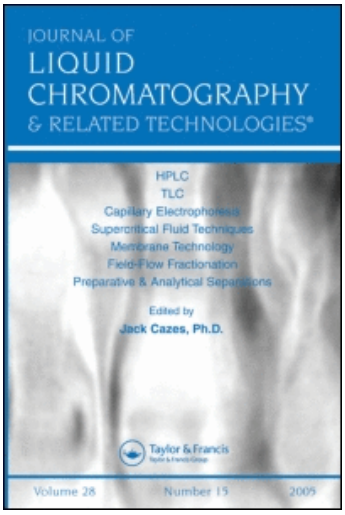

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ABSTRACT

A simple method is presented for the preparative isolation of seven major cannabinoids from *Cannabis sativa* plant material. Separation was performed by centrifugal partition chromatography (CPC), a technique that permits large-scale preparative isolations. Using only two different solvent systems, it was possible to obtain pure samples of the cannabinoids; (−)-Δ^9-(*trans*)-tetrahydrocannabinol (Δ^9-THC), cannabidiol (CBD), cannabinol (CBN), cannabigerol (CBG), (−)-Δ^9-(*trans*)-tetrahydrocannabinolic acid-A (THCA), cannabigerolic acid (CBGA), and cannabidiolic acid (CBDA). A drug-type and a fiber-type cannabis cultivar were used for the isolation. All isolates were shown to be more than

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90% pure by gas chromatography. This method makes acidic cannabinoids available on a large scale for biological testing. The method described in this report can also be used to isolate additional cannabinoids from cannabis plant material.

Key Words: *Cannabis sativa*; Cannabinoids; Isolation; Preparative scale; Centrifugal partition chromatography.

INTRODUCTION

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. Research on the cannabis plant and on the patients using cannabis products demands reference compounds in the form of purified cannabis constituents. Although more than 400 compounds have been identified in cannabis,[1] most studies focus on the effects of the cannabinoids, in particular, (−)-Δ^9-(*trans*)-tetrahydrocannabinol (Δ^9-THC). Most of the effects of cannabis have been attributed to Δ^9-THC, and synthetic Δ^9-THC (dronabinol, Marinol©) has been approved for some medical applications. However, in several medical studies, the effect of Δ^9-THC or dronabinol alone could not match the effect of a total cannabis preparation,[2] indicating there might be other active compounds present. More than 60 cannabinoids have been found in cannabis,[1] and occasionally new cannabinoids are still being discovered.[3] Only a few of the known cannabinoids have been studied in some detail, although many of these have been shown to possess some biological activity (reviewed by Ref.[4]).

Although it seems justified to investigate cannabinoids other than Δ^9-THC alone, the biggest obstacle is the availability of sufficient amounts of highly pure reference standards for calibration of analytical tools and for medical studies. Only a few of the naturally occurring cannabinoids are commercially available today: Δ^9-THC, Δ^8-THC, cannabidiol (CBD), and cannabinol (CBN). In fresh plant material of cannabis, most cannabinoids are present in the form carboxylic acid known as acidic cannabinoids.[5] The free phenolic forms of the cannabinoids are also known as neutral cannabinoids. Of the acidic cannabinoids, only (−)-Δ^9-(*trans*)-tetrahydrocannabinolic acid-A (THCA) has been studied biologically to some extent,[6] as far as we know. Although it is the most abundant cannabinoid found in drug-type cannabis, it is not yet commercially available. For THCA and other acidic cannabinoids, several isolation methods or synthetic routes have been described, but most of these methods were inefficient, time-consuming, or not suitable for preparative isolations.[7–11]

In this study, centrifugal partition chromatography (CPC) was tested for the large-scale isolation of cannabinoids. It is a countercurrent liquid–liquid partitioning chromatography technique in which the stationary phase is immobilized by centrifugal force, while the mobile phase is pumped through at high flow rates. During the separation, sample components are partitioned between the mobile and the stationary phases, and are separated on the basis of differences in their partition coefficients. The CPC offers particular advantages in the isolation of compounds; there is no irreversible retention, it can cover a broad scale of polarities, and it has a very high capacity because of the large volume of stationary phase involved in the separation process. The CPC can be used on a preparative scale with an injection size up to several grams. The method was first described by Murayama et al.[12] and the theoretical aspects were discussed by Foucault.[13] Another countercurrent chromatography technique, direct counter current chromatography (DCCC) was used for the first isolation of THCA as a complex with dimethylformamide.[14]

The isolated cannabinoids were analyzed for purity by GC and additional analysis was done by HPLC and thin layer chromatography (TLC). Purity of all isolates was more than 90%. The isolated cannabinoids are suitable as standards for quantification experiments or as reference compounds in biological assays. The use of different cannabis cultivars for the isolation of additional cannabinoids is discussed.

EXPERIMENTAL

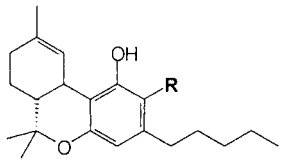
Standards and Solvents

A standard of Δ^8-tetrahydrocannabinol (Δ^8-THC) was obtained from Sigma (St. Louis, MO). Standards of CBD and CBN were a gift of the Dutch Forensic Institute (NFI, Rijswijk, The Netherlands). Reference compounds of cannabigerol (CBG), Δ^9-THC, cannabigerolic acid (CBGA), cannabidiolic acid (CBDA), and THCA were isolated previously in our laboratory by using preparative HPLC and identified as described below. Figure 1 shows the structures of these cannabinoids.

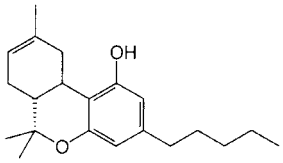
All organic solvents (analytical or HPLC reagent grade) were purchased from J. T. Baker (Deventer, The Netherlands).

Plant Material

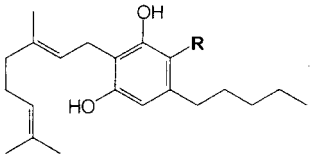
C. sativa L. plant material of the drug-type (cultivar SIMM02) was obtained from Stichting Institute for Medical Marijuana (SIMM) in Rotterdam,



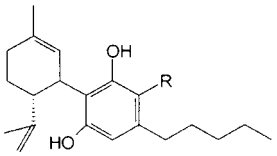
R = H: Δ^9-tetrahydrocannabinol (Δ^9-THC)
R = COOH: Δ^9-tetrahydrocannabinolic acid A (THCA)



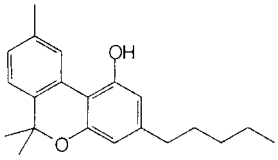
Δ^8-tetrahydrocannabinol (Δ^8-THC)



R = H: Cannabigerol (CBG)
R = COOH: Cannabigerolic acid (CBGA)



R = H: Cannabidiol (CBD)
R = COOH: Cannabidiolic acid (CBDA)



Cannabinol (CBN)

Figure 1. Structures of the cannabinoids.

The Netherlands. After harvest, the plant material was air-dried in the dark under constant temperature and humidity for 4 weeks. Fiber-type cannabis (cultivar Kompolti) was grown outdoors in the garden of our institute. Plant material was harvested in October 2002, and air-dried in the dark at ambient temperature for 4 weeks. No pesticides or other chemicals were applied to the plants.

For isolation of cannabinoids, only female flower tops were used. These were manicured to remove other plant parts like leaves and stems, and subsequently sealed under vacuum in plastic bags. The plant material was then stored at $-20^{\circ}C$ until used.

TLC

Samples were manually spotted on $10 \times 20\,cm^2$ reversed phase (C_{18}) silica gel plates F254 No. 105559 (Merck, Darmstadt, Germany), and developed in saturated normal chambers (saturation time 15 min). The eluent was methanol : 5% acetic acid, 19 : 1 (v/v). After development, visual inspection was done under UV 254 nm. General visualization of compounds was done by spraying with modified anisaldehyde–sulphuric acid spray reagent.[15] For selective visualization of cannabinoids, the TLC plate was sprayed with 0.5% fast blue B salt (Sigma) in water, followed by 0.1 M NaOH.[16] Reference standards were used for identification of chromatographic spots.

High-Performance Liquid Chromatography

The HPLC profiles were acquired on a Waters (Milford, MA) HPLC system consisting of a 626 pump, a 717 plus autosampler, and a 2996 diode-array detector (DAD), controlled by Waters Millennium 3.2 software. The profiles were recorded at 285 nm to keep a stable baseline during the gradient. Full spectra were recorded in the range of 200–400 nm. The analytical column was a Vydac (Hesperia, CA) C_{18}, type 218MS54 ($4.6 \times 250\,mm^2$, 5 μm), with a Waters Bondapak C_{18} ($2 \times 20\,mm^2$, 50 μm) guard column. The mobile phase consisted of a mixture of methanol–water containing 25 mM of formic acid in gradient mode; methanol : water in ratios from 65 : 35 to 100 : 0 over 25 min, then isocratic to 28 min. The column was re-equilibrated under initial conditions for 4 min. Flowrate was 1.5 mL/min and total runtime was 32 min. All determinations were carried out at ambient temperature.

Gas Chromatography (GC)-FID and Gas Chromatography–Mass Spectrometry (GC–MS)

The GC-FID profiles were generated 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), 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 temperatures were maintained at 280°C and 290°C, respectively. Nitrogen was used as the carrier gas at a pressure of 70 kPa. Air and hydrogen were used as detector gases. The injection split ratio was 1/50.

To obtain mass-spectral data of isolated compounds, gas chromatography–mass spectrometry (GC–MS) analyses were performed on a Varian (Bergen op Zoom, The Netherlands) 3800 gas chromatograph, coupled to a Varian Saturn 2000 mass spectrometer operating in the electron impact (EI) mode. The GC was fitted with a Varian VA5MS capillary column (30 m × 0.25 mm inner diameter) coated with DB1 at a film thickness of 0.25 μm. The oven temperature was programmed as described above. Helium was used as the carrier gas at a pressure of 65 kPa. The injection split ratio was 1/50. The system was controlled by Varian Saturn GC/MS workstation version 5.2 software. All GC–MS samples were analyzed without prior derivatization.

Extraction

Dried flower tops of SIMM02 (50 g) and Kompolti (100 g) were extracted three times by maceration with 1.25 L of *n*-hexane for several hours. Each extraction was started by 5 min of sonication. Finally, the three sequential extracts were combined and filtered over a glass-filter.

Separation of Acidic and Neutral Cannabinoids

A glass-filter (mesh size 2 mm) of about 5 cm in diameter and 7 cm in height was filled 2/3 with acid-washed sea-sand (Sigma) and topped with glass pearls (±1 mm diameter). Before use, the sand was sequentially washed with 200 mL of hexane, ethanol, and water. Cannabis hexane extract was concentrated to about 5 mL of hexane, placed dropwise on top of the sand filter, and evaporated by using a hot air blower. The sand filter

was then placed onto a suction Erlenmeyer, and acidic cannabinoids were eluted by washing the sand filter under vacuum with a 0.1 M NaOH solution. The elution was continued until the eluate turned from deep-orange to colorless. Neutral cannabinoids and other compounds were then eluted with ethanol (200 mL), followed by hexane (200 mL). Acidic cannabinoids were precipitated in the aqueous eluate by adding HCl until the pH reached 2, and then filtered through the sand filter. The precipitate that remained on top of the sand filter was finally eluted with ethanol (200 mL). Neutral and acidic cannabinoid fractions were both concentrated into a small volume by evaporation under reduced pressure, and analyzed by GC and HPLC.

CPC Apparatus

A Sanki (Kyoto, Japan) centrifugal partition chromatograph (type LLB-M), equipped with a 100 mL cartridge was used. It was connected to a Shimadzu LC-10ADvp pump, a Rheodyne (Cotati, CA) manual injector with a 5 mL loop, and a Pharmacia (Roosendaal, The Netherlands) FRAC-100 fraction collector. Pressure was limited to 100 bar.

Isolation of Acidic Cannabinoids

For the isolation of THCA and CBGA by CPC, the two-phase system hexane/methanol/water, 5 : 3 : 2 (v/v/v, solvent system 1) was used. The aqueous phase of the solvent system was acidified with 25 mM of formic acid. During the run the methanol/water ratio of the mobile phase was linearly increased from 3 : 2 to about 4.5 : 0.5, to speed up the elution of retained compounds. The upper hexane-rich layer was used as stationary phase, while the lower aqueous layer was used as mobile phase, so the CPC was operated in descending mode. The flowrate was set at 4 mL/min and rotation speed was 500 rpm. The volume of stationary phase was 70 mL under these conditions. The sample (2.5 g of the acidic cannabinoids fraction of SIMM02) was dissolved in the upper layer to a final volume of 5 mL for injection. Fraction size was 10 mL, and 50 fractions were collected before eluting the stationary phase. Each fraction was analyzed by TLC and selected fractions were further analyzed by HPLC. Fractions containing a high proportion ($>90\%$) of THCA or CBGA were combined and evaporated to dryness. The final sample was redissolved in 5 mL of ethanol and kept at -20°C for qualitative analysis.

CBDA was isolated from the acidic cannabinoid fraction of Kompolti extract as described above, using the same CPC two-phase system (solvent system 1).

Isolation of Neutral Cannabinoids

Slightly different methods were used to isolate the neutral cannabinoids Δ^9-THC, CBN, CBD, and CBG. For the isolation of CBN, previously isolated THCA (600 mg) was decarboxylated by heating. The sample was placed in a heat-resistant open glass vial and ethanol was evaporated by flushing with nitrogen gas. The vial was then placed into a preheated oven at 135°C overnight. The color of the extract darkened considerably during heating. Total decarboxylation of THCA was confirmed by HPLC after the heating period. The resulting mixture of CBN, Δ^8-THC, and Δ^9-THC was fractionated by CPC.

For the isolation of CBD, the acidic cannabinoids fraction of Kompolti extract was used. After evaporation of the solvent, 600 mg was heated at 180°C for 10 min in an open glass container. Total decarboxylation of CBDA was confirmed by HPLC after the heating period. The resulting mixture of CBD and other neutral cannabinoids was fractionated by CPC. Isolation of CBG was performed according to the same protocol using 1.0 g of the acidic cannabinoids fraction of SIMM02 extract.

Isolation of Δ^9-THC was done from the neutral cannabinoids fraction of SIMM02 extract. After evaporation of the solvent, 510 mg of the neutral cannabinoids fraction of SIMM02 extract was directly fractionated by CPC.

Fractionation of neutral cannabinoids was performed by CPC using the two-phase system hexane/acetone/acetonitrile, 5 : 2 : 3 (v/v/v, solvent system 2). The acetonitrile-rich lower phase was used as stationary phase and the hexane-rich upper phase was used as mobile phase, so the CPC was operated in ascending mode. The flowrate was set at 5 mL/min and rotation speed was 600 rpm. The volume of stationary phase was 65 mL under these conditions. The sample was dissolved to a final volume of 5 mL of upper phase for injection. Fraction size was 10 mL and 50 fractions were collected before eluting the stationary phase. Each fraction was analyzed by TLC and selected fractions were further analyzed by HPLC. Fractions containing a high proportion (>90%) of the desired compound were combined and subsequently evaporated under reduced pressure. The final sample was redissolved in 5 mL of ethanol and kept at −20°C for qualitative analysis.

Confirmation of Identity and Purity of Isolated Cannabinoids

The identity of isolated cannabinoids was confirmed by comparing retention times (HPLC and GC) and spectroscopical data (UV, MS) with reference compounds and literature data.[9–11,17,18] Purity of isolated cannabinoids was determined by GC-FID at a concentration of 1 mg/mL (by weight). To visualize compounds that cannot be detected by GC, samples were also

qualitatively analyzed by HPLC and TLC (visualization with anisaldehyde spray reagent).

RESULTS AND DISCUSSION

For the isolation of seven different cannabinoids, two different types of *C. sativa* L. were used. The structures of the isolated cannabinoids are shown in Fig. 1. Analysis of the hexane extracts by HPLC showed that the main compounds of SIMM02 were THCA and CBGA, while CBDA was the main compound for the Kompolti cultivar (Fig. 2). *n*-Hexane was chosen as the extraction solvent because it is easy to evaporate and it did not extract chlorophyll, which interferes with most of the chromatography techniques. The extraction yields for the drug-type cannabis SIMM02 and the fiber-type cannabis Kompolti, after hexane extraction of dried plant material, were 17% and 3%, respectively. Exploiting the solubility of acidic cannabinoids in water under basic conditions, the acidic cannabinoids could efficiently be separated from neutral cannabinoids and other plant compounds in the hexane extract, as shown by HPLC analysis (Fig. 3). The plant material of

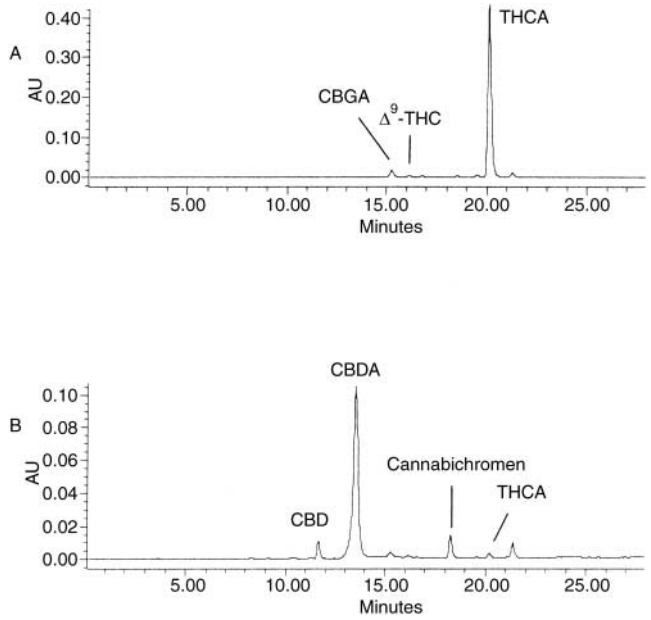


Figure 2. HPLC-chromatograms of the hexane extract of cannabis cultivars SIMM02 (A) and Kompolti (B). Main cannabinoid peaks are indicated.

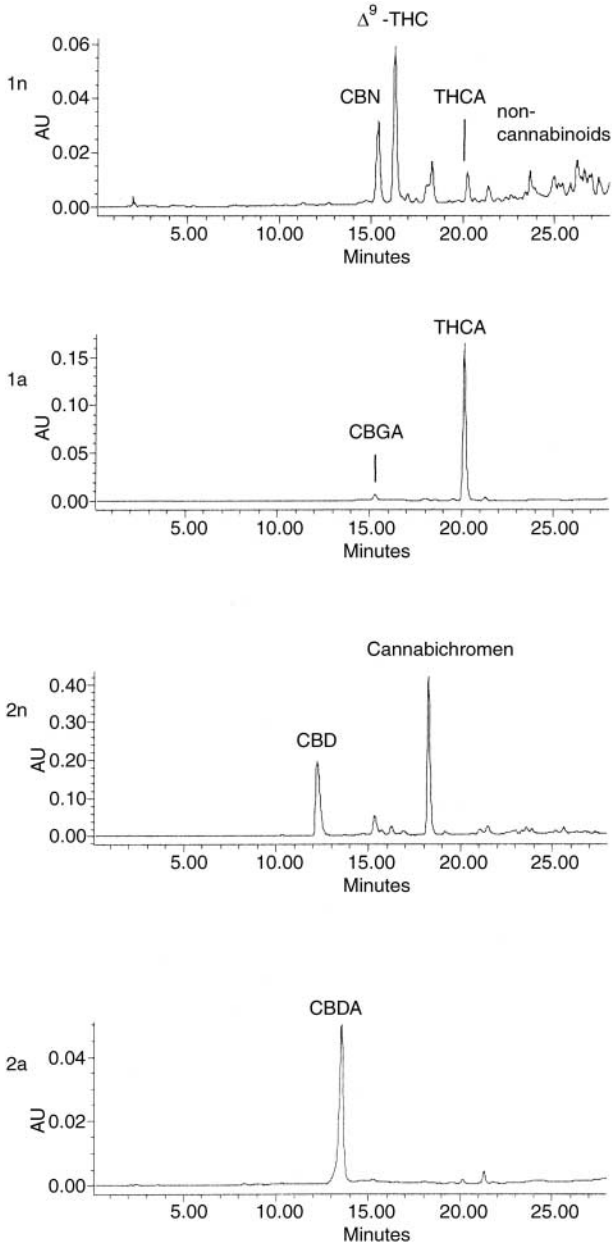


Figure 3. HPLC-chromatograms of neutral (n) and acidic (a) cannabinoid fraction after sand filter fractionation of hexane extracts. 1: SIMM02; 2: Kompolti.

Kompolti was considerably older than the SIMM02 plant material, resulting in a relatively larger amount of neutral cannabinoids present in the Kompolti extract.

The acidic cannabinoids fraction, resulting from the sand filter separation, was the preferred starting material for the isolation of cannabinoids, because it is clear of interfering compounds like lipids or terpenoids, and it contains the highest yield of extracted cannabinoids. About 2/3 of the weight of the total hexane extract was retrieved in the acidic cannabinoids fraction. A schematic overview of the isolation of the different cannabinoids can be seen in Fig. 4.

The CPC two-phase systems used in this study were selected based on their polarity, stability, and absence of (very) toxic solvents. The performance of selected CPC systems was evaluated according to Ingkaninan et al.[19] It should be noted that the retention volume in CPC is strongly dependent on the size of the injection sample, i.e., a higher amount results in a larger retention volume. Therefore, only the relative elution order of the cannabinoids in the used solvent systems is shown in Fig. 5. The separation of cannabinoids by CPC is based on polarity. Interestingly, the elution order of neutral cannabinoids in solvent system 2 is similar to the elution order in HPLC, but only

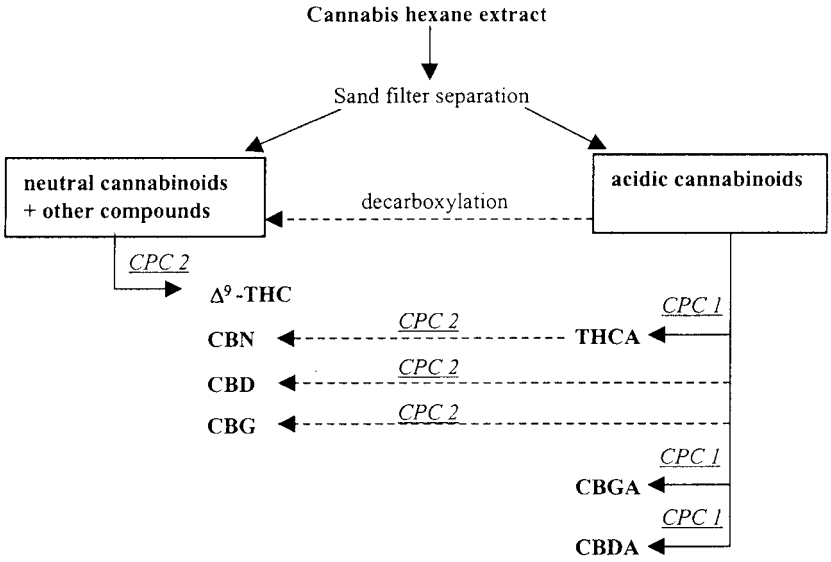


Figure 4. Scheme of the preparative scale isolation of cannabinoids from *C. sativa* hexane extract. CPC: separation by centrifugal partition chromatography using the indicated solvent system; CPC 1: hexane/methanol/water/formic acid; CPC 2: hexane/acetone/acetonitrile.

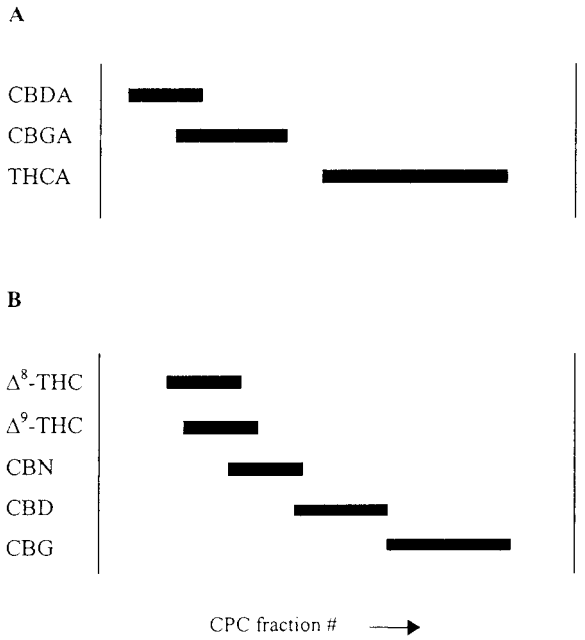


Figure 5. Schematic overview of the elution order of cannabinoids in CPC. (A) Acidic cannabinoids in CPC solvent system 1; (B) neutral cannabinoids in CPC solvent system 2.

in the reversed order, because in CPC the non-polar phase (hexane-rich) is used as mobile phase. Probably, the retention volume of any neutral cannabinoid could be predicted by comparison with the relative retention-time in the used HPLC system. This might facilitate the isolation of additional cannabinoids in the future. The amount of each cannabinoid isolated per gram of dry-weight plant material and the total amount isolated in this study are shown in Table 1.

Using CPC solvent system 1, the acidic cannabinoids THCA and CBGA could be well separated in a single experiment. This solvent system has the advantage that the concentration of methanol in the mobile phase can be increased during the run. In this way, the retention volume of the strongly retained THCA could be reduced from more than 800 mL (isocratic CPC, data not shown) to about 500 mL (gradient CPC). Because CBDA was the major compound in the Kompolti extract, it was fairly simple to isolate. Increasing the methanol concentration of the mobile phase could reduce the elution volume of CBDA considerably. It was reported that THCA can

Table 1. Identification, yields and purity of the isolated cannabinoids.

Isolated cannabinoid	Isolated in this study (mg)	Relative yield (g/100 g of dry weight plant material)	MW[a]	UV-match[b]	Purity by GC[c] (%)
Δ^9-THC	90.0	0.83	314	+	93.1
THCA	1590	8.34	358	+	94.0
CBD	232	0.46	314	+	92.7
CBDA	326	0.65	358	+	90.2
CBG	40.3	0.54	316	+	92.2
CBGA	37.9	0.46	360	+	92.9
CBN	99.4	1.38	310	+	95.0

[a]Molecular weight as determined by GC–MS.
[b]UV spectrum of the isolated cannabinoid matches with reference compound or literature data.
[c]Determined at a concentration of 1 mg/mL, 2 μL injected.

be stored at least for 1 year at −20°C,[10] so the isolated acidic cannabinoids were kept in ethanol at −20°C. Preliminary data (HPLC) shows all isolated cannabinoids to be stable for at least 6 months under these conditions (data not shown).

For the isolation of the neutral cannabinoids, different methods were used. Neutral cannabinoids can be obtained by heating acidic cannabinoids to produce their corresponding neutral analogs by decarboxylation. This method is commonly used for the analysis of the total cannabinoids content in cannabis samples by HPLC.[20] The heating temperature is about 180°C and samples are heated for several minutes.

To obtain the neutral cannabinoid Δ^9-THC, initially a small amount of THCA was decarboxylated at 180°C for 5 min in an oven. However, after analysis by GC, it was found that a considerable amount of Δ^8-THC had formed during the heating process. The structural isomers Δ^8- and Δ^9-THC could not be well separated by the CPC system used (data not shown). It was also noted that an increasing amount of CBN was formed during the heating period because of oxidation. Subsequently Δ^9-THC was isolated directly from the neutral cannabinoids fraction. Given the low abundance of neutral cannabinoids in the extracts, only a small amount of Δ^9-THC could be isolated. The degradation of THCA into CBN was exploited for the isolation of CBN, since the plant material used was naturally very low in CBN or CBNA content.

For the isolation of CBG, the acidic cannabinoids fraction of SIMM02 was heated, resulting in a mixture of several neutral cannabinoids. Because

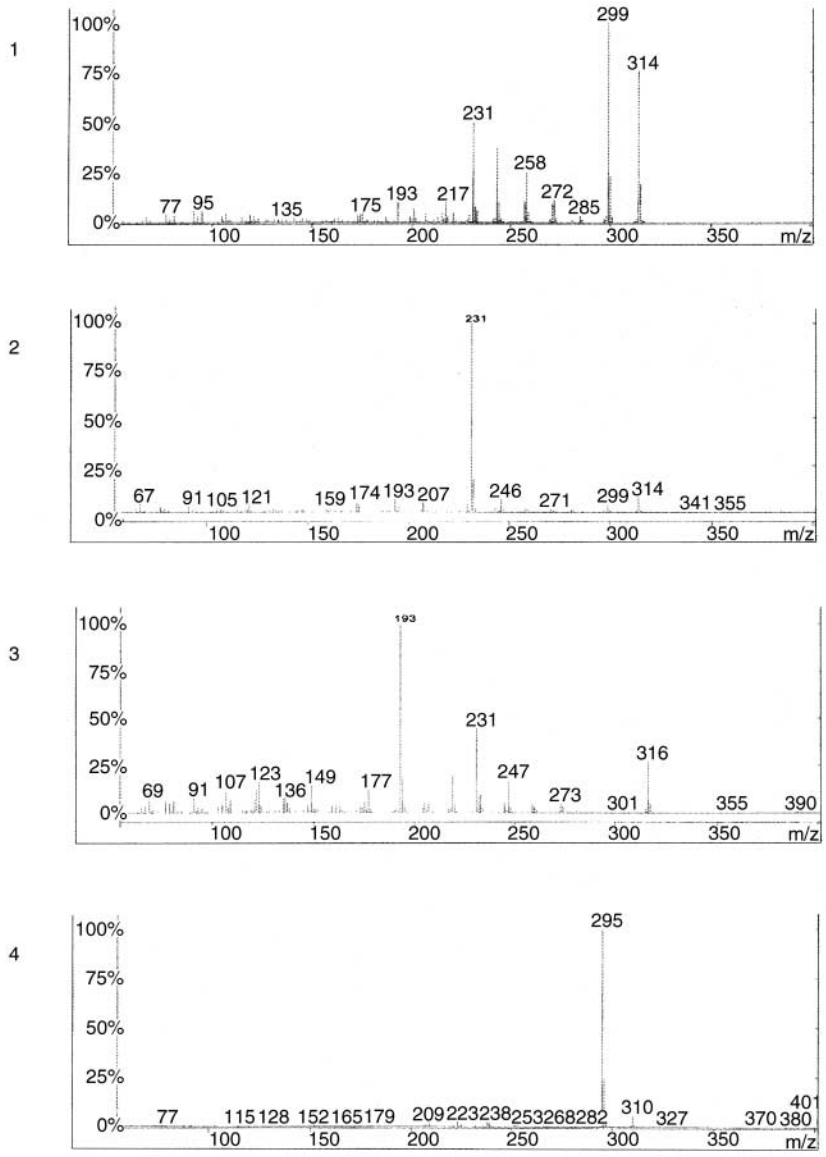


Figure 6. GC–MS spectra of the isolated cannabinoids. Only spectra of the neutral cannabinoids are shown. Acidic cannabinoids are decarboxylated in the GC-injector, and MS-spectra similar to the corresponding neutral cannabinoids are obtained. 1: Δ^9-THC and THCA; 2: CBD and CBDA; 3: CBG and CBGA; 4: CBN.

CBG is very well separated from the other neutral cannabinoids by CPC system 2 (see Fig. 5), CBG could be isolated directly from the mixture. Therefore, an amount of the acidic cannabinoids fraction was heated directly (without prior removal of THCA and other cannabinoids) and separated by solvent system 2. Because of its high abundance in Kompolti extract, CBD could be isolated in the same way.

All isolates could be positively identified by comparison with reference compounds and literature data. The GC–MS spectra of the isolated cannabinoids are shown in Fig. 6. The MS-spectra of acidic cannabinoids and their corresponding neutral cannabinoids are similar because of decarboxylation of acidic cannabinoids in the injector-part of the GC. The purity of isolated cannabinoids was determined by GC-FID (Fig. 7), and expressed as percentage of peak area compared to the total peak area in the chromatogram (Table 1). All

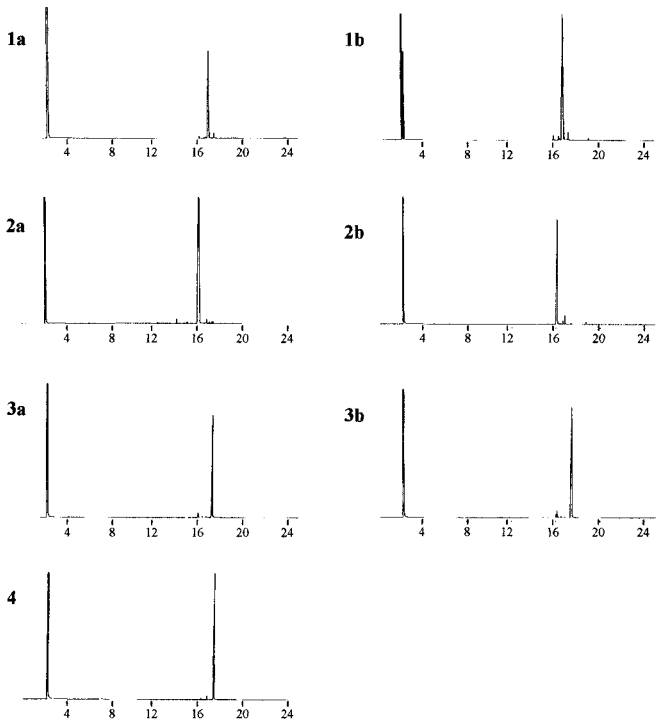


Figure 7. GC-chromatograms of the isolated cannabinoids. Purity is expressed as percentage of peak area compared to the total peak area in the chromatogram. 1a: Δ^9-THC; 1b: THCA; 2a: CBD; 2b: CBDA; 3a: CBG; 3b: CBGA; 4: CBN.

isolated cannabinoids could be well separated by the GC system used. No additional impurities could be detected in the samples after qualitative analysis by HPLC and TLC, as shown in Figs. 8 and 9, respectively.

CONCLUSION

Preparative isolation of seven different major cannabinoids could be achieved by using CPC as the single technique, with two different solvent systems. The quality of the isolated cannabinoids (>90% pure by GC-FID) is sufficient for many purposes. Additional HPLC and TLC data support the

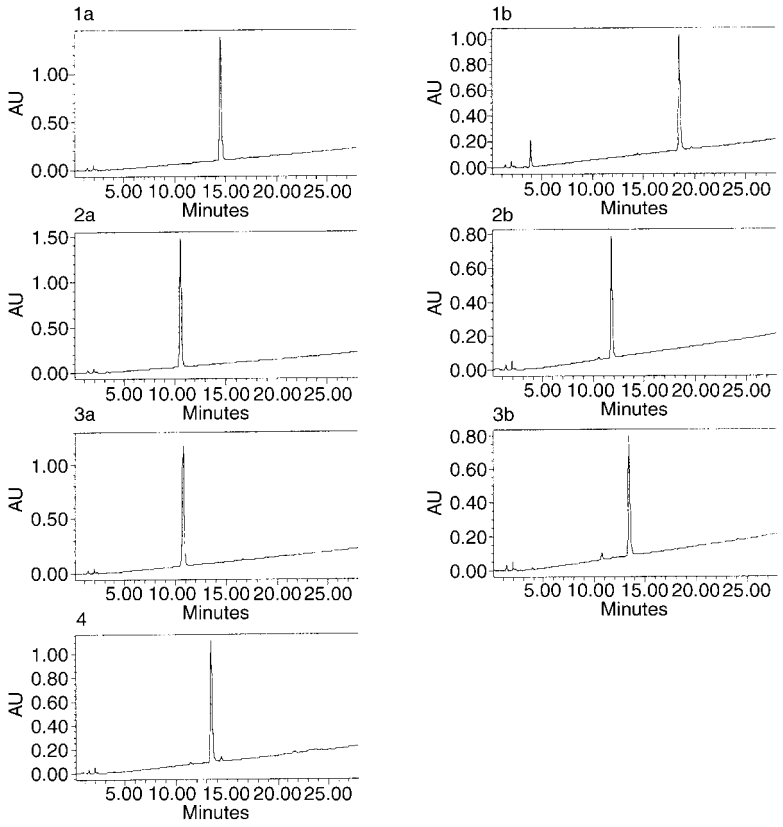


Figure 8. HPLC-chromatograms of the isolated cannabinoids. UV was monitored at maximum absorption between 200 and 400 nm. 1a: Δ^9-THC; 1b: THCA; 2a: CBD; 2b: CBDA; 3a: CBG; 3b: CBGA; 4: CBN.

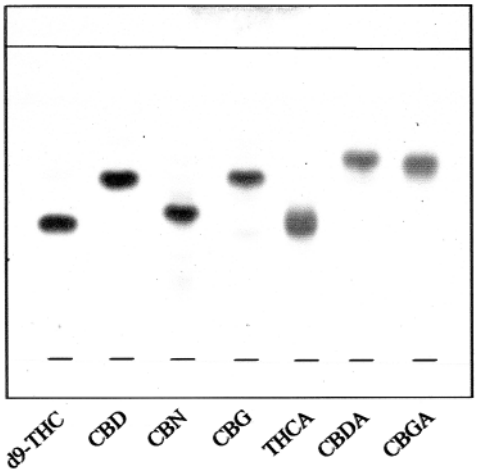


Figure 9. TLC of the isolated cannabinoids. Compounds were visualized by spraying the plates with modified anisaldehyde–sulphuric acid spray reagent to visualize cannabinoids as well as non-cannabinoids.

purity of the isolated compounds. This method can make the isolated cannabinoids available for biological testing on a large scale. Also, other cannabinoids can probably be isolated in this way by choosing a cannabis variety with a high content of the desired cannabinoid and, simultaneously, a low content of cannabinoids that are known to overlap with the desired cannabinoid in the CPC separation. The vast diversity in cannabis varieties should make it possible to find a suitable variety for most cannabinoid isolations. It should be possible to isolate several cannabinoids in just one chromatographic run, but the efficiency depends on peak overlap and contamination of the sample with non-cannabinoids. To ensure a high yield, the acidic cannabinoid fraction of a cannabis extract should be used.

Recently, we developed a 1H-NMR method for the quantification of cannabinoids, without the need for cannabinoid reference standards.[21] This makes it possible to easily obtain quantified solutions of the cannabinoids that were isolated here for which no commercial standard is available (i.e., CBG, CBGA, CBDA, and THCA).

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