN). Detection limits were 15 pg mg^{-1} for THC, and 0.5 pg mg^{-1} for CBD and CBN (n = 5, S/N = 3). Quantification limits were 20 pg mg^{-1} (THC) and 1 pg mg^{-1} (CBD and CBN) (n = 5, S/N = 10), and lower than the cut-off value for THC (100 pg mg^{-1}) [35], assuring applicability of the method in analysis of real samples.

Compared with earlier SPME–GC–MS methods for analyses of THC, CBD and CBN in hair [17–21], the proposed technique offers a shorter extraction time than those reported by Musshoff et al. [19], de Oliveira et al. [20] and Sporkert and Pragst [21] (33, 40 and 30 min, respectively). Good absolute recoveries (4.4–8.9%) were achieved by the present method compared to those previously obtained of 0.3–7.5% [19] and 4.0–11.2% [20]. Limits of detection in the range 1–20 pg mg^{-1} were better than those found for THC, CBD and CBN by Nadulski and Pragst [17] (37–48 pg mg^{-1}), Strano-Rossi and Chiarotti [18] (100–200 pg mg^{-1}), Musshoff et al. [19] (50–140 pg mg^{-1}) and De Oliveira et al. [20] (120 pg mg^{-1}).

3.4. Application

The validated method was used for the analysis of THC, CBD and CBN in hair samples obtained from 23 patients (males aged between 18 and 49 years) attending a drug dependency rehabilitation center, who reported using *Cannabis* products at a frequency of between 1 and 35 joints per week, over a period of between 1 and 27 years. Table 6 provides the quantitative results obtained for each patient, together with consumption frequency, the last usage of the drug, the number of days hospitalized, length and color of hair, and information on usage of other drugs.

CBN was detected in all of the samples, either alone (n = 12), in association with CBD (n = 11), or together with THC and CBD (n = 5). These observations could be explained by the conversion of THC to CBD by pyrolytic degradation of THC to CBN when *Cannabis* is smoked [18]. THC and CBD were detected in 22% (n = 5) and 48% (n = 11) of the cases analyzed, respectively. The concentrations of CBD, THC and CBN varied in the ranges 1–18 pg mg^{-1} (mean 10 pg mg^{-1}), 20–232 pg mg^{-1} (mean 69 pg mg^{-1}) and 9–107 pg mg^{-1} (mean 21 pg mg^{-1}), respectively.

According to the Society of Hair Testing [35], which recommends a limit value for THC of 100 pg mg^{-1}, the results for patient 9 (THC = 232 pg mg^{-1}) would be indicative of regular exposure to cannabis (either external contamination by cannabis smoke, or genuine self-administration). Results for patients 3 (63 pg mg^{-1} of THC) and 6 (84 pg mg^{-1} of THC), who both reported high consumption of the drug (25–30 joints per week, respectively), would be considered as testing positive according to the criteria of Pragst and Nadulski [36], who proposed a lower limit value of 50 ng mg^{-1} of THC in hair, since they observed that many occasional *Cannabis* users were not identified using a limit of 100 pg mg^{-1}.

From the data in Table 6, lack of detection of THC, together with low concentrations of CBD and CBN, could be due to lengthy internment and consequently long periods of abstinence. Other factors that could influence the concentrations of *Cannabis* components include cosmetic treatment [37], or exposure to sunlight and humidity [38]. Patients 1, 4, 5, 10, 14 and 15 showed a period of abstinence from the drug that was greater than that corresponding

Table 5
Validation data for the proposed method.

Compound	Linear range (pg mg^{-1})	R^2	Spiked amount (pg mg^{-1})	% RSD[a]		Absolute recovery (%)	LOD[b] (pg mg^{-1})	LOQ[c] (pg mg^{-1})
				Intra-day	Inter-day			
CBD	1–500	0.9971	1	6.6	6.9	4.8 ± 1.3	0.5	1
			500	3.3	4.4	4.4 ± 1.1		
THC	20–500	0.9939	20	5.7	6.7	8.9 ± 1.5	15	20
			500	7.8	11.9	7.6 ± 1.2		
CBN	1–500	0.9971	1	8.9	13.7	8.2 ± 1.4	0.5	1
			500	7.6	12.9	7.7 ± 0.9		

[a] RSD: relative standard deviation.
[b] LOD: limit of detection.
[c] LOQ: limit of quantification.

to the length of hair collected. For instance, assuming an average hair growth rate of 1 cm per month, analysis of a 6 cm hair sample, cut close to the scalp, should provide an indication of any cannabis consumption within the last 6 months. Since THC, CBD and CBN are lipophilic compounds, these substances could be deposited in adipose tissues, with gradual subsequent release increasing the time during which cannabinoids could be incorporated into the hair, as reported by Pragst and Balikova [4]. The color of the hair samples varied from gray to dark brown. The transfer of drugs is associated with their affinity for melanin, and various studies have shown that the melanin content is important for the binding of basic drugs, such as cocaine or amphetamine. However, there are no data in the literature indicating that cannabinoid uptake is influenced by hair color [39].

The high sensitivity of the method enabled detection of the cannabinoids in hair samples even after periods of abstinence exceeding 3 years (reported by patient 10). The technique's selectivity provided chromatograms that were free of any interference close to the retention times of the cannabinoids, even in the presence of different chemical class drugs of abuse (Table 6). Fig. 7C shows a typical chromatogram for a sample obtained from a patient testing positive. Positivity suggests either that a very long hair shaft has been analyzed, or that the subjects did not report their actual cannabis use. It is also possible that the results could reflect external contamination, rather than past consumption.

4. Conclusions

An analytical method based on hollow fiber liquid-phase microextraction and gas chromatography–tandem mass spectrometry was developed and validated for determination of THC, CBN and CBD in human hair. Due to its simplicity and low cost, the fiber can be discarded after each extraction, eliminating any possibility of carryover. A factorial design was employed to optimize the method

Table 6
Self-reported drug use histories for 23 cannabis users.

Patient	Frequency (joints) per week	Last use (days) approximately	Internment days	Hair length (cm)	Color of hair	CBD, THC and CBN concentration in hair (pg mg^{-1})	Consume of other drugs
1	20	365	30	6	Gray	18; < LOQ; 58	Amphetamine, barbiturates, cocaine, crack, ecstasy, LSD
2	10	28	18	2	Dark	< LOQ; <LOQ; 17	Crack, cocaine
3	25	14	14	2	Dark	6; 63; 14	Crack, cocaine
4	7	365	75	8	Dark	n.d.; n.d.; 13	Alcohol, cocaine, crack
5	7	150	10	4	Dark	1; n.d.; 12	Alcohol, cocaine, crack
6	30	7	5	6	Blond	6; 84; 57	Cocaine, crack, LSD
7	9	120	2	8	Brown	n.d.; n.d.; 18	Crack
8	8	28	5	4	Gray	< LOQ; n.d.; 13	Alcohol, cocaine, crack
9	35	15	7	2	Dark	14; 232; 107	Alcohol, cocaine
10	10	1095	38	5	Dark	< LOQ; n.d.; 12	Crack
11	10	150	120	5	Dark	n.d.; n.d.; 11	Alcohol, cocaine, crack
12	14	90	960	7	Brown	6; n.d.; 18	Alcohol, cocaine, crack
13	7	28	8	4	Dark	6; n.d.; 18	Alcohol, amphetamine, cocaine, crack, psylocibin
14	15	365	5	8	Brown	n.d.; n.d.; 13	Alcohol, amphetamine, cocaine, crack
15	30	365	365	7	Gray	n.d.; n.d.; 16	Alcohol, cocaine, crack, flunitrazepam, psylocibin
16	1	30	15	3	Dark	n.d.; n.d.; 11	Alcohol, crack
17	10	30	28	6	Dark	n.d.; n.d.; 13	Alcohol, cocaine
18	20	150	90	5	Dark	n.d.; n.d.; 11	Cocaine, crack
19	7	120	28	5	Brown	n.d.; n.d.; 11	Alcohol, amphetamine, benzodiazepines, cocaine, crack, morphine
20	12	365	14	13	Brown	7.; n.d.; 11	Alcohol, amphetamine, cocaine, crack, psylocibin
21	7	90	45	4	Brown	n.d.; n.d.; 10	Alcohol, cocaine, crack
22	10	45	30	3	Blond	n.d.; n.d.; 9	Alcohol, cocaine, crack,
23	15	120	120	5	Brown	n.d.; n.d.; 9	Crack

n.d.: not detected.
<LOQ: Below limit of quantitation.
LSD: lysergic acid diethylamide.

variables, which reduced the number of optimization experiments. Method validation showed that selectivity, sensitivity, linearity, precision, absolute recovery, and detection and quantification limits were comparable or superior to existing methods. Results for samples obtained from *Cannabis* users indicated that the technique could be used for analyses of cannabinoids in human hair, as well as for monitoring of drug dependent individuals in rehabilitation clinics.

Acknowledgements

The authors thank Obra Social Fazenda da Esperança, Casa de Saúde Santa Maria and its members for the kind provision of samples. Financial support was provided by Fundação de Amparo à Pesquisa e à Inovação Tecnológica do Estado de Sergipe (FAPITEC, in the form of a studentship), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Process no 479687) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

References

[1] E. Gallardo, J.A. Queiroz, Biomed. Chromatogr. 22 (2008) 795.
[2] M.A. Huestis, E.J. Cone, in: S.B. Karch (Ed.), Drug Abuse Handbook, CRC Press, Boca Raton, 1997, p. 811.
[3] A.M. Baumgartner, P.F. Jones, W. Baumgartner, C.T. Black, J. Nuclear Med. 20 (1979) 748.
[4] F. Pragst, M.A. Balikova, Clin. Chim. Acta 370 (2006) 18.
[5] M.M. Radwan, M.A. ElSohly, D. Slade, S.A. Ahmed, L. Wilson, A.T. El-Alfy, I.A. Khan, S.A. Ross, Phytochemistry 69 (2008) 2627.
[6] M.M. Radwan, M.A. ElSohly, D. Slade, S.A. Ahmed, I.A. Khan, S.A. Ross, J. Nat. Prod. 72 (2009) 906.
[7] A.A.M. Stolker, J. van Schoonhoven, A.J. de Vries, I. Bobeldijk-Pastorova, W.H.J. Vaes, R. van den Berg, J. Chromatogr. A 1058 (2004) 143.
[8] B. De Backer, B. Debrus, P. Lebrun, L. Theunis, N. Dubois, L. Decock, A. Verstraete, P. Hubert, C. Charlier, J. Chromatogr. B 877 (2009) 4119.
[9] M. Uhl, H. Sachs, Forensic Sci. Int. 145 (2004) 144.
[10] M.J. Baptista, P.V. Monsanto, E.G.P. Marquesa, A. Bermejo, S. Ávila, A.M. Castanheira, C. Margalho, M. Barroso, D.N. Vieira, Forensic Sci. Int. 128 (2002) 66.
[11] V. Cirimele, H. Sachs, P. Kintz, P. Mangin, J. Anal. Toxicol. 20 (1996) 13.
[12] J.Y. Kim, S. Suh, M.K. In, K.J. Paeng, B.C. Chung, Arch. Pharm. 28 (2005) 1086.
[13] V. Cirimele, P. Kintz, C. Jamey, B. Ludes, J. Anal. Toxicol. 23 (1999) 349.
[14] O. Quintela, A.M. Bermejo, M.J. Tabernero, S. Strano-Rossi, M. Chiarotti, A.C.S. Lucas, Forensic Sci. Int. 107 (2000) 273.
[15] J. Wicks, L.M.T. Tsanaclis, Ann. Toxicol. Anal. 17 (2005) 120.
[16] V. Cirimele, P. Kintz, R. Majdalani, P. Mangin, J. Chromatogr. B 673 (1995) 173.
[17] T. Nadulski, F. Pragst, J. Chromatogr. B 846 (2007) 78.
[18] S. Strano-Rossi, M. Chiarotti, J. Anal. Toxicol. 23 (1999) 7.
[19] F. Musshoff, H.P. Junker, D.W. Lachenmeier, L. Kroener, B. Madea, J. Anal. Toxicol. 26 (2002) 554.
[20] C.D.R. de Oliveira, M. Yonamine, R.L.M. Moreau, J. Sep. Sci. 30 (2007) 133.
[21] F. Sporkert, F. Pragst, Forensic Sci. Int. 107 (2000) 129.
[22] F. Musshoff, D.W. Lachenmeier, L. Kroener, B. Madea, Forensic Sci. Int. 133 (2003) 32.
[23] J. Zhang, H.K. Lee, J. Chromatogr. A 1117 (2006) 31.
[24] Z.S. Ferrera, C.P. Sanz, C.M. Santana, J.J.S. Rodríguez, Trends Anal. Chem. 23 (2004) 470.
[25] E. Psillakis, N. Kalogerakis, Trends Anal. Chem. 22 (2003) 573.
[26] S. Pedersen-Bjergaard, K.E. Rasmussen, Anal. Chem. 71 (1999) 2650.
[27] K.E. Rasmussen, S. Pedersen-Bjergaard, Trends Anal. Chem. 23 (2004) 2.
[28] S. Pedersen-Bjergaard, K.E. Rasmussen, J. Chromatogr. A 1184 (2008) 133.
[29] A. Sarafraz-Yazdi, A. Amiri, Trends Anal. Chem. 29 (2010) 10.
[30] F. Pena-Pereira, I. Lavilla, C. Bendicho, Spectrochim. Acta, Part B 64 (2009) 4.
[31] F.J.M. de Santana, A.R.M. de Oliveira, P.S. Bonato, Anal. Chim. Acta 538 (2005) 96.
[32] F.T. Peters, M. Hartung, M. Herbold, G. Schmitt, T. Daldrup, F. Musshoff, Toxichem. Krimtech 71 (2004) 146.
[33] E. Psillakis, N. Kalogerakis, J. Chromatogr. A 999 (2003) 148.
[34] A. Saleh, Y. Yamini, M. Faraji, S. Shariati, M. Rezaee, J. Chromatogr. B 877 (2009) 1761.
[35] Society of Hair Testing, Forensic Sci. Int. 145 (2004) 84.
[36] F. Pragst, T. Nadulski, Ann. Toxicol. Anal. 17 (2005) 237.
[37] C. Jurado, P. Kintz, M. Menéndez, M. Repetto, Int. J. Legal Med. 110 (1997) 159.
[38] G. Skopp, L. Potsch, M. Mauden, Clin. Chem. 46 (2000) 1846.
[39] G. Skopp, P. Strohbeck-Kuehner, K. Mann, D. Hermann, Forensic Sci. Int. 170 (2007) 49.