Journal of Chromatography B, 877 (2009) 4115–4124

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Innovative development and validation of an HPLC/DAD method for the qualitative and quantitative determination of major cannabinoids in cannabis plant material

Benjamin De Backer[a,*], Benjamin Debrus[b], Pierre Lebrun[b], Laetitia Theunis[a], Nathalie Dubois[a], Lies Decock[c], Alain Verstraete[c], Philippe Hubert[b], Corinne Charlier[a]

[a] Laboratory of Clinical, Forensic and Environmental Toxicology, CIRM, CHU Sart-Tilman, University of Liège, B-4000 Liège, Belgium
[b] Laboratory of Analytical Chemistry, CIRM, Department of Pharmacy, University of Liège, B-4000 Liège, Belgium
[c] Department of Clinical Chemistry, Microbiology and Immunology, Ghent University, Ghent, Belgium

ARTICLE INFO

Article history:
Received 28 August 2009
Accepted 1 November 2009
Available online 6 November 2009

Keywords:
HPLC
Cannabis sativa
Cannabinoids
THC
CBD
CBG

ABSTRACT

GC is commonly used for the analysis of cannabis samples, e.g. in forensic chemistry. However, as this method is based on heating of the sample, acidic forms of cannabinoids are decarboxylated into their neutral counterparts. Conversely, HPLC permits the determination of the original composition of plant cannabinoids by direct analysis. Several HPLC methods have been described in the literature, but most of them failed to separate efficiently all the cannabinoids or were not validated according to general guidelines. By use of an innovative methodology for modelling chromatographic responses, a simple and accurate HPLC/DAD method was developed for the quantification of major neutral and acidic cannabinoids present in cannabis plant material: Δ9-tetrahydrocannabinol (THC), THC acid (THCA), cannabidiol (CBD), CBD acid (CBDA), cannabigerol (CBG), CBG acid (CBGA) and cannabinol (CBN). Δ8-Tetrahydrocannabinol (Δ8-THC) was determined qualitatively. Following the practice of design of experiments, predictive multilinear models were developed and used in order to find optimal chromatographic analytical conditions. The method was validated following an approach using accuracy profiles based on β-expectation tolerance intervals for the total error measurement, and assessing the measurements uncertainty. This analytical method can be used for diverse applications, e.g. plant phenotype determination, evaluation of psychoactive potency and control of material quality.

1. Introduction

Cannabis can be considered as the most controversial plant in our society: next to the important medical use, cannabis is also the most frequently consumed drug of abuse in Europe. It has been estimated that about four million European adults (~1% of all 15- to 64-year-olds) are using cannabis each day or almost daily; and that around 23 million Europeans (~7% of all 15- to 64-year-olds) have consumed cannabis at least one time during the past year [1]. The plant *Cannabis sativa* L. constitutes the basic material of all cannabis products. *C. sativa* L. belongs to the family of the Cannabinaceae. The current systematic classification of cannabis is listed in Table 1 [2,3].

1.1. Cannabinoids

The chemistry of cannabis has been studied extensively: approximately 500 compounds have been identified. The most interesting among these constituents are the cannabinoids; terpenophenolic compounds unique to cannabis and concentrated in a resinous secretion produced by the trichomes of the plant. These trichomes are particularly concentrated at specific parts of the female inflorescence [2].

The cannabinoids form a group of related compounds of which about 70 are known [2,4]. Of the major cannabinoids in *C. sativa* L., Δ9-tetrahydrocannabinol (THC) is generally accepted to be the compound that possesses the psychoactive properties [5,6]. In plant tissues, cannabinoids are biosynthesized in an acidic (carboxylated) form. The most common types of acidic cannabinoids found are Δ_9-tetrahydrocannabinolic acid A (THCA-A), cannabidiolic acid (CBDA) and cannabigerolic acid (CBGA). THC acid exists under two forms: THCA-A and THCA-B. However, only traces of THCA-B can be detected in cannabis samples [3], THCA-A is the major form and will be further referred to as THCA. CBGA is the direct pre-

* Corresponding author at: Service de Toxicologie Clinique, Médicolégale, Environnementale et en Entreprise, Centre Hospitalier Universitaire Sart-Tilman B 35, B-4000 Liège, Belgium. Tel.: +32 43 668095; fax: +32 43 668889.
E-mail address: b.debacker@ulg.ac.be (B. De Backer).

1570-0232/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2009.11.004

Table 1
Current systematic classification of *Cannabis sativa* L. [2,3].

Division	Angiosperms
Class	Dicotyledon
Subclass	Archichlamydeae
Order	Urticales
Family	Cannabinaceae
Genus	*Cannabis*
Species	*sativa* L.

cursor of THCA, CBDA and cannabichromenic acid (CBCA) (Fig. 1). The carboxyl group is not very stable and is easily lost as CO_2 under influence of heat or light, resulting in the corresponding neutral cannabinoids: THC, cannabidiol (CBD) and cannabigerol (CBG) [2,7]. These are formed during heating and drying of harvested plant material, or during storage and when cannabis is smoked [6,8,9].

The variable conditions during all stages of growing, harvesting, processing, storage and use also induce the presence of breakdown products of cannabinoids. The most commonly found degradation product in aged cannabis is cannabinol (CBN), produced by oxidative degradation of THC under the influence of heat and light [2,10]. THC can also be transformed by isomerization to Δ_8-THC, which is an artefact. In order to quantify the "total THC content" once present in the fresh plant material, the concentrations of degradation products have to be added to THCA and THC contents.

1.2. Phenotypes

Hillig and Mahlberg [8] identified three chemotypes (chemical phenotypes) of cannabis: drug-type plants (chemotype I) show a

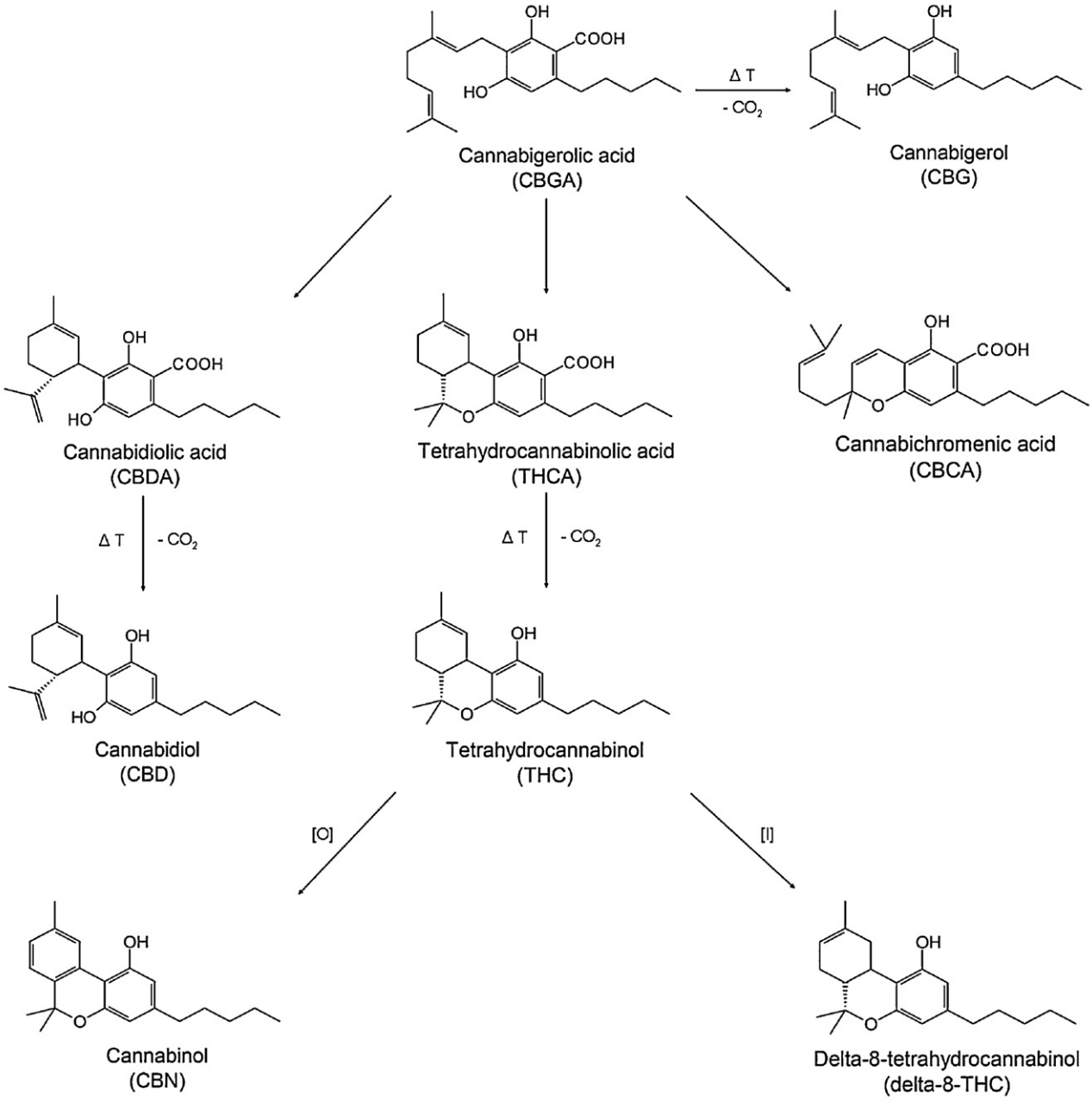

Fig. 1. Biosynthetic pathway for the production of cannabinoids and main breakdown products of THC. (ΔT = heating, [O] = oxidation, [I] = isomerization).

high [total THC/total CBD] ratio (≫1.0), intermediate type plants (chemotype II) have an intermediate ratio (close to 1.0), and fibre-type plants (chemotype III) exhibit a low [total THC/total CBD] ratio (≪1.0). For forensic and legal purposes, the most important classification of cannabis types is that into the drug-type and the fibre-type. The latter, usually called "hemp", refers to varieties that have low THC concentrations but generally contain other non-psychoactive cannabinoids as major compounds, like CBD or CBG. In many countries, hemp cultivation is prohibited by legislation because of the presence of the psychoactive compounds. In countries where hemp cultivation is allowed, the cultivars are tested in order to verify that the psychoactive potency is below a minimum acceptable level [5]. In Europe, the maximum THC content allowed for the cultivation of hemp is either 0.2% or 0.3% of the weight of dry matter, in function of the country.

1.3. Analytical methods

The analysis of the original composition of plant material is necessary for diverse purposes as phenotype determination and quality control of medicinal cannabis used in therapeutic treatment. In addition, it has been repeatedly suggested that the effects of THC or other single cannabinoids are not equal to that of whole cannabis preparations [11,12]: some of the bio-activity observed for these preparations could be due to acidic cannabinoids [13]. That way, a method allowing the qualitative and quantitative determination of neutral as well as acidic cannabinoids in plant material must be available [2].

Gas Chromatography (GC) is the most commonly used method for the analysis of cannabis products [5,8,14–18], but it does not permit the determination of acidic cannabinoids due to decarboxylation into their neutral forms during analysis. Furthermore, this thermal conversion of acidic cannabinoids seems to be incomplete [19]. In order to quantify neutral cannabinoids by GC, a time-consuming derivatization step is mandatory. On the contrary, High Performance Liquid Chromatography (HPLC) allows the determination of the neutral forms since no heating occurs during separation. Use of HPLC is thereby the simplest method for the determination of the original composition in cannabinoids of plant material. Raharjo and Verpoorte [15] reviewed different HPLC methods for the analysis of cannabinoids. However, most of them were not validated according to the new guidelines using the total error approach, or were not able to separate efficiently all the major cannabinoids [2,3,20,21]. Because of the complex composition of plant material, the analysis of major cannabinoids is not easily achieved and overlap of peaks occurs (between CBD/CBG and CBN/CBGA) [2,20]. Consequently, Hazekamp et al. [2,20] had to combine HPLC with a secondary analysis by GC in order to identify and quantify all major cannabinoids. The use of mass spectrometry coupled to HPLC may be a solution in order to resolve all peaks in a single analytical run [10,20]. However, this method is expensive and not routinely available to most laboratories.

The goal of the present study was therefore to develop and to validate a simple HPLC/DAD method, allowing a good separation followed by a qualitative and quantitative determination of major neutral and acidic cannabinoids present in plant material. Determination was performed on cannabinoids of potential interest for the medicinal research community and cannabinoids used for the classification of cannabis phenotypes and for monitoring of the psychotropic potency: THC, THCA, CBD, CBDA, CBG, CBGA, CBN and Δ_8-THC. The method was validated within broad ranges of concentrations adapted to the levels found in the three cannabis plant phenotypes.

2. Materials and methods

2.1. Chemicals and reagents

Cannabinoid reference standards for THC, CBD, CBN and Δ-8-THC were purchased from LGC Standards (Molsheim, France). Reference standards for THCA, CBDA, CBGA and CBG were purchased from Echo Pharmaceuticals BV (Weesp, The Netherlands). All standards had a purity of ≥98%. Prazepam was purchased from Certa (Braine-l'Alleud, Belgium). For extractions, HPLC grade methanol and chloroform were purchased from LabScan (Dublin, Ireland). For the mobile phase, HPLC quality methanol was purchased from Biosolve (Valkenswaard, The Netherlands); ultrapure distilled water and deionized water were prepared in-house and filtered prior to use; ammonium formate and formic acid were purchased from Sigma (Bornem, Belgium). All reagents were at least of analytical grade.

2.2. Cannabis samples

Eight samples of drug-type cannabis and one sample of non-psychotropic cannabis were provided by police (confiscated samples). Two other samples of fibre-type cannabis were generously provided by the laboratory of Ecophysiology and Plant Breeding of the Université catholique de Louvain.

2.3. Sample preparation

Plant material samples were dried for 24 h in a 35 °C forced ventilation oven. Crumbly samples were then grinded and mixed. 200 mg of this fine powder were weighed in a flask and extracted with 20 mL of a mixture methanol/chloroform (v/v: 9/1) by agitation during 30 min. The extract was filtered and appropriately diluted in a small test tube. A 100 μL aliquot of the dilution was evaporated under a gentle stream of nitrogen and redissolved in 100 μL of a mixture of water/methanol (v/v: 5/5). Prazepam (100 mg/L) was used as internal standard.

2.4. HPLC equipment and chromatographic conditions

All chromatographic runs were carried out using an Hewlett-Packard (HP) HPLC System (Agilent Technologies, Böblingen, Germany), consisting of a G1311A quaternary solvent pump (1200 series), a G1322A solvent degasser (1200 series), a G1313A autosampler (1100 series) and a G1316A column compartment (1100 series). A Waters (Zellik, Belgium) 2996 photodiode-array detector (DAD) was used for detection. Full spectra were recorded in the range 200–400 nm. Chromatographic separations were achieved using a Waters XTerra® MS C18 analytical column (5 μm, 250 mm × 2.1 mm i.d.), protected by a Waters XTerra® MS C18 guard column (5 μm, 10 mm × 2.1 mm i.d.). Equipment control, data acquisition and integration were performed with Empower Pro 2.0 software.

The mobile phase consisted of a mixture of methanol/water containing 50 mM of ammonium formate (adjusted to pH 5.19). Initial setting was 68% methanol (v/v), which was linearly increased to 90.5% methanol over 25 min, then increased to 95% in 1 min. After maintaining this condition for 3 min, the column was set to initial condition in 1 min and re-equilibrated under this condition for 6 min. The total runtime was 36 min. Flow-rate was set to 0.3 mL/min, the injection volume was 30 μL. All experiments were carried out at 30 °C.

2.5. Method validation

In accordance to ISO17025 and the guidelines of the French Society of Pharmaceutical Sciences and Techniques (SFSTP), the present

Table 2
Description of the three factors involved in the experimental design.

	Factors		
	pcI (%)	pH	T_G (min)
Levels		2.6	
	5	4.45	10
	40	6.3	20
	75	8.15	30
		10.0	

method was fully validated using total error approach [22–25]. The e.noval software V2.0 (Arlenda, Liège, Belgium) was used to compute all validation results and build the accuracy profiles.

3. Results

3.1. Method optimization

3.1.1. Experimental design

Three HPLC factors have been investigated: the percentage of methanol at the beginning of the gradient (pcI), the pH of the aqueous part of the mobile phase (pH) and the gradient time to reach 95% of methanol (T_G). Table 2 shows the levels of these three factors. Design of experiments (DoE) methodology has been used and a full factorial design was selected, which is convenient to explore the space of factors. As such, a total of 45 experimental conditions were defined and a chromatogram was recorded at each of these. At the central point (pcI = 40%, pH 6.3 and T_G = 20 min), two independent repetitions (preparation of new buffer) were carried out to estimate the reproducibility of the system.

3.1.2. Statistical models

In the resulting chromatograms, the peaks were detected and indexed at their beginnings, apexes and ends. The retention factors ($\log(k)$) have been used to create a multivariate responses surface model. Fig. 2 illustrates the fit of the observed retention times versus the predicted retention times using the statistical models. Residuals are mainly located into the interval [−2, 2] min. As the adjusted r^2 of each model were higher than 0.95, the overall quality of the fit is good although some outliers are observed.

3.1.3. Optimization — Design Space

The minimal separation (separation of the critical pair) is optimized using the methodology presented by Lebrun et al. [26]. The separation is defined as the difference between the beginning of a peak and the end of the preceding peak. Consequently to the response (retention times) modelling, the experimental domain is investigated to encounter an optimal separation. The propagation of the predictive error through the criterion (the separation, S) was analyzed to give confidence in this optimum. The Design Space (DS) is defined as the set of factor conditions that are likely to provide satisfactory results in the future use of the analytical method (e.g. routine). Mathematically, the DS applied in this case is defined as in Eq. (1),

$$DS = \{x_0 \in \chi | E_\theta[P(\min(S) > \lambda | \theta] \geq \pi\} \quad (1)$$

where x_0 is the set of factor conditions belonging to the experimental domain χ, for which the expected probability to have a separation (S) higher than λ is higher than π, given the uncertainty of the estimation of the parameters θ of the model. A separation of at least 0 min (λ = 0 min) should be obtained. Monte Carlo simulations are performed to propagate uncertainty from parameters to responses and criterion. A summary of the optimal values of factors (the best probabilities to achieve a minimal separation of at least 0 min; baseline-resolved peaks) is shown in Table 3. Fig. 3 shows the probability surfaces in different directions of the space of factors, around the optimal solution and with, for each graph, two factors that are fixed at optimal values.

Table 3
Optimal factor setting maximizing the separation of the compounds.

	pcI (%)	pH	T_G (min)
Optimal values P(separation > 0) > 0.4	68	5.2	30

The chromatograms predicted at the conditions described in Table 3 can be seen in Fig. 4. Despite the poor DS probability (40%), a good agreement between the predicted chromatogram and the real processed chromatogram is observed. Separation of all of the compounds is well achieved within the DS. A chromatogram experimentally obtained with cannabinoid standards is shown in Fig. 5. Qualitative HPLC profiles of herbal cannabis and cannabis resin samples are provided in Figs. 6 and 7, respectively.

3.2. Method validation

3.2.1. Selectivity

The selectivity of detection of each compound was ensured by the determination of the retention times and the recording of the complete UV spectra of the cannabinoids. Spectra are shown in Fig. 8.

3.2.2. Linearity

The response function is, within a certain range, the relationship between the response observed and the concentration of the analyte in the sample [27]. Calibration curves were obtained from standard solutions in methanol containing eight different concentrations for each cannabinoid from 0.15 to 20% (percentage of weight of dry plant material), corresponding to 0.375 to 50 μg/mL. The concentration levels were chosen in order to cover the different contents in cannabinoids in plant materials of diverse types. Each calibration point was analyzed in duplicate on two consecutive days. Calibration curves were calculated using unweighted linear regression analysis and linearity was expressed by the r^2-value. The calibration parameters were stable with regression coefficients always >0.99 for each cannabinoid studied. The regression coeffi-

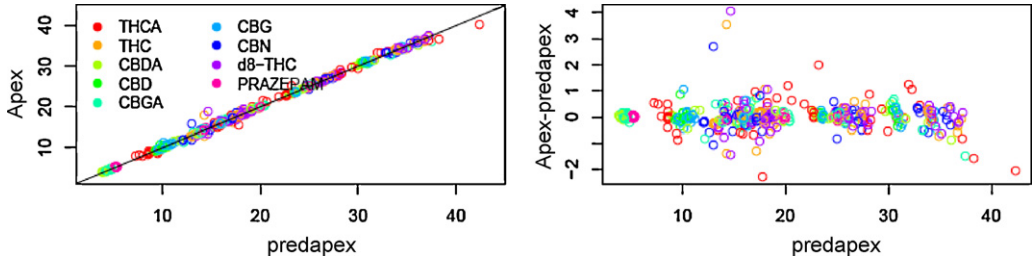

Fig. 2. Actual original responses (Apex) versus predicted one (predapex). On the right are the residuals.

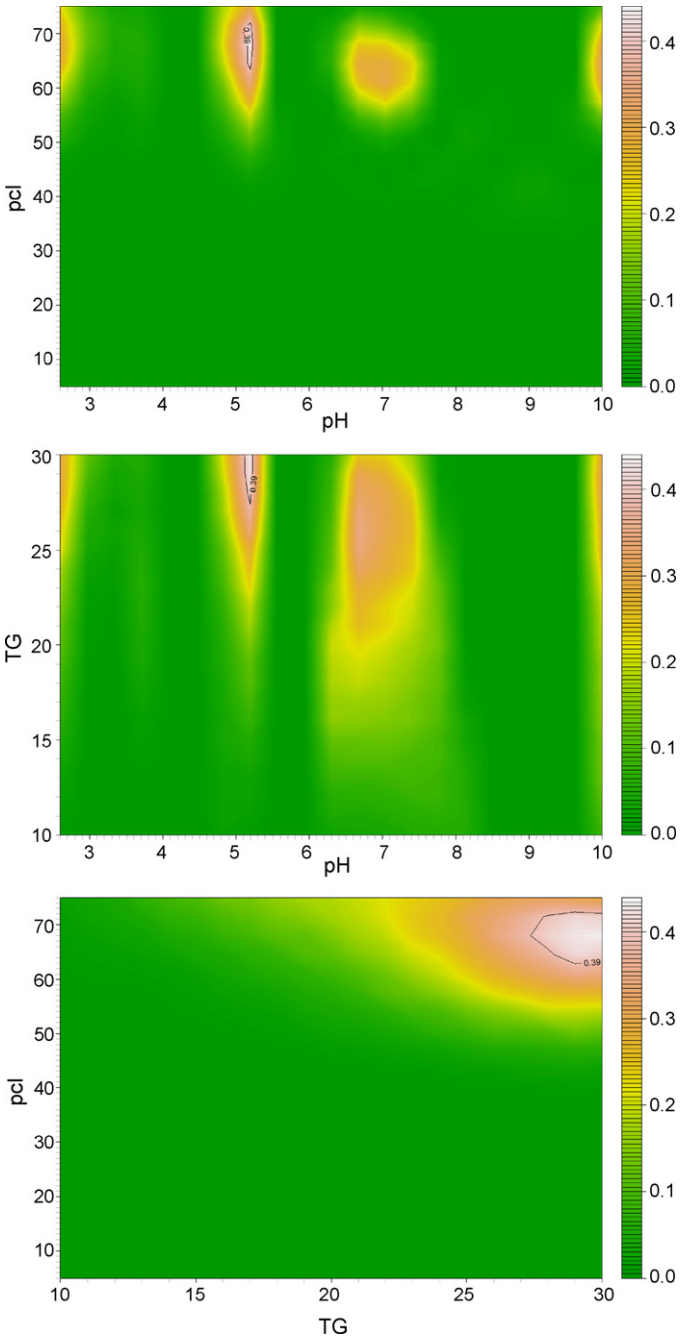

Fig. 3. Representation of the Design Space of the method on the experimental domain. Inside black lines, the expected probability to have well-separated peaks is 0.9.

cients for each analyte are listed in Table 4. The curves were linear in the concentration range studied for each analyte.

3.2.3. Limit of quantification (LOQ), limit of detection (LOD)

The LOQ were experimentally determined by analyzing standard solutions at 0.025, 0.05, 0.075 and 0.1% (corresponding to 0.0625, 0.125, 0.1875 and 0.25 μg/mL). The lower LOQ was determined as the concentration which provided measurements with an accuracy within the acceptance limits (±20%) from their nominal values. The LOD was determined as the smallest dilution that gave a good correlation between the compound UV–vis spectrum and the spectra library. LOQ and LOD for each cannabinoid are listed in Table 4.

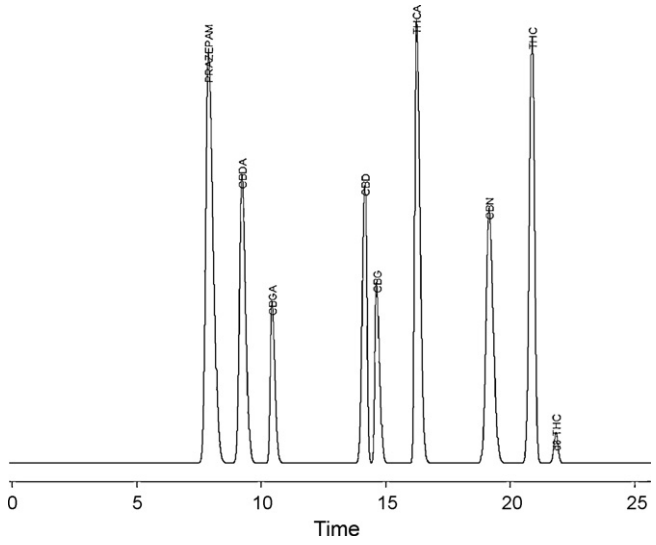

Fig. 4. Predicted chromatogram at optimal solution.

Table 4
Linearity (expressed by the regression coefficient values, r^2), limits of quantification (LOQ), limits of detection (LOD).

	r^2	LOQ (%)	LOD (%)
THCA	0.9969	0.05	0.025
THC	0.9940	0.05	0.025
CBDA	0.9939	0.05	0.05
CBD	0.9951	0.075	0.075
CBGA	0.9948	0.05	0.05
CBG	0.9959	0.15	0.1
CBN	0.9917	0.05	0.025

3.2.4. Trueness, precision and accuracy

A statistical approach based on the total error measurements including both bias and standard deviation was applied to validate the method.

Validation standards: because cannabis without major cannabinoids was not available, the validation standards were prepared by spiking samples of nettle (*Urtica dioica*, which belongs to the same order as *C. sativa* L.) with an extract of cannabis resin. This hashish extract contained all the cannabinoids of interest in significant amounts, except Δ_8-THC. The method was therefore not validated for the quantification of Δ_8-THC. The extract of hashish was quantified and used to prepare three validation standards. The volumes added correspond to different concentrations for each cannabinoid, in function of the quantity initially present in the hashish sample. The concentrations spiked for each cannabinoid are listed in Table 5. Each validation standard was analyzed in triplicate on three consecutive days. The concentrations of the validation standards were back-calculated from the obtained results to determine the mean relative bias, the

Table 5
Mean introduced concentrations (%).

	Concentration level 1 (%)	Concentration level 2 (%)	Concentration level 3 (%)
THCA	1.83	3.06	6.12
THC	1.15	1.92	3.84
CBDA	1.00	1.67	3.33
CBD	0.549	0.917	1.834
CBGA	0.134	0.217	0.434
CBG	0.092	0.154	0.308
CBN	0.158	0.264	0.528

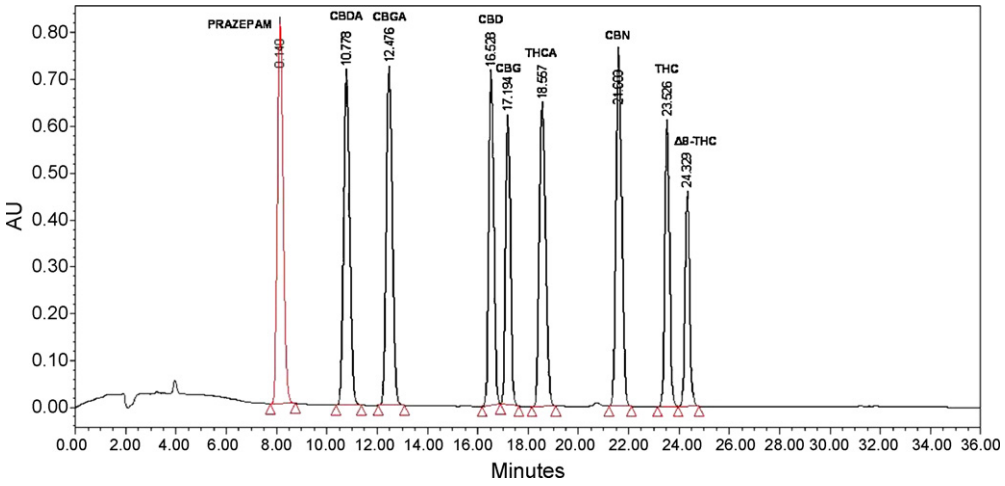

Fig. 5. Example of chromatogram determined experimentally (with a concentration of 10% in each cannabinoid) and retention times of the compounds (prazepam is used as internal standard).

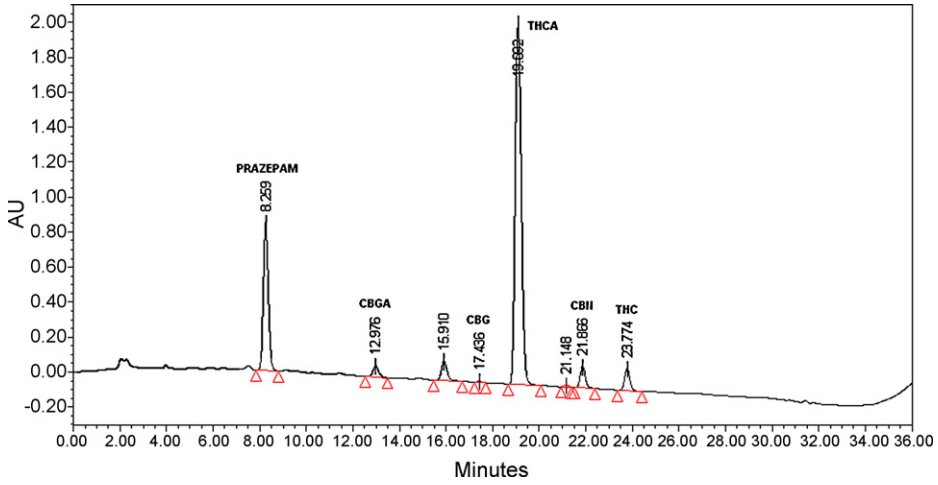

Fig. 6. Qualitative HPLC profile of herbal cannabis sample.

standard deviation for intermediate precision and finally the upper and lower β-expectation tolerance limits at the 17.5% level.

Trueness and precision give information on respectively systematic and random errors. Trueness refers to the closeness of agreement between the exact concentration in spiked material and the obtained main results. Trueness is expressed in terms of relative bias (%) and was calculated from the validation standards for each compound [24,28]. Trueness was acceptable for all cannabinoids, since the relative bias (%) were always smaller than 10%. Results are presented in Table 6.

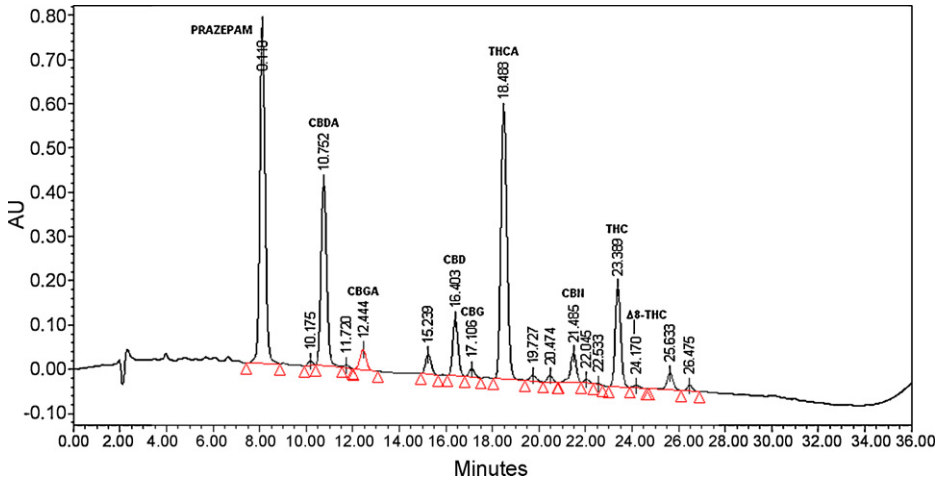

Fig. 7. Qualitative HPLC profile of cannabis resin sample.

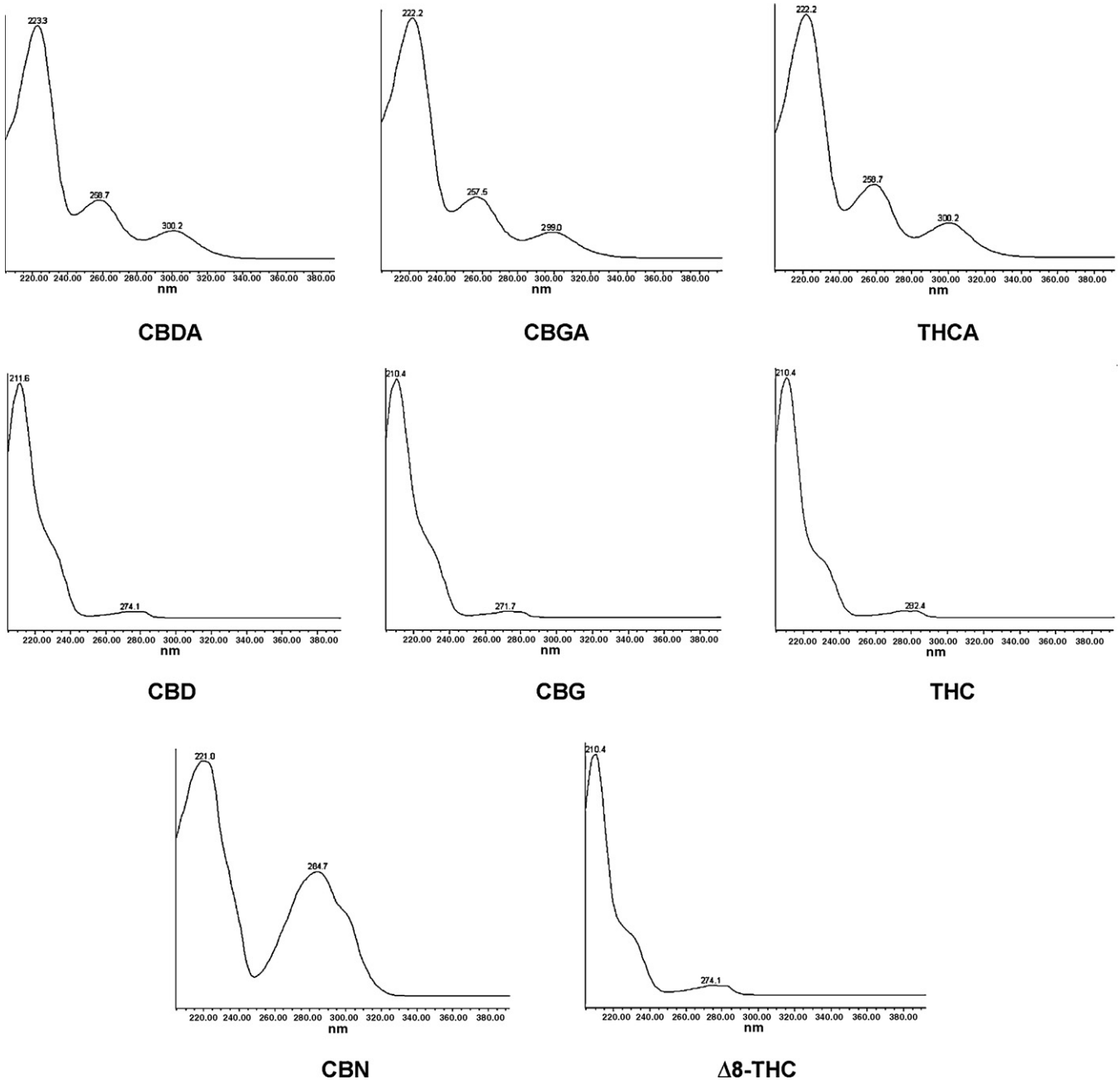

Fig. 8. UV spectra of studied cannabinoids.

The precision of the method was determined by computing the Relative Standard Deviations (RSDs) for repeatability and time-different intermediate precision at each concentration level of the validation standards [24,25,28], and did not exceed 11% for all of the cannabinoids (Table 6).

Accuracy takes into account the total error (sum of the systematic and random errors) of the test results [24,25,28]. It refers to the closeness of agreement between the test results and the acceptance reference value. The acceptance limits were set at ±30% as recommended [22]. As shown in Fig. 9, the relative upper and lower β-expectation tolerance intervals (%) did not exceed the acceptance limits (±30%) for each cannabinoid and each concentration level. The β-expectation tolerance limits are listed in Table 6. The approach used guarantees that each further measurement of unknown samples will be included within the tolerance limits at the 17.5% level.

3.2.5. Recovery

The absolute recoveries of THCA, THC, CBDA, CBD, CBGA, CBG and CBN were determined at the three different concentrations listed in Table 5 [22,29]. The mean recoveries are shown in Table 7. Those absolute recoveries were calculated by comparing peak areas of each cannabinoid obtained from freshly prepared matrix samples treated according to the described procedure with those found after the direct injection on the analytical column of standard solutions at the same concentrations. All the recoveries were good demonstrating the high extraction efficiency of the method.

3.2.6. Uncertainty assessment

The uncertainty characterizes the dispersion of the values that could reasonably be attributed to the measurand. Several uncertainty results were generated. The expanded uncertainty represents an interval around the results where the unknown true

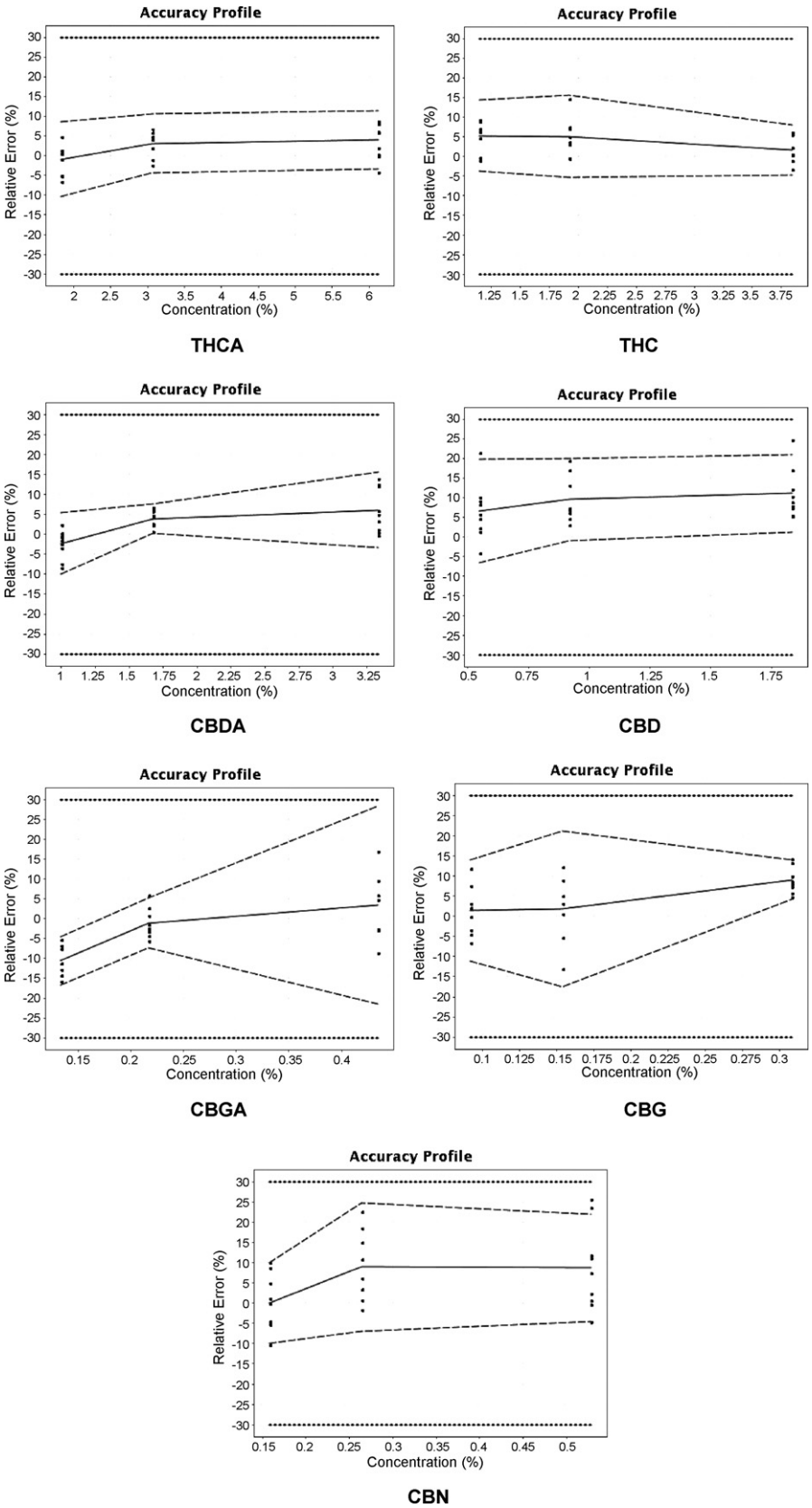

Fig. 9. Accuracy profiles of the cannabinoids. The plain line is the relative bias, the dashed lines are the β-expectation tolerance limits and the dotted lines represent the acceptance limits (30%). The dots represent the relative back-calculated concentrations and are plotted with respect to their targeted concentration.

Table 6
Method validation for seven cannabinoids in plant material. Trueness, precision, accuracy and uncertainty.

	Level	THCA	THC	CBDA	CBD	CBGA	CBG	CBN
Trueness								
Relative bias (%)	1	−0.9	5.3	−2.3	6.6	−10.6	1.4	0.1
	2	3.1	5.1	3.9	9.5	−1.1	1.8	8.9
	3	4.0	1.6	6.1	11.1	3.4	9.1	8.8
Intra-assay precision								
Repeatability (*RSD* %)	1	1.03	1.31	2.15	5.79	3.04	2.39	3.82
	2	1.01	2.21	2.30	4.71	3.23	3.35	3.98
	3	2.39	1.94	4.98	6.28	3.00	1.93	6.09
Between-assay precision								
Intermediate precision (*RSD* %)	1	3.97	3.80	3.89	7.52	3.64	5.34	4.46
	2	3.14	4.32	2.30	6.01	3.79	7.78	6.84
	3	3.26	2.12	5.69	6.28	10.94	1.93	6.09
Accuracy								
β-*Expectation tolerance limits* (%)	1	[1.64, 1.99]	[1.11, 1.32]	[0.90, 1.05]	[0.51, 0.66]	[0.11, 0.13]	[0.08, 0.10]	[0.14, 0.17]
	2	[2.93, 3.38]	[1.82, 2.22]	[1.68, 1.80]	[0.91, 1.10]	[0.20, 0.23]	[0.13, 0.19]	[0.25, 0.33]
	3	[5.91, 6.82]	[3.66, 4.15]	[3.22, 3.85]	[1.86, 2.22]	[0.34, 0.56]	[0.32, 0.35]	[0.50, 0.64]
Uncertainty								
Relative expanded uncertainty (%)	1	9.1	8.7	8.8	16.5	7.9	12.1	9.6
	2	7.2	9.8	4.8	13.2	8.2	17.7	15.4
	3	7.2	4.6	12.3	13.2	25.1	4.1	12.8

Table 7
Mean recoveries of cannabinoids.

	Number of repetition (*n*)	Recovery ± SD (%)
THCA	3	102.1 ± 2.6
THC	3	104.0 ± 2.1
CBDA	3	102.6 ± 4.3
CBD	3	109.1 ± 2.3
CBGA	3	97.2 ± 7.2
CBG	3	104.1 ± 4.3
CBN	3	105.9 ± 5.1

value can be observed with a confidence level of 95%. The relative expanded uncertainties (%) are obtained by dividing the corresponding expanded uncertainties with the corresponding introduced concentrations. Values for each cannabinoid are presented in Table 6 and were between 4.1 and 25.1%.

3.3. Analysis

The present method was applied for the analysis of different cannabis products. The preparation of these samples was the same as described above. Samples 1–8 were police confiscates of drug-type cannabis in which THCA and THC are the main cannabinoids. Samples 9 and 10 are fibre-type cannabis, respectively Fedora 17 and Santhica 27 varieties. Fedora 17 is a "classical" fibre-type variety containing CBDA and CBD as major cannabinoids. Santhica 27 is a new variety of hemp in which the biogenesis of cannabinoids seems to have stopped precociously: CBGA and CBG are the main cannabinoids [17]. Sample 11 is a non-psychotropic cannabis which grew wild (seeds coming from bird food). Cannabinoid concentrations of these samples of herbal cannabis are listed in Table 8.

4. Discussion

Gas Chromatography (GC) is the most commonly used method for the analysis of cannabis products, e.g. in forensic chemistry [5,8,14–18]. However, as this method is based on heating the sample, thermal conversion occurs and the acidic forms of cannabinoids are converted into their decarboxylated counterparts. In order to determine neutral cannabinoids, a time-consuming derivatization step is mandatory. Conversely, High Performance Liquid Chromatography (HPLC) permits the determination of the original composition of the cannabinoids in the plant by direct analysis. In contrast to GC, no decomposition of the cannabinoids occurs during analysis by HPLC. Furthermore, THCA decarboxylation during GC analysis is often supposed to be complete [5,14]; but Dussy et al. [19] demonstrated in 2005 that this conversion is only partial. Various analytical conditions were studied and a maximal conversion of about 67% was obtained at an injector temperature of 220 °C. Laboratories quantifying total THC by HPLC, building the sum of THCA and the already present THC in the plant, get therefore a higher value than those who quantify THC by GC [19].

Several HPLC methods have been described in the literature [2,3,20,21], reviewed in 2004 by Raharjo and Verpoorte [15], but most of them failed to separate efficiently all the cannabinoids or were not validated according to the new guidelines using total error approach. Some methods were not validated for acidic cannabinoids as these were, until recently, not commercially available.

Table 8
Cannabinoid concentrations found in different types of herbal cannabis products.

Sample	Drug/fibre-type	THCA (%)	THC (%)	CBN (%)	Total THC (%)	CBDA (%)	CBD (%)	CBGA (%)	CBG (%)
1	Drug	20.24	1.55	*<LOD*	21.79	*<LOD*	*<LOD*	0.72	<LOQ
2	Drug	5.40	3.23	0.06	8.69	*<LOD*	*<LOD*	0.12	<LOQ
3	Drug	22.92	2.58	*<LOD*	25.51	*<LOD*	*<LOD*	1.89	0.28
4	Drug	15.68	1.56	*<LOD*	17.24	*<LOD*	*<LOD*	0.30	<LOQ
5	Drug	15.81	1.21	*<LOD*	17.02	*<LOD*	*<LOD*	0,43	*<LOD*
6	Drug	15.53	1.29	*<LOD*	16.82	*<LOD*	*<LOD*	0.41	*<LOD*
7	Drug	8.18	4.05	0.33	12.56	*<LOD*	*<LOD*	0,17	<LOQ
8	Drug	10.79	3.20	0,08	14.08	<LOQ	*<LOD*	0.38	<LOQ
9	Fibre	0.09	<LOQ	*<LOD*	0.09	2.37	0.10	0.10	<LOQ
10	Fibre	<LOQ	*<LOD*	*<LOD*	<LOQ	*<LOD*	*<LOD*	2.58	0.16
11	Fibre	0.57	0.10	*<LOD*	0.67	*<LOD*	*<LOD*	<LOQ	*<LOD*

Hazekamp et al. [2,20] described two methods (acidic or basic eluent) who did not permit a full separation of peaks for either CBGA/CBN or CBD/CBG. A secondary analysis by GC was necessary to quantify those cannabinoids, causing a waste of time. Another solution is the coupling of the HPLC system with a mass spectrometer. However, mass spectrometry is not routinely available to most laboratories.

The selectivity of the compounds can be modified by adjusting the pH of the eluent. The relative retention times of the acidic cannabinoids are influenced by changing the pH, while the order of elution and the relative retention times for the neutral cannabinoids remain the same [2,20]. By adjusting the pH of our eluent (pH 5.19) and the gradient elution slope, thanks to the optimization method, we were able to modify precisely the relative retention times of the compounds in order to fully separate each of them (however, CBD and CBG may yet slightly overlap if present in high concentrations, >10%). Consequently, the method developed and validated allows a good separation of eight major cannabinoids of interest in a single run of 25 min (36 min with re-equilibration).

5. Conclusion

Using original tools, a simple and accurate HPLC method for the quantification of major cannabinoids in cannabis plant material has been developed and validated. This analytical method can be used for diverse applications, e.g. plant phenotype determination, evaluation of psychoactive potency and control of medicinal sample quality. It could also be an aid for checking the identity of cannabis specimen of different origin, next to other techniques as determination of microelements or stable isotopes of carbon and nitrogen [30]. In addition, quantification of total CBG can be useful for the identification of different types of fibre hemp analyzed [17].

Acknowledgments

The authors acknowledge the Belgian Science Policy (Belspo) for the financial support (GEOCAN project DR-00-48) and are very grateful to the reviewers for providing important comments that led to significant improvements of this article.

References

[1] European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), Annual Report 2008: The State of the Drug Problem in Europe, Office for Official Publications of the European Communities, Luxembourg, 2008.
[2] A. Hazekamp, Cannabis; extracting the medicine, PhD thesis, Universiteit Leiden, The Netherlands, 2007.
[3] T. Lehmann, R. Brenneisen, J. Liq. Chromatogr. 18 (1995) 689.
[4] M.A. Elsohly, D. Slade, Life Sci. 78 (2005) 539.
[5] A.K. Hewavitharana, G. Golding, G. Tempany, G. King, N. Holling, J. Anal. Toxicol. 29 (2005) 258.
[6] C. Giroud, Chimia 56 (2002) 80.
[7] G.A. Thakur, R.I. Duclos Jr., A. Makriyannis, Life Sci. 78 (2005) 454.
[8] K.W. Hillig, P.G. Mahlberg, Am. J. Bot. 91 (2004) 966.
[9] N.J. Doorenbos, P.S. Fetterman, M.W. Quimby, C.E. Turner, Ann. N. Y. Acad. Sci. 191 (1971) 3.
[10] A.A. Stolker, J. van Schoonhoven, A.J. de Vries, I. Bobeldijk-Pastorova, W.H. Vaes, R. van den Berg, J. Chromatogr. A 1058 (2004) 143.
[11] E.B. Russo, J.M. McPartland, Psychopharmacology (Berl.) 165 (2003) 431.
[12] E. Russo, G.W. Guy, Med. Hypotheses 66 (2006) 234.
[13] K.C. Verhoeckx, H.A. Korthout, A.P. van Meeteren-Kreikamp, K.A. Ehlert, M. Wang, J. van der Greef, R.J. Rodenburg, R.F. Witkamp, Int. Immunopharmacol. 6 (2006) 656.
[14] H. Stambouli, A. El Bouri, M.A. Bellimam, T. Bouayoun, N. El Karni, Ann. Toxicol. Anal. 17 (2005) 79.
[15] T.J. Raharjo, R. Verpoorte, Phytochem. Anal. 15 (2004) 79.
[16] D.W. Lachenmeier, L. Kroener, F. Musshoff, Anal. Bioanal. Chem. 378 (2004) 183.
[17] G. Fournier, O. Beherec, S. Bertucelli, Ann. Toxicol. Anal. 16 (2004) 128.
[18] N. Fucci, Forensic Sci. Int. 138 (2003) 91.
[19] F.E. Dussy, C. Hamberg, M. Luginbühl, T. Schwerzmann, T.A. Briellmann, Forensic Sci. Int. 149 (2005) 3.
[20] A. Hazekamp, C. Giroud, A. Peltenburg, R. Verpoorte, J. Liq. Chromatogr. Rel. Technol. 28 (2005) 2361.
[21] V. Gambaro, L. Dell'Acqua, F. Farè, R. Froldi, E. Saligari, G. Tassoni, Anal. Chim. Acta 468 (2002) 245.
[22] C.T. Viswanathan, S. Bansal, B. Booth, A.J. DeStefano, M.J. Rose, J. Sailstad, V.P. Shah, J.P. Skelly, P.G. Swann, R. Weiner, AAPS J. 9 (2007) E30.
[23] B. Boulanger, P. Chiap, W. Dewé, J. Crommen, P. Hubert, J. Pharm. Biomed. Anal. 32 (2003) 753.
[24] P. Hubert, J.J. Nguyen-Huu, B. Boulanger, E. Chapuzet, P. Chiap, N. Cohen, P.A. Compagnon, W. Dewé, M. Feinberg, M. Lallier, M. Laurentie, N. Mercier, G. Muzard, C. Nivet, L. Valat, J. Pharm. Biomed. Anal. 36 (2004) 579.
[25] P. Hubert, J.J. Nguyen-Huu, B. Boulanger, E. Chapuzet, N. Cohen, P.A. Compagnon, W. Dewé, M. Feinberg, M. Laurentie, N. Mercier, G. Muzard, L. Valat, E. Rozet, J. Pharm. Biomed. Anal. 45 (2007) 82.
[26] P. Lebrun, B. Govaerts, B. Debrus, A. Ceccato, G. Caliaro, P. Hubert, B. Boulanger, Chemometr. Intell. Lab. Syst. 91 (2008) 4.
[27] E. Rozet, C. Hubert, A. Ceccato, W. Dewé, E. Ziemons, F. Moonen, K. Michail, R. Wintersteiger, B. Streel, B. Boulanger, P. Hubert, J. Chromatogr. A 1158 (2007) 126.
[28] E. Rozet, A. Ceccato, C. Hubert, E. Ziemons, R. Oprean, S. Rudaz, B. Boulanger, P. Hubert, J. Chromatogr. A 1158 (2007) 111.
[29] Guidance for Industry: Bioanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER), 2001, May.
[30] E.K. Shibuya, J.E.S. Sarkis, O. Negrini-Neto, J.P.H.B. Ometto, J. Braz. Chem. Soc. 18 (2007) 205.