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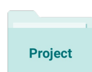

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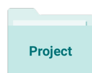

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Quantitative Analysis of Cannabinoids from *Cannabis sativa* Using ^{1}H-NMR

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A ^{1}H-NMR method has been developed for the quantitative analysis of pure cannabinoids and for cannabinoids present in *Cannabis sativa* plant material without any chromatographic purification. The experiment was performed by the analysis of singlets in the range of δ 4.0—7.0 in the ^{1}H-NMR spectrum, in which distinguishable signals of each cannabinoid are shown. Quantitation was performed by calculating the relative ratio of the peak area of selected proton signals of the target compounds to the known amount of the internal standard, anthracene. For this method no reference compounds are needed. It allows rapid and simple quantitation of cannabinoids with a final analysis time of only 5 min without the need for a pre-purification step.

Key words cannabinoid; ^{1}H-NMR spectroscopy; quantitative analysis; *Cannabis sativa*

The cannabis plant has been of medicinal interest for centuries. In recent years a lot of research on the medical applications of *Cannabis sativa* L. has been initiated, as several, mostly European, countries move towards a more liberal view on the use of Cannabis as a medicine. Many different pharmacological properties have been associated with cannabis use, including increased heart rate, drop of body temperature, ataxia and a loss of time-space perception.[1] Amongst the constituents of *Cannabis sativa*, the cannabinoids have been recognized as the active constituents for most clinical activities. The cannabinoids make up a large family of closely related C_{21} compounds and their carboxylic acids and are unique to the cannabis plant.[2] Pharmacological activites of the cannabinoids are very diverse, ranging from analgetic and antiemetic to the treatment of glaucoma and multiple sclerosis.[3,4] Only four of the 66 known natural cannabinoids[5] are currently commercially available as certified reference standards, *i.e.*: (−)-delta-9-tetrahydrocannabinol (Δ^9-THC or THC), (−)-delta-8-tetrahydrocannabinol (Δ^8-THC), cannabidiol (CBD) and cannabinol (CBN). There are indications that also these reference compounds have to be re-quantified regularly because of degradation and differences between batches during production.[5]

Recently, our laboratory developed a method for the large scale isolation of highly pure cannabinoids from *Cannabis sativa* flower tops.[7] For the quantitative analysis of these compounds, GC with FID or other detection has been widely used, but this method can not distinguish between cannabinoids and their carboxylic counterparts without prior derivatization.[8,9] HPLC with UV detection is more suitable for simultaneous analysis of these compounds, but it proves to be very difficult to separate all components in a single chromatographic run[10,11] and some contaminations cannot be detected because they lack UV absorbance. Furthermore, both methods are sensitive to impurities in the sample, *e.g.* chlorophyll or lipids, and they usually require a sample clean-up step prior to analysis. Most importantly, the reference compounds needed for the preparation of a calibration curve are not available for many cannabinoids. A review of methods for cannabinoids analysis in biological materials is given by Raharjo.[12]

To solve the problems associated with these analytical techniques, the development of a reliable and easy method is required as alternative to the conventional analyses. In this study, we developed an analytical method using ^{1}H-NMR for cannabinoids without the need for any chromatographic purification. Quantitative NMR has been shown to be very accurate and highly reproducible, within a very short analysis time. The usefulness of quantitative NMR for the validation of natural product reference compounds as well as theoretical aspects have been shown by Maniara and Pauli.[13,14]

The developed method was applied on the quantitative analysis of five different isolated cannabinoids. A similar method has been recently described by our laboratory for the quantitative analysis of bilobalide and ginkgolides in Ginkgo biloba leaves and products.[15] The usefulness of this method was further shown by quantitation of THCA in four different types of *Cannabis sativa* plant material.

Experimental

Plant Material *Cannabis sativa* plant material was obtained from Stichting Institute for Medical Marijuana (SIMM) in Rotterdam, The Netherlands, and from Bedrocan BV, The Netherlands. Four different cannabis cultivars were used. After harvest the plant material was air-dried in the dark under constant temperature and humidity for 4 weeks. Only flowertops of female plants were used. These were manicured to remove other plant material like leaves and stems, and stored at −20 °C.

Solvents and Chemicals Anthracene was purchased from Sigma (St. Louis, MO, U.S.A.). Deuteriated chloroform ($CDCl_3$, 99.8%) was obtained from Eurisotop (Gif-sur-Yvette, France). All organic solvents were analytical grade and obtained from Merck Biosolve Ltd. Valkenswaard, The Netherlands.

Pure cannabinoids were previously isolated and identified by us.[7] They were stored as ethanolic solutions at −20 °C. The following isolated cannabinoids were used for quantitation: (−)-delta-9-tetrahydrocannabinol (THC), (−)-delta-9-tetrahydrocannabinolic acid A (THCA), cannabidiol (CBD), cannabidiolic acid (CBDA) and cannabinol (CBN), (Fig. 1). Commercially obtained certified standard of THC was from Cerilliant, (Round Rock, TX, U.S.A.), while CBD and CBN were obtained from Sigma.

^{1}H-NMR Parameters ^{1}H-NMR spectra were recorded in $CDCl_3$ using a Bruker DPX 300 spectrometer, equiped with an Indy Silicon Graphics computer. For each sample, 64 scans were recorded with the following parameters: 32 K data points, pulse width of 4.0 μs and relaxation delay of 1 second. FID's were Fourier transformed with LB of 0.5 Hz. For quantitative analysis, peak area was used after baseline correction.

Determination of Accuracy Certified cannabinoid standards were used to evaluate the accuracy of the developed method. From newly opened vials containing THC (1.0 mg/ml), CBD (0.99 mg/ml) and CBN (0.98 mg/ml) 100 μl was mixed with 1.00 mg of anthracene as internal standard (all in triplicate). These samples were evaporated using a vacuum centrifuge and redissolved in 1 ml of $CDCl_3$ for NMR analysis.

An aliquod of the ethanolic solutions of cannabinoid standards, isolated

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by our own lab (isolates), were diluted in ethanol to a concentration of about 0.5 mg/ml (based on weight after extensive evaporation of solvent). After this they were quantified as described above.

Evaluation of Recovery of Cannabinoids Fivehundred milligram of cellulose filter paper (Schleicher & schuell, GmbH, Cassel, Germany) was cut into pieces of *ca.* 0.5 cm diameter and placed in the extraction vessel. Each isolated cannabinoid (1.0 mg in ethanol) was spiked into the filter paper disks and the spiked samples were dried at room temperature for 24 h before extraction.

Extraction and Quantification of Cannabinoids For each analysis plant material (350 μg dry weight) or recovery control was extracted two times for 10 min with 15 ml methanol/chloroform (9 : 1, v/v). Extractions were started by 2 min of ultrasonication and were performed at 4 °C. Both extracts were combined and the volume was brought to 50 ml with extraction solvent. Then 0.5 ml of extract was mixed with 1.00 mg of anthracene as internal standard. These samples were evaporated using a vacuum centrifuge and redissolved in 1 ml of $CDCl_3$ for ^{1}H-NMR analysis. All experiments were based on triplicates. For the plant materials, only the amount of THCA was determined.

To evaluate the linearity between sample size of the plant material and the quantification result, different amounts of plantmaterial (100, 300, 500 μg, all in triplicate) were extracted and quantified.

Gas Chromatography (GC) Quantification of THCA or CBDA, using a certified standard of THC or CBD was performed with a Chrompack (Middelburg, The Netherlands) CP9000 gas chromatograph, fitted with a Durabond fused silica capillary column (30 m×0.25 mm inner diameter) coated with DB-1 (J&W scientific Inc., Rancho Cordova, CA, U.S.A.) at a film thickness of 0.1 μm. The (FID) signal was recorded on a Shimadzu (Kyoto, Japan) CR3A integrator. The oven temperature was programmed from 100 °C to 280 °C at a rate of 10 °C/min. The oven was then kept at 280 °C until the end of the runtime of 30 min. The injector and the detector temperature were maintained at 280 °C and 290 °C, respectively. Nitrogen was used as the carriergas at a pressure of 70 kPa. Air and hydrogen were used as detector gasses. The injection split ratio was 1/50.

Results and Discussion

In this study we tried to develop a ^{1}H-NMR method for the quantitative analysis of pure cannabinoids and cannabinoids present in *Cannabis sativa* plant material, in order to perform quantitative analysis of cannabinoids without the need of chromatographic separation or the use of certified reference standards. The ^{1}H-NMR spectra of the studied cannabinoids have been published.[16,17] The cannabinoids most commonly found in Cannabis plant materials were used for this study. However it must be noted that one major cannabinoid, cannabichromenic acid (CBCA, Fig. 1) was not studied because there was no reference standard available for this compound. CBCA is commonly found in fiber-type, as well as drug-type Cannabis.

The proton signals selected for this study were in the range of δ 4.0—7.0, as this is the range where the ^{1}H-NMR spectra are most distinguishable. As internal standard anthracene was selected because it is a very stable compound with a simple ^{1}H-NMR spectrum consisting of a singlet (δ 8.43) and two quartets (δ 8.01, δ 7.48). These signals do not overlap with signals of the cannabinoids that were used in this study. For the quantification experiments, the singlet of anthracene was always used. Based on the chemical structure of the molecule the most suitable proton signals for quantification were selected for each cannabinoid. For some cannabinoids more than one proton signal was evaluated. Using known amounts of certified standards for THC, CBD and CBN, the developed method was shown to be highly accurate, as can be seen in Table 1. Following this, the studied cannabinoids were quantified by preparing a solution of about 0.5 mg/ml in $CDCl_3$ (based on weight after extensive drying to remove solvent) and performing a preliminary quantification of these solutions using the described ^{1}H-NMR method. The NMR spectra obtained in these experiments are shown in Fig. 2.

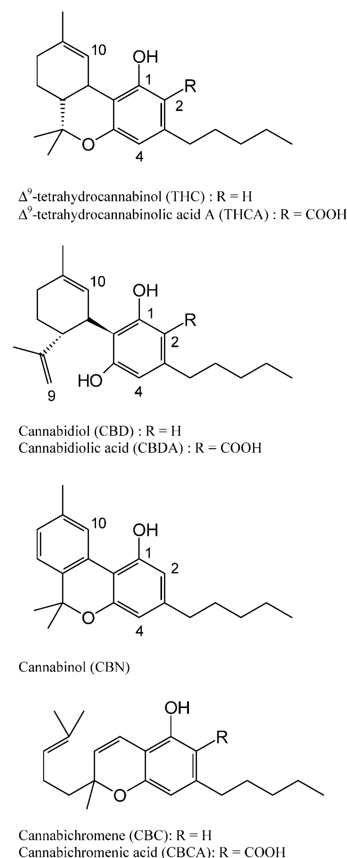

Fig. 1. Structures of the Cannabinoids

Table 1. Quantitation of Known Amounts of Commercially Obtained Cannabinoid Standards

Cannabinoid	Added (μg)	Calculated (μg)
THC	100	99 (±2.9%)
CBD	99	99 (±2.0%)
CBN	98	99 (±1.2%)

For each cannabinoid a calibration curve was determined in the concentration ranges as shown in Table 2, in order to evaluate the accuracy of this method depending on the different concentrations. The highest concentration used was at least two times higher than the 0.5 mg/ml used for the preliminary quantification. The calibration curves were made using the ratio of the peak integral of the compound and the internal standard. The linearity of the calibration curves was determined by plotting the least squares regression lines (Table 2). All calibration curves were highly linear with a r^2-value of more than 0.99. Because all preliminary quantifications were well within the linear range of this method, we can conclude that these values were accurate. It should be noted that these calibration curves are not needed for quantitation of the compounds in future experiments, because the integral is always proportional to the amount of the compound and the same for all compounds.

For testing the recovery of cannabinoids from a plant ma-

trix (consisting mainly of cellulose) during the extraction step, 1.0 mg of each compound was extracted from cellulose papers onto which the compounds were adsorbed.[15,18] The extraction procedure was kept as simple as possible and needed no sample clean-up steps before analysis. The recovery of each cannabinoid with the used method was more than 92%, as shown in Table 3.

Finally, extracts of four different *Cannabis sativa* cultivars were analyzed for their THCA content using the ^{1}H-NMR method developed in this study (Fig. 3). THCA was a major component of three of these extracts, as shown by HPLC analysis (data not shown). The fiber-type cannabis contained almost no THCA, but a high level of CBDA. For this type CBDA was quantified. The results were found to be very reproducible with a standard deviation of less than 6%. The results could be confirmed by gas chromatography (see Table 4). Because THCA or CBDA is converted into THC or CBD because of the heat applied in GC, a calibration curve of certified THC standard was used for a calibration curve. Table 5 shows the high linearity between the amount of cannabis plant material used for extraction and the THCA quantification results (not done for CBDA).

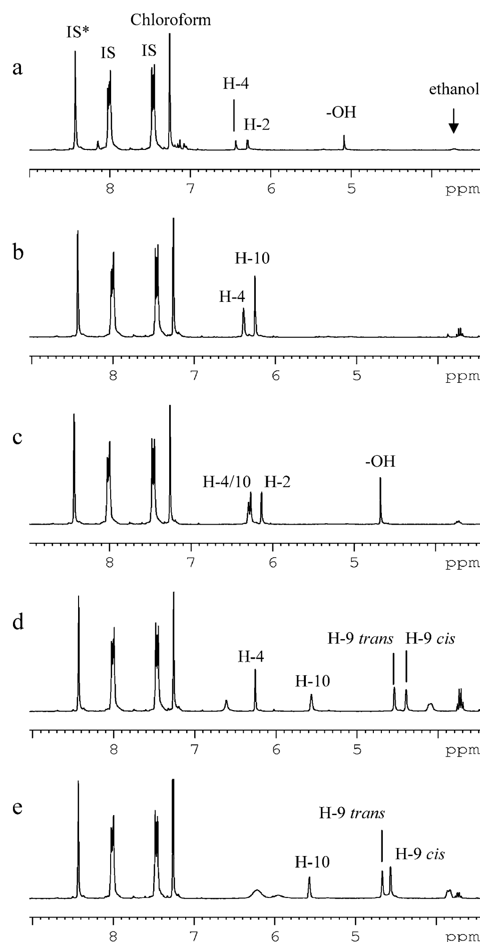

Fig. 2. ^{1}H-NMR Spectra of 0.5 mg (by Weight) of Each Cannabinoid together with 1 mg of Anthracene as Internal Standard

Quantitation was performed by calculating the ratio of the peak area of selected proton signals of the targetcompounds to the singlet of anthracene (*). In some spectra a residue of ethanol is visible. a: CBN, b: THCA, c: THC, d: CBDA, e: CBD, IS = signals of internal standard.

Conclusion

The content of the major cannabinoid of *Cannabis sativa* plant material and in isolated cannabinoid preparations could be analyzed with a simple method and in a short time for the final analysis, only 5 min, which is much shorter than conventional chromatographic methods. Moreover, cannabinoids could be quantified which are not available as reference compounds and can therefore not be quantified by other methods (*i.e.*: CBDA and THCA). Preliminary results show that this method is also suitable for the quantitation of cannabigerol (CBG) and cannabigerolic acid (CBGA) and probably additional cannabinoids. The ^{1}H-NMR method for the quantitative analysis of cannabinoids has the additional advantage that an overall profile is obtained of the extract and the purity of an isolated cannabinoid can be determined as well as the identity of impurities.

It seems clear that the quantitation of cannabinoids in iso-

Table 3. Recovery of the Cannabinoids (%) after Extraction from Filterpaper with Methanol/Chloroform, 9 : 1 (v : v)

THC	CBD	CBN	THCA	CBDA
99.2 (±6.7)	98.0 (±6.7)	92.1 (±4.2)	99.6 (±5.1)	100.4 (±6.2)

Each experiment was performed in triplicate.

Table 2. Linearity of the Calibration Curves of the Cannabinoids

Cannabinoid	Conc. range	Proton signal	δ in ppm	Linearity
CBN	0.1—1.0	H-4	6.44	0.9985
		H-10	8.16	Overlaps with internal standard
THCA-A	0.2—4.0	H-4	6.39	0.9996
		H-10	6.24	0.9998
THC	0.1—1.0	H-2	6.14	0.9993
		H-4/H-10	6.27/6.29	0.9999 Partially overlapping
CBDA	0.2—4.0	H-4	6.26	0.9999
		H-10	5.55	0.9999
		H-9 *trans*	4.54	0.9999
		H-9 *cis*	4.40	0.9999
CBD	0.2—4.0	H-10	5.57	0.9992
		H-9 *trans*	4.66	Interaction with –OH
		H-9 *cis*	4.56	Interaction with –OH

Listed are the concentration range of the calibration curves and the proton signals that were tested. The linearity of each calibration curve was determined by plotting the least squares regression line. The preferred proton signals for quantitation are presented in bold. Each sample was measured in duplicate.

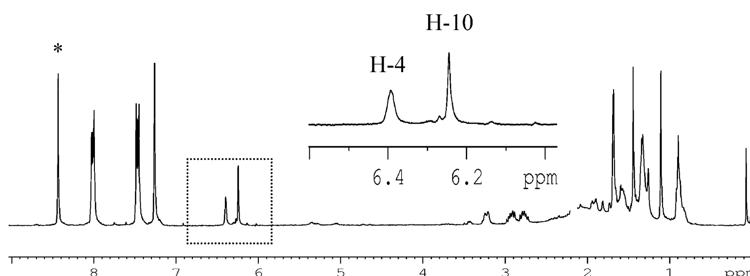

Fig. 3. ^{1}H-NMR Spectrum of a Drug Type Cannabis Extract together with 1 mg of Anthracene as Internal Standard

Part of the spectrum is enlarged to show the overlap of proton signals of THCA with signals of minor compounds. For quantitation the singlet of anthracene (*) and H-4 of THCA were used.

Table 4. Quantitation of the Amount of THCA in Four Different Cannabis Types (mg/g of Dry Weight Plant Material), Calculated from the Amount of THCA in the Extracts with NMR and GC

	Cultivar type	THCA by NMR (mg/g dry weight)	Used proton signal	THCA by GC (mg/g dry weight)
Extract 1	Drug	179 (±10)	H-4	198 (±3)
Extract 2	Drug	229 (±1)	H-4	234 (±14)
Extract 3	Intermediate	118 (±3)	H-4	103 (±6)
Extract 4	Fiber	Too low	H-4	0.88 (0.09)
		CBDA by NMR (mg/g dry weight)		CBDA by GC (mg/g dry weight)
Extract 4	Fiber	22.0 (±1.4)	H-4	21.4 (±1.9)

For the fiber type also CBDA was quantified. Each experiment was performed in triplicate.

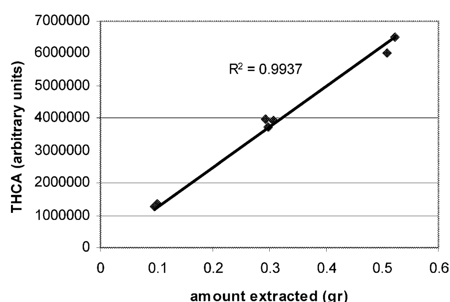

Fig. 4. Linearity between the Amount of Extracted Cannabis Plant Material and the Amount of THCA (in Arbitrary Units) after Quantification

lated samples or simple mixtures can easily and quickly be performed by quantitative ^{1}H-NMR. However, for the quantification in complex plant extracts, the preferred proton signal for quantification should be a singlet which shows a high linearity in the measured concentration range. Furthermore it should not overlap with a proton signal of another component of the extract. Because the composition of extracts can be variable, the most suitable proton signal should be selected after inspection of the total ^{1}H-NMR spectrum.

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