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PAPER IN FOREFRONT

Identification and quantification of cannabinoids in *Cannabis sativa L.* plants by high performance liquid chromatography-mass spectrometry

Oier Aizpurua-Olaizola · Jone Omar · Patricia Navarro · Maitane Olivares · Nestor Etxebarria · Aresatz Usobiaga

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Abstract High performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) has been successfully applied to cannabis plant extracts in order to identify cannabinoid compounds after their quantitative isolation by means of supercritical fluid extraction (SFE). MS conditions were optimized by means of a central composite design (CCD) approach, and the analysis method was fully validated. Six major cannabinoids [tetrahydrocannabinolic acid (THCA), tetrahydrocannabinol (THC), cannabidiol (CBD), tetrahydrocannabivarin (THCV), cannabigerol (CBG), and cannabinol (CBN)] were quantified (RSD < 10%), and seven more cannabinoids were identified and verified by means of a liquid chromatograph coupled to a quadrupole-time-of-flight (Q-ToF) detector. Finally, based on the distribution of the analyzed cannabinoids in 30 *Cannabis sativa L.* plant varieties and the principal component analysis (PCA) of the resulting data, a clear difference was observed between outdoor and indoor grown plants, which was attributed to a higher concentration of THC, CBN, and CBD in outdoor grown plants.

Keywords Cannabinoid analysis · Liquid chromatography · Mass spectrometry · Fingerprinting

Introduction

The widespread consumption of *Cannabis sativa L.* as a recreational drug competes with a more interesting and promising use of it as a medicinal plant. In fact, Cannabis has been applied as a therapeutical drug in many diseases such as multiple sclerosis, chronic pain, glaucoma, asthma, etc. [1].

The fact that the plant has more than 500 compounds makes it a complex matrix, even though the main focus of interest of this plant is in the content and distribution of the phytocannabinoids, which increases the complexity of any herbal extract [2–5]. Moreover, the varieties of species (*sativa*, *indica*, and *rudelaris*), all the hybridized strains are spread all over the world; the uptake ways and the posologies of it complicated the use of standard protocols. In addition, cannabinoids are biosynthesized in an acidic form, among which the most abundant are cannabidiolic acid (CBDA) and Δ^9-tetrahydrocannabinolic acid A (THCA-A). However, these acidic cannabinoids are not stable since they may decompose in the presence of light or heat. Overall, acidic cannabinoids are decarboxylated to their neutral homologues, as in the case of tetrahydrocannabinolic acid (THCA), which is decarboxylated to tetrahydrocannabinol (THC) [6].

THC is accepted to be the main psychoactive agent and it possesses analgesic, anti-inflammatory, appetite stimulant, and antiemetic properties; it can also protect the brain from cognitive deficits at very low doses [7, 8]. However, regular use of the plant may cause cognitive deficits at least in adolescents, since until the early 30s they have significant neurodevelopmental changes [9, 10]. The neurocognitive effects of extended use in adults are still somewhat inconsistent [9]. Cannabidiol (CBD) can modulate euphoric effects of THC and has antipsychotic, neuroprotective, anticancer, antidiabetic, and other effects such as reducing the anxiety induced by fear or reducing cigarette consumption in tobacco smokers [11–16]. Other minor cannabinoids present in cannabis are known to have diverse biological activities. Cannabigerol (CBG), for example, has antibacterial, antiproliferative, and bone-stimulant properties. Cannabinol (CBN)

O. Aizpurua-Olaizola (✉) · J. Omar · P. Navarro · M. Olivares · N. Etxebarria · A. Usobiaga
Analytical Chemistry Department, University of the Basque Country (UPV/EHU), Barrio Sarriena s/n, 48940 Leioa, Basque Country, Spain
e-mail: oier.aizpurua@ehu.es

has a sedative or stupefying effect. Tetrahydrocannabivarin (THCV) has anorexic and antiepileptic effects and, also, it may be clinically effective in migraine treatment [12, 14]. Thus, cannabis preparations may provide advantages over other single-compound synthetic drugs. The therapeutic effects of major constituents may be enhanced by other cannabinoids or non-cannabinoids whereas some unwanted side effects may be mitigated [12, 17–21].

As mentioned before, one of the major drawbacks of using cannabis plants medically is the lack of standardization. As an example, a study developed by Hazekamp and Fischedick reported that the nominal concentrations of target cannabinoids obtained for the same plant variety but from different coffee shops varied by more than 25% [22]. To overcome this problem, there are at least two approaches: one leans towards the tight control of the varieties and strains and the way they are grown in order to assure the highest homogeneity in the final plants; the other approach focuses on the blending of extracts to offer the desired products [22, 23].

To address the extraction of bioactive compounds, supercritical fluid extraction (SFE) is one of most suitable techniques [24] attributable to the safe use of CO_2 as the main solvent and ethanol as a co-solvent compared with other commonly used solvents in the extraction of cannabinoids [24, 25]. Moreover, it assures the stability of thermo-labile and light-sensitive compounds and is scalable up to industrial size [24]. In addition, SFE can be used with cannabis samples with very good yields [26].

In addition to the development of more efficient methods to extract major and trace elements from complex plant matrices, great effort has been made to develop robust and sensitive chromatographic methods capable of resolving complex mixtures. Two separation methods (gas-chromatography (GC) and high performance liquid chromatography (HPLC)) have been mainly used in most researches dealing with the analysis of cannabinoids [23, 26–28]. Some works have pointed out that one dimension GC does not offer enough resolution to analyze such complex mixtures. In this sense, hyphenated techniques such as comprehensive two-dimensional gas chromatography (GCxGC) have already been successfully employed to establish chemical profiles of different cannabis and heroin samples [29, 30]. Nonetheless, GC analysis requires a derivatization step to measure the thermo-labile acidic cannabinoids (due to the decarboxylation process in the injection port). Conversely, these compounds can be directly analyzed by means of HPLC without any derivatization step [28], which is a major advantage for obtaining a more complete chemical profile of the cannabis samples. In addition, other techniques, such as supercritical fluid chromatography (SFC), have been used to analyze acidic cannabinoids. SFC with photodiode array detection (PDA) is less sensitive than GC or HPLC coupled to mass spectrometry (MS) but the analysis time is much shorter [31].

The performance of HPLC-MS/MS depends on the type of mass analyzer used in the analysis [32]. Nowadays, the most widely used analyzers are the triple quadrupole instruments (QqQ), which have excellent sensitivity and selectivity to quantify analytes. However, they do not allow structural identification of non-target compounds as they provide only nominal mass measurements. In this respect, time-of flight analyzers (ToF) are often used in order to obtain the structural information of the target compounds [33, 34]. Coupled to a quadrupole mass filter, Q-ToF mass spectrometers provide accurate mass identification (<5 ppm, accepted accuracy threshold for confirmation of elemental composition) for both the precursor and the product ions [32]. This allows differentiating between two different compounds with the same nominal mass but with different elemental composition.

In the framework of a full characterization of ecologically grown Cannabis plants of 30 different varieties under tight control of the strains and growing conditions, the main aim of this work was to develop and validate an HPLC-MS/MS method to quantify the major cannabinoids, and to identify and extract the fingerprints of the less abundant cannabinoids by a HPLC-qToF approach.

Experimental

Materials and reagents

Cannabinoid standard compounds (THCA, CBG, and THCV) were purchased from Echo Pharmaceuticals BV (Weesp, The Netherlands) and reference standards (THC, CBD, CBN, and THC-d3) were purchased from Cerilliant (Round Rock, Texas, USA). HPLC grade EtOH and MeOH were obtained from Panreac (Barcelona, Spain), LC/MS grade formic acid from Thermo Fisher Scientific (Erembodegem, Belgium), leucine enkephalin acetate hydrate from Sigma-Aldrich Chemie (Steinheim, Germany), 99.9995% purity CO_2 from Air Liquide (Madrid, Spain), and washed thin sea sand from Scharlau (Sentmenat, Spain). Deionized water was generated with a Milli-Q water purification system Element 10 from Millipore (<0.057 $S \cdot cm^{-1}$ Milli-Q model; Millipore, Billerica, MA, USA).

Samples

Cannabis plant material was provided by Ganjazz Art Club (Donostia, Basque Country, Spain). Ten types of plants were chosen to develop and validate the analytical method (MW: *Medicine Woman*, AM: *Amnesia*, GW: *Great White Sarck*, TI: *Tijuana*, and SO: *Somango* from indoor and BU: *Bubba Kush*, BL: *Blueberry* SS: *Super Skunk*, GR: *Grapefruit*, and TR: *Trainwreck X HP* from outdoor), and 20 more for fingerprinting (*Parmir*, *Power plant*, *AK 47*, *N.Y.C Diesel*, *Jaggen*,

Cheese, *Chocolope*, *Deep Chunk*, *OG Kush*, *Soul Diesel*, *Skunk Green*, *Super Lemon Haze*, *Super Silver Haze* and *Neviles Haze* from indoor and *Amnesia*, *Critical*, *Chocolope*, *Cream Caramel*, *Super Lemon Skunk*, *Trainwreck*, and *Grapefruit* from outdoor).

The plants were grown from clones of a mother plant and each one had its optimum flowering growth time. For indoor plants, vegetative growth was carried out from the October 1, 2012 to October 25, 2012, and the flowering growth until January 7, 2013. This last phase was developed under 600 watt lamps with 70,000 l×. They were dried in closets during 15 d and then carried in wood boxes to an air-tight container and stored in a cool dry place during at least another 15 d. Though no pesticide was used, the occurrence of plants diseases like mildew was less than 5%. In the case of outdoor plants, their vegetative growth was carried out indoors from July 15, 2013 to October 15, 2013 under a complete lamp supply with micro- and macro-organisms but without pesticides. The flowering phase was developed outdoors in greenhouses, and plants were pulverized with neem oil and other nonchemical preventatives like *Bacillus turigensis* against larvae. The mildew level was between 10% and 15% depending on cannabis variety.

The plant samples were cryo-milled under liquid nitrogen at 660 rpm during 4 min (SPEX SamplePrep, 6770 Freezer/Mill, Madrid, Spain) and stored frozen (–20 °C) in amber glass vials until their analysis (maximum 1 mo).

SFE of cannabis plants

SFE was performed on a Thar SFC, Waters Company (Saint-Quentin, France) Method Station SFC system, consisting of a Fluid Delivery Module (CO_2 pump and solvent pump), a high speed Alias autosampler, an analytical-2-Prep column, a photodiode array detector (PAD, Waters 2998), an automated back pressure regulator (ABPR), and a high pressure extraction vessel of 1 mL (EV-1 Jasco). Extraction conditions employed were optimized by our research group in a previous work [26]. Briefly, Cannabis plant was accurately weighed (0.05 g) in the extraction vessel and 1 g of sea sand was added to fill it. CO_2 was used as extraction solvent and EtOH (20%) as co-solvent in order to modify polarity. Samples were extracted for 10 min at 35 °C with a total flow of 1 mL/min at a fixed pressure of 100 bar. Extracts were collected in amber glass vials with 4 mL of EtOH and kept at –20 °C until their analysis (maximum 1 wk).

Liquid chromatography tandem mass spectrometry with triple quadrupole detection (HPLC-MS/MS)

The HPLC-MS/MS analyses were performed using an Agilent Technologies (Santa Clara, CA, USA) Infinity liquid 1260 chromatographic system, consisting of an autosampler, a column thermostat, and a binary solvent management system coupled to a triple quadrupole (Agilent Technologies 6430) equipped with an atmospheric pressure chemical ionization ion source (APCI).

The chromatographic separation was achieved using a Kinetex C18 column (2.6 μm, 150 mm×3 mm i.d.) with a guard column (0.5 μm depth filter×0.1 mm) (Phenomenex, Torrance, CA, USA) and a binary A/B gradient (solvent A: Milli Q water with 0.1% formic acid, and solvent B: MeOH with 0.1% formic acid). The gradient program was established as follows: initial conditions were 50% B, raised to 80% B over the first min, held at 80% B until 11 min, increased to 95% B over the next 2 min, held at 95% B until 16 min, decreased to 50% B over the next 2 min, and held at 50% B until 28 min for re-equilibration of the system prior to the next injection. A flow rate of 0.25 mL/min was used; the column temperature was 30 °C and the injection volume was 10 μL.

MS acquisition was carried out in the APCI positive ionization mode. The conditions were set as follows: corona discharge current of 5 μA, capillary voltage of 3500 V, heated vaporizer at 280 °C, a nitrogen flow rate of 7 L/min, a source temperature of 210 °C, and nebulizer pressure of 32 psi. For the quantification of the target cannabinoids, multiple reaction monitoring (MRM) mode was used and two transitions were monitored (one used as quantifier and the other as qualifier) (see Table 1).

The quantification of the extracts by means of LC-MS/MS was performed with external calibration (i.e., a set of standards containing target compounds at concentrations ranging from 0.5 to 1000 ng/mL in MeOH were analyzed in the same conditions of the samples). System fluctuations were corrected with an isotopically labeled standard (THC-d3) used as internal standard.

No reference material was available to validate the HPLC-MS/MS analysis method, so it was decided to compare it with a SFC method. SFC analyses were carried out in the same Thar SFC system used for extraction. The compounds were separated in a Kromasil normal phase analytical DIOL column (5 μm, 250 mm×4.6 mm i.d.) (Teknokroma, Spain). Five μL were injected in the loop injection system. MeOH was chosen as co-solvent to increase the polarity of the supercritical CO_2. Different MeOH percentages (2%–18%) and flow (1.0–1.5 mL/min) were tried in order to fit the best separation of the target compounds. Under optimum conditions, the samples were analyzed at 40 °C using 15% MeOH at a flow of 1.5 mL/min. System pressure was fixed at 150 bar and compounds were monitored at 220 nm in the PDA. The analysis time was 7 min.

Calibration curves were built in MeOH in the range of 0.5-5 μg/mL for THC and 2–50 μg/mL for THCA. Under these conditions, dilution of the samples (1:50 in MeOH) was required in order to avoid chromatographic column saturation.

Table 1 MRM transitions, optimized potentials, qualifier/quantifier area ratios, and retention times of the analytes and the internal standard for HPLC-MS/MS analysis. Mass/charge ratio of the precursor ion (Q1), mass/charge ratio of the fragment ion (Q2), fragmenter potential (FP), collision energy (CE), qualifier/quantifier ratio (Q/Q), retention time (t_R)

Analyte	Q1 (u)	Q2 (u)	FP (V)	CE (eV)	Q/Q (%)	t_R (min)
Known compounds						
CBD	315.1	192.8	40	20	67	8.7
	315.1	259.0	40	15	67	8.7
THCV	287.1	165.0	80	20	28	8.9
	287.1	231.0	80	15	28	8.9
CBG	317.2	193.0	120	10	24	8.9
	317.2	123.0	120	25	24	8.9
CBN	311.0	222.9	50	15	48	10.8
	311.0	293.0	50	10	48	10.8
THC	315.0	193.0	70	20	38	12.1
	315.0	259.0	70	20	38	12.1
THCA	315.1	193.0	100	20	49	17.0
	315.1	259.1	100	15	49	17.0
Unknown compounds						
C1	372.9	316.9	60	10	23	6.9
	372.9	180.7	60	10	23	6.9
C2	359.0	341.0	100	10	22	7.7
	359.0	218.8	100	30	22	7.7
C3	375.0	209.0	100	20	68	8.7
	375.0	251.0	100	5	68	8.7
C4	315.0	193.0	100	20	45	9.3
	315.0	258.9	100	15	45	9.3
C5	317.1	193.1	100	10	28	10.8
	317.1	123.0	100	30	28	10.8
C6	375.0	251.0	80	5	81	11.3
	375.0	209.0	80	20	81	11.3
C7	287.2	165.1	120	20	44	12.7
	287.2	123.1	120	30	44	12.7
Internal standard						
THC-d3	317.8	195.9	120	25	54	12.0
	317.8	262.0	120	20	54	12.0

Ultra-performance liquid chromatography-quadrupole time of flight mass spectrometry (UPLC-qTOF)

The identification of unknown cannabinoids was performed using an ACQUITY UPLC system from Waters (Milford, MA, USA), equipped with a binary solvent delivery pump, an autosampler, and a column compartment. The same phase column and pre-column used in low resolution analysis were used at 30 °C for separation of cannabinoids. Flow rate was 0.25 mL/min and injection volume was 10 μL. Mobile phases consisted of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). Separation was carried out in 22 min. Initial conditions were 50% B, raised to 100% B over 15 min, held at 100% B until 17 min, decreased to 50% B over the next 2 min, and held at 50 B until 22 min for re-equilibration of the system prior to the next injection. All samples were kept at 4 °C during the analysis.

All MS data acquisitions were performed on a SYNAPT G_2 HDMS with a Q-ToF configuration (Waters, Milford, MA, USA) equipped with an APCI source operating at 450 °C in positive mode. The capillary voltage was set to 0.7 kV and corona discharge to 5 μA. Nitrogen was used as the desolvation and cone gas at flow rates of 800 L/h and 20 L/h, respectively. The source temperature was 120 °C and the desolvation temperature was 300 °C. Leucine-enkephalin solution was used for the lock mass correction, monitoring the ions at mass-to-charge ratio (*m/z*) 556.2771 and 278.1141. All of the acquired spectra were automatically corrected during acquisition based on the lock mass. Data were acquired in the mass range 50–1200 u in resolution mode (FWHM ≈ 20,000). Before analysis, the mass spectrometer was calibrated with a sodium formate solution.

Low collision energy MS experiments and high collision energy MS/MS assays were performed over a single experimental run using an acquisition mode called MS^E. In this way,

molecular ions data and fragment ions data were obtained in the same run, essential for structure elucidation. In positive mode, only the protonated molecules were able to form adducts with mobile phase species, so their presence in the low collision energy spectra provide an unequivocal identification of the $[M + H]^+$ ions. The sodium adducts $[M + Na]^+$ at 22 u above the proposed protonated molecule were also used for this purpose.

Data treatment

Based on the analytical results obtained from the 30 plant samples, a multivariate data treatment was carried out to assess the difference between varieties of *Cannabis sativa L.* Unsupervised pattern recognition was accomplished with the statistical software The Unscrambler (9.7 Camo Asa, Oslo, Norway) in order to identify the main variation sources and the distribution of the collected samples. LC-MS/MS data were normalized sample-wise in order to avoid any systematic trend due to higher or lower concentrations and then treated by principal component analysis (PCA). PCA uses an orthogonal transformation to convert a number of possibly correlated variables into linearly uncorrelated variables called principal components. The first principal component accounts for as much of the variability in the matrix data as possible and the next principal component accounts for as much of the remaining variability as possible. This way, the dimensionality of the data set can be reduced and the underlying variables identified.

First, a PCA was performed with only quantified cannabinoid concentration data (30 sample×6 analytes), and second, a PCA with all cannabinoids corrected area data (30 sample×13 analytes) was performed to see whether any difference between them exists. Both models were built with leverage correction.

Results and discussion

Optimization of HPLC-MS/MS analysis

According to previous works, it is well-known that different solvent combinations can be employed for the separation and analysis of cannabinoids in HPLC-MS/MS [35, 36]. In this work, different solvent modifiers were tested: (1) water and MeOH both with 2-10 mM NH_4Ac; (2) water and MeOH both with 0.1%–0.2% (v/v) formic acid; (3) water and MeOH both with 0.1%–0.2% (v/v) formic acid and 2-10 mM NH_4Ac. The influence of flow rate (0.1–0.25 mL/min) and injection volume (2-20 μL) were also tested. The most appropriate conditions for target analytes separation taking into account the best peak shape and the highest sensitivity were obtained using a mobile phase consisting of a mixture of water with 0.1% formic acid (A) and MeOH with 0.1% formic acid (B), a flow rate of 0.25 mL/min and 10 μL of injection volume. The column temperature was maintained constant at 30 °C during the run.

The effect of MS acquisition parameters was optimized by means of a central composite design (CCD) approach covering the following factor spaces: capillary voltage 1120–5880 V, heated vaporizer temperature 155–485 °C, nitrogen flow 1.05–12.95 L/min, nitrogen temperature 65–350 °C, and nebulizer pressure 3–60 psi. Peak areas obtained for a standard mixture containing CBD, THC, and CBN at 1 μg/mL were used as the design responses. Though a CCD with five variables is a rather demanding approach, all the experiments (i.e., 45 experiments including three replicates of the central point) were carried out without the analytical column and, therefore, they were accomplished during the same day. As a result, we were able to build and analyze the response surface to find the maximum sensitivity and the highest resolution. The precision, in terms of relative standard deviation (RSD) was estimated from the three replicates of the central point (RSD <2% for all the target compounds) and the data were treated with The Unscrambler software in order to build the response surfaces by multiple linear regression (MLR).

MLR data revealed that the significant factors affecting the peak areas were the heated vaporizer temperature and the nebulizer pressure (p-level<0.05) for all the studied compounds. As shown in Fig. 1 for CBD, the local maximum was found at the low-medium heated vaporizer values and medium nebulizer values within the factor space. This pattern was also observed for the rest of the analytes. The conditions of the nonsignificant parameters were established also according the obtained response surfaces for each compound. In this way, MS parameters optimal conditions were fixed as follows: 3500 V of capillary voltage, 280 °C of vaporizer temperature, 7 L/min of nitrogen at 210 °C, and 32 psi of nebulizer. Capillary voltage and nitrogen flow and temperature were set at the medium value as they were not significant and for practicality. Once these parameters were optimized, the influence of corona discharge intensity was assessed in the 2–10 μA range, and the maximum sensitivity was attained at 5 μA of corona for the majority of the target compounds.

Regarding the MRM detection mode, it was fully optimized using a standard solution of each target compound at 500 ppb in MeOH in order to find out the optimum precursor ions, which corresponded to the most intense ions with the highest *m/z* ratio, the product ions, and the collision energies. Eight different collision energies were assessed in the 5–40 eV range in order to opt for the most intense product ions. Two different transitions were selected accordingly; one used for quantification purposes and the second one for qualification purposes (see Table 1).

Figure of merits

HPLC-MS/MS analysis method was fully validated (see Table 2) over a range of 0.5–2000 ng/mL. The calibration

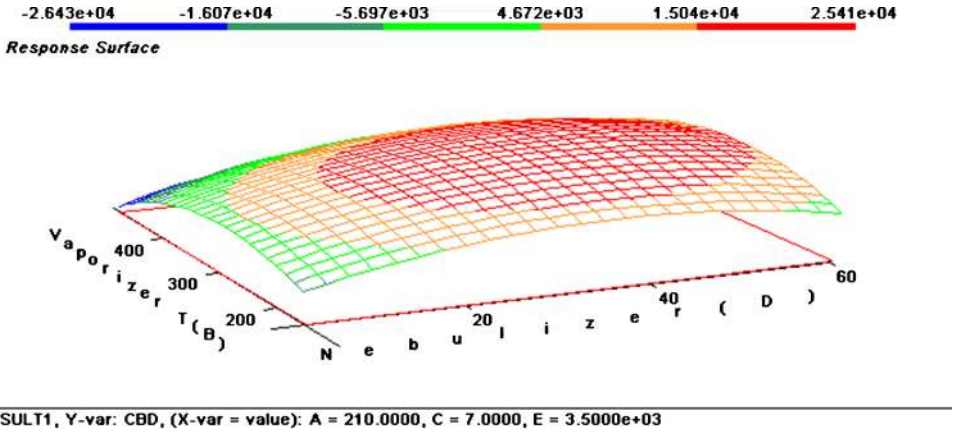

Fig. 1 Response surface of vaporizer temperature (°C) and nebulizer pressure (psi) for the MS parameter optimization for CBD analyte

curves were linear (R^2>0.999) in the concentration ranges studied for each analyte.

The instrumental limits of detection (LODs) were set at the lowest concentration where the signal-to-noise ratios of the analytes were higher than 3. All the obtained LODs were between 0.02 and 0.2 ng/mL.

To check the trueness of the developed method, different aliquots of MeOH were spiked at two different concentration levels within the calibration curve range: three replicates in the low concentration range (10 ng/mL) and another three spiked samples in the high concentration range (1500 ng/mL). Trueness, expressed in terms of relative bias (%), was acceptable for all cannabinoids at both low and high concentration levels (see Table 2).

The precision of the whole method was assessed by calculating the relative standard deviations (RSDs) for repeatability and reproducibility. Repeatability was quantified by intra-day variation carrying out SFE extractions from the same sample (*Somango*) and analyzing them (n=5), and reproducibility by inter-day variation, extracting the same sample in triplicate on three different days. The RSD values ranged from 5% to10% in the case of the samples analyzed on the same day, whereas the RSD values obtained from the analyses performed in different days varied from 2% to 7%.

As no reference material was available, the accuracy of the developed method was assessed comparing the concentration of cannabinoid compounds in real cannabis plants with SFC-PDA. To this end, 10 varieties of cannabis were analyzed in triplicate by SFC-PDA and the results were compared with the HPLC-MS/MS method. Unfortunately, due to the lower sensitivity of the SFC-PDA method (i.e., instrumental limits of detection higher than 500 ng/mL), the concentration of the major cannabinoids (i.e., THC and THCA) only were determined. The concentration of these analytes obtained by both methods was statistically comparable (p-level<0.05) as can be seen in Fig. 2.

Identification of the cannabis extracts by HPLC-MS/MS and UPLC-qToF

Since more than 70 cannabinoids can be found in the extracts of cannabis [3, 5], and the distribution of these cannabinoids can be extended in a very wide range of concentrations, sample extracts were initially analyzed by HPLC-MS/MS in scan mode in order to obtain their mass spectra with complete information, and so, ease the compound identification. In the first approach, two different distributions were observed in the *Somang*o variety of a real cannabis plant. On the one hand, some analytes were found at concentrations close to the limit of detection such as CBD or THCV. On the other hand, major cannabinoids such as THC and THCA were found at high concentrations, and they could saturate the detector. Therefore, three different dilutions (i.e., 1:10, 1:500, and 1:5000) of the sample extracts were injected in the HPLC-

Table 2 Correlation coefficients (R^2), low and high concentration range trueness expressed in terms of bias, instrumental limits of detection (LOD), and instrumental and procedural precisions expressed with relative standard deviations (RSD) of SFC and HPLC-MS/MS analysis methods

Analyte	R^2	Trueness (%)		LOD (ng/mL)	Precision (%)	
		Low	High		Repeatability	Reproducibility
CBD	0.9998	4.9	0.1	0.2	10	7
THCV	0.9999	2.9	0.7	0.05	6	2
CBG	0.9998	4.2	3.2	0.02	7	2
CBN	0.9998	1.0	1.6	0.05	7	4
THC	0.9998	1.2	1.7	0.05	5	5
THCA	0.9992	1.9	1.5	0.2	5	4

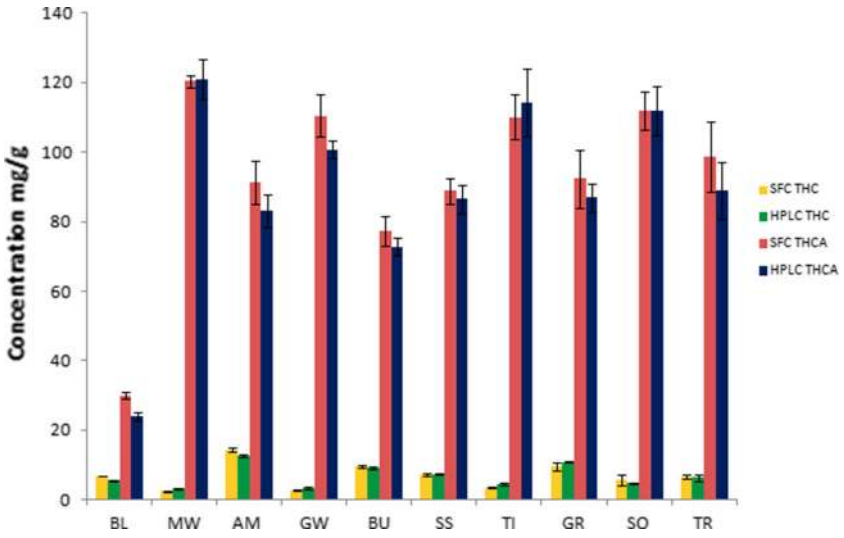

Fig. 2 Representation of THC and THCA concentrations (mg/g) of different cannabis plants extracted by SFC and analyzed by HPLC-MS/MS with the corresponding standard deviations (n=3). BL: Blueberry; MW: Medicine Woman; AM: Amnesia; GW: Great White Sarck; BU: Bubba Kush; SS: Super Skunk; TI: Tijuana; GR: Grapefruit; SO: Somango; TR: Trainwreck X HP

MS/MS system in order to properly detect all the analytes of interest. When less diluted samples were injected, the most concentrated analytes (i.e., THCA and THC) were sent to waste after the chromatographic separation and, therefore, they did not enter the detector. Although they passed though the column, no saturation or carryover problem after a sample cleaning injection with MeOH was observed.

As can be seen in Table 1, six different cannabinoids were identified and quantified using pure standards in the *Somango* cannabis plant. Besides the known compounds, seven other unknown compounds were detected based on the mass spectra collected in the scan mode (see Fig. 3) and based on the information found in the literature [5, 37]. In the latter case, after the identification of the precursor ions ($[M + H]^+$) of the seven unknown cannabinoids, the fragmentation pattern at different collision energies was assessed in order to identify the unknown cannabinoids.

The identification of unknown compounds (C1–C7) was verified by means of UPLC-qToF, which provides high resolution and accurate mass measurements of the precursor and fragments ions [32]. Moreover, the formation of positively charged adducts $[M + Na]^+$ instead of the precursor ion $[M + H]^+$ is often observed in the positive ionization mode. These sodium adducts are valuable to confirm the $[M + H]^+$ identification because of the 23 u higher molecular mass. This strategy was successfully applied to identify the unknown seven cannabinoids. Their experimental theoretical masses and the suggested molecular formula as well as the compound name are shown in Table 3. The errors obtained in the identification for all the compounds were ≤1.2 mDa.

C1 was identified as cannabicoumaric acid as $[M + Na]^+$ reassured the molecular weight, and among all known cannabinoids the $C_{22}H_{29}O_5$ formula is unique for this compound. C2 was identified as cannabichromenic acid (CBCA) as it showed a loss of 44 u (359→315) and $[M + Na]^+$ aduct

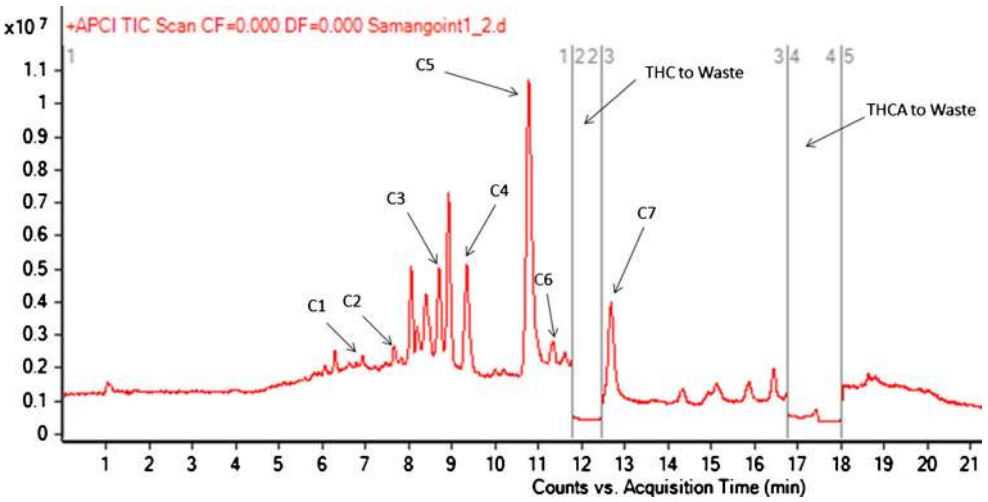

Fig. 3 Scan chromatogram of *Somango* (SO) sample extract (dilution 1:10) where THCA and THC were not analyzed

Table 3 UPLC-qToF verification in the identification of unknown (C1-C7) cannabinoid compounds

	$[M + H]^+$	Calc. mass	% Error mDa	% Error ppm	Molecular formula	Compound name
C1	373.2011	373.2015	0.4	1.1	$C_{22}H_{29}O_5$	Cannabicoumaric acid
C2	359.2113	359.2222	0.9	2.5	$C_{22}H_{31}O_4$	CBCA
C3	375.2537	375.2535	0.2	0.5	$C_{23}H_{35}O_4$	10-Ethoxy-9-hydroxy-Δ^6a-THC
C4	373.2375	373.2380	0.5	1.3	$C_{23}H_{33}O_4$	4-Acetoxycannabichrome
C5	361.2367	361.2379	1.2	3.3	$C_{22}H_{32}O_4$	CBGA
C6	365.2538	375.2535	0.3	0.8	$C_{23}H_{35}O_4$	CBGAM
C7	345.2064	345.2066	0.2	0.6	$C_{21}H_{29}O_4$	THCA-C4

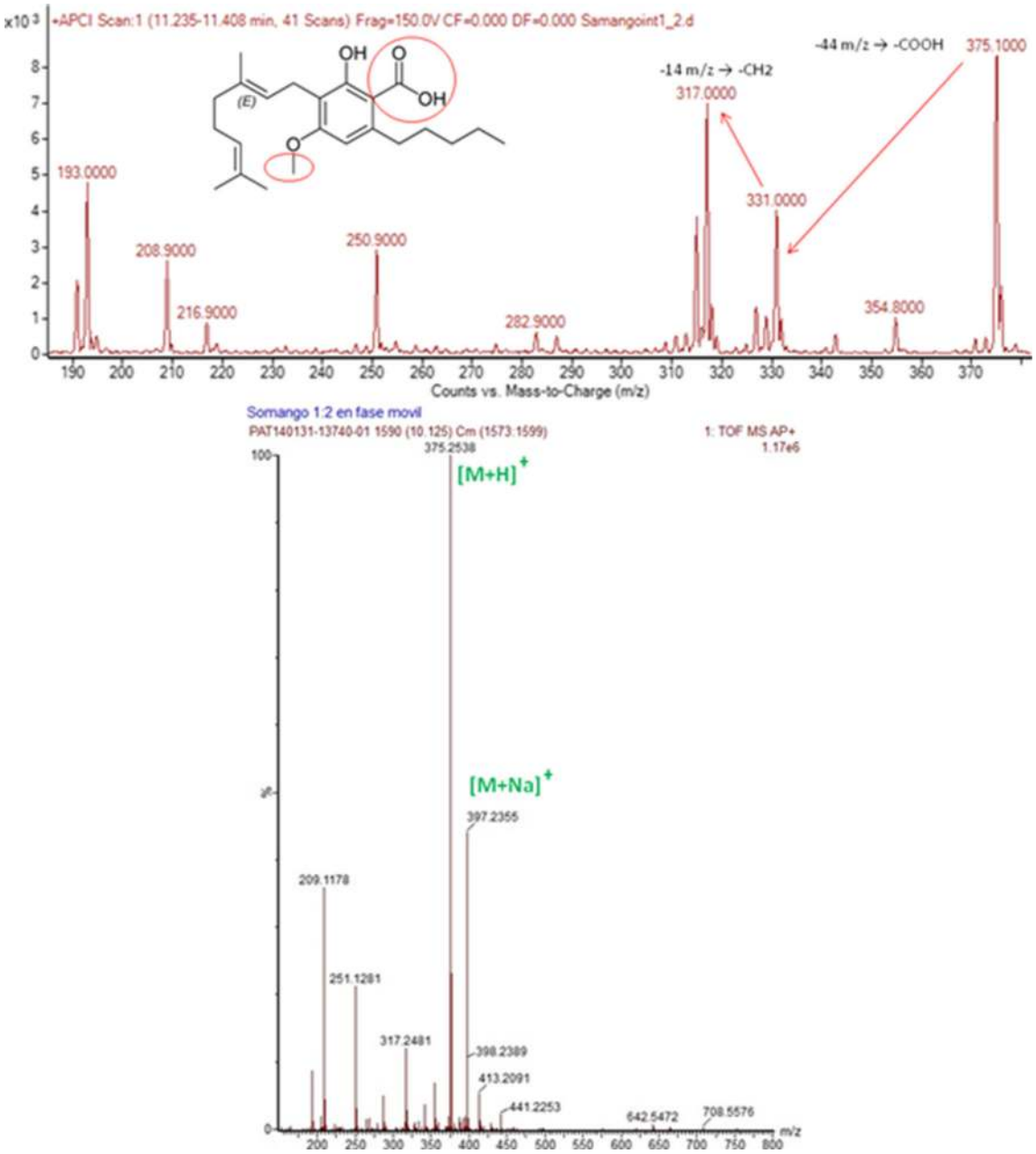

Fig. 4 C6 compound, identified as cannabigerolic acid monomethylether (CBGAM). Up spectra is from HPLC-MS/MS and down spectra from UPLC-qToF

confirmed the molecular weight. The loss of 44 u is due to the loss of carboxyl groups of the acid cannabinoids, which are not very stable since their carboxyl group is cleaved off as CO_2 under the influence of heat or light, resulting in their corresponding neutral cannabinoids. The mass of the molecular ion could have been attributed to cannabidiolic acid (CBDA) but the fragmentation spectrum is quite different from its neutral compound, CBD, and moreover, in female flowers the presence of CBD-type cannabinoids is expected to be low. C5 and C6 were identified also as acid cannabinoids because all of them showed a loss of 44 u. Their fragmentation pathways were similar to CBG and their quasimolecular ion $[M + H]^+$, reassured by the sodium adducts, proved that the unknown compounds C5 and C6 were cannabigerolic acid (CBGA) and cannabigerolic acid monomethylether (CBGAM), respectively. The loss of the methyl group (331→317 *m/z*) also supported this identification (Fig. 4). C3 showed the same chemical formula of C6 but based on the different fragmentation patterns, this compound was identified as10-ethoxy-9-hydroxy-Δ6a-THC. The chemical formula assigned to C4 was also unique among all known cannabinoids, which enabled the identification of this compound as 4-Acetoxycannabichrome. Finally, C7 was identified as THCA-C4 because its fragmentation patterns were very similar to THCA, which was also supported by the $[M + H]^+$ adduct.

Quantification of the cannabis extracts by HPLC-MS/MS and statistical data treatment

Once the analysis method was validated and the unknown cannabinoids were identified in *Somango*, 30 different

Table 4 Quantification results of cannabinoids in HPLC-MS/MS of different *Cannabis sativa L.* varieties

Sample	Indoor/Outdoor	THCA (mg/g)	THC (mg/g)	CBG (mg/g)	THCV (μg/g)	CBN (μg/g)	CBD (μg/g)
Parmir	I 1	81±4	2.6±0.2	0.37±0.03	35±2	11.2±0.8	1.6±0.2
Great White Sarck	I 2	99±5	3.7±0.2	0.37±0.03	56±3	7.5±0.5	2.9±0.3
Power Plant	I 3	107±5	2.0±0.1	0.39±0.03	70±4	11.6±0.8	1.8±0.2
AK 47	I 4	74±4	1.2±0.1	0.30±0.02	33±2	7.4±0.5	0.67±0.07
N.Y.C. Diesel	I 5	114±6	2.2±0.1	1.14±0.08	35±2	7.0±0.5	2.4±0.2
Jaggen	I 6	91±5	2.9±0.2	0.67±0.05	62±4	10.2±0.7	2.1±0.2
Medicine Woman	I 7	119±6	3.6±0.2	1.23±0.08	60±4	11.1±0.8	2.5±0.2
Amnesia	I 8	117±6	2.7±0.2	1.04±0.07	97±6	18±1	3.9±0.4
Cheese	I 9	70±4	1.1±0.1	0.54±0.04	13.7±0.8	4.6±0.3	1.5±0.2
Chocolope	I 10	94±5	2.9±0.2	0.55±0.04	12.4±0.8	10.9±0.8	3.4±0.3
Deep Chunk	I 11	71±4	1.3±0.1	0.16±0.01	31±2	6.1±0.4	1.9±0.2
OG Kush	I 12	67±3	1.8±0.1	0.34±0.02	27±2	2.4±0.2	1.9±0.2
Soul Diesel	I 13	70±4	1.4±0.1	0.19±0.01	26±2	4.5±0.3	2.5±0.2
Skunk Green	I 14	80±4	2.0±0.1	0.076±0.005	38±2	15±1	2.8±0.3
Super Lemon Haze	I 15	69±3	3.5±0.2	0.30±0.02	310±20	13.0±0.9	3.6±0.4
Super Silver Haze	I 16	105±5	3.2±0.2	0.53±0.04	134±8	9.1±0.06	3.5±0.4
Tijuana	I 17	92±5	3.6±0.2	0.73±0.05	135±8	13.0±0.9	4.5±0.4
Neviles Haze	I 18	63±3	1.9±0.1	0.067±0.005	63±4	5.9±0.4	2.2±0.2
Somango	I 19	86±4	4.6±0.3	0.68±0.05	240±10	10.0±0.07	3.7±0.4
Amnesia	O 1	91±5	16±1	0.74±0.05	94±6	91±6	9.1±0.9
Critical	O 2	112±6	7.6±0.5	0.38±0.03	153±9	61±4	5.0±0.5
Blueberry	O 3	30±2	6.5±0.4	0.100±0.007	28±2	60±4	3.3±0.3
Chocolope	O 4	80±4	25±2	0.75±0.05	5.8±0.3	84±6	14±1
Cream Caramel	O 5	113±6	10.8±0.7	1.17±0.08	103±6	63±4	6.9±0.7
Bubba Kush	O 6	69±3	9.1±0.5	0.018±0.001	52±3	61±4	6.0±0.6
Super Lemon Skunk	O 7	51±3	17±1	0.54±0.04	4.5±0.3	91±6	10±1
Super Skunk	O 8	76±4	5.0±0.3	0.39±0.03	69±4	59±4	6.0±0.6
Trainwreck	O 9	65±3	22±1	0.48±0.03	3.6±0.2	73±5	12±1
Trainwreck X HP	O 10	71±4	6.0±0.4	0.33±0.02	98±6	58±4	3.4±0.3
Grapefruit	O 11	73±4	9.6±0.6	0.39±0.03	107±6	470±30	10±1

I: Indoor; O: Outdoor

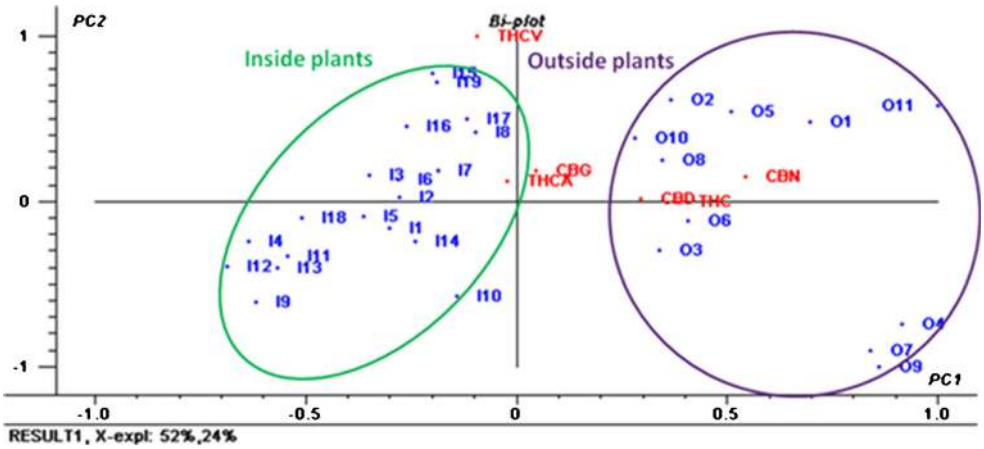

Fig. 5 Score and loadings biplot (PC1 vs. PC2) of *Cannabis sativa L.* samples performed by principal components analysis according to the concentration of the main quantified cannabinoid compounds. (I: refers to indoor grown plants; O: refers to outdoor grown plants)

Cannabis sativa L. varieties were subsequently analyzed. Six major cannabinoids were quantified (Table 4) and the other previously identified seven compounds were qualified correcting the areas with the areas of the deuterated internal standard THC-d3. Relative standard deviations were calculated carrying out five extractions from the *Somango* cannabis extract sample (RSD<10%) (Table 2).

As it can be observed in Table 4 that overall, indoor grown plants have less concentration of CBN. This may be attributed to the controlled ambient conditions in which the indoor plants were grown because it is known that CBN is produced when THC is exposed to air and consequently degraded to CBN [2]. In the same way, outdoor plants have also higher degradation of acidic cannabinoids to their corresponding neutral compounds. Owing to this, they have more THC and CBD than indoor plants. Conversely, the adverse meteorological conditions do not affect the growth of indoor plants, so that they retain the resin better where cannabinoids are in higher concentration, resulting in a lower degradation [2]. Nonetheless, concentrations found for THCA, CBG, and THCV varied a lot from one variety to another.

PCA was carried out to reduce the dimensionality of the data set and identify better the variation of the cannabinoid concentrations between plant varieties. Thus, the PCA analysis of the 30 samples required two PCs to explain up to 76% of the total variance when the quantified cannabinoids were considered, and 62% when all the cannabinoids (quantified and qualitatively identified compounds) were included.

Figure 5 shows the score plot of the PCA model with the quantified cannabinoid data-set and, as can be seen, the indoor and outdoor plants are clearly distinguished mostly by CBN. As mentioned before, it can be observed that outdoor grown plants have more CBN, THC, and CBD, probably due to the effect of the weather alterations. However, the distribution of THCA, THCV, and CBG did not offer any meaningful pattern, probably because the differences between varieties are much higher than the degradation differences. Moreover, differences between *Sativa* and *Indica* varieties were studied but no clear results were obtained from the treated data. This can be because the majority of the plants are not purely *Sativa* or *Indica* and, moreover, there is a lack of cannabis plant varieties standardization [22].

As six cannabinoids were quantified and another seven identified, this methodology provided accurate cannabinoid profiles and can be easily used for differentiate between plant varieties as it has been done in other works [23, 26].

Conclusions

An HPLC-MS/MS method was fully optimized and validated to determine the major cannabinoids present in extracts obtained by supercritical fluid extraction of *Cannabis Sativa L.* plants. In addition, the identification of seven minor cannabinoids was achieved by means of UPLC-qToF. This methodology can be useful in establishing an accurate cannabinoid profile of cannabis varieties in order to correlate to therapeutic effectiveness.

Based on the application of both analytical techniques, the analysis of 30 different cannabis strains grown under controlled conditions was carried out. The first results showed that the distribution patterns of indoor grown and outdoor grown plants were different enough for their differentiation. The found difference was attributed to a higher concentration in THC, CBN, and CBD in outdoor grown plants.

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Oier Aizpurua-Olaizola obtained a Chemistry degree from the University of the Basque Country (UPV/EHU). Currently, he is pursuing his PhD at the same university. His research interests deal with new extraction, detection, and microencapsulation methodologies for bioactive compounds and also with the development of analytical approaches for cannabinoids in different matrixes.

Maitane Olivares is lecturer in the Department of Analytical Chemistry of the Faculty of Science and Technology of the University of the Basque Country (UPV/EHU). She is an analytical chemist with experience in the development of analytical approaches for organic compounds in environmental chemistry and cultural heritage fields, and has published around 30 novel manuscripts in these areas.

Jone Omar Onaindia received her PhD degree in analytical chemistry from the University of the Basque Country (UPV/EHU) in 2013 and is currently a postdoctoral fellow in analytical chemistry at the Joint Research Centre (JRC-IRMM). Her research interests are the development of analytical methodologies for Feed Additives by means of spectroscopy and data treatment by means of chemometrics.

Nestor Etxebarria is Professor in the Department of Analytical Chemistry at the University of the Basque Country. He also works at the Plentzia Marine Station (PIE). His research interests are the development of analytical methods and environmental chemistry, especially focused on the fate and the effects of contaminants.

Patricia Navarro is a postdoctoral researcher in the Analytical Chemistry Department of the University of the Basque Country. During the time she was pursuing her PhD, her work was focused on the development of analytical methodologies for environmental samples analysis. Later, she researched also in the organometallic compounds field. Her research interest deals with new extraction and detection methodologies for organic pollutants. She has about 20 publications in these areas.

Aresatz Usobiaga is an assistant professor in the Department of Analytical Chemistry of the Faculty of Science and Technology of the University of the Basque Country (UPV/EHU) since 2004. She is an analytical chemist and, although her first research works were in the area of electrochemistry during the time she was pursuing her PhD, she has experience in the development of analytical approaches for organic compounds in the last 10 years, mainly in the environmental and natural product research fields.