

Determination of retention behavior of cannabis extract by column screening



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SUMMARY

The need of high purity cannabinoids is continuously growing. For this reason, it is of fundamental importance to find high selective and efficient methods for their purification. In this application, the separation of six cannabinoids contained in a crude CBD extract has been evaluated on six analytical columns with four different mobile phase compositions under normal phase conditions. The information obtained could be useful for the set-up of following purification steps.

INTRODUCTION

In the last years, the growing interest of cannabis and its related products for both medicinal and recreational use has led to the development of different techniques able to separate and purify cannabinoids. Among all phytocannabinoids, (-)-trans- Δ^9 -tetrahydrocannabinol (Δ^9 -THC) and (-)-trans-cannabidiol (CBD) are the most abundant and studied. Δ^9 -THC is known to have psychoactive effects, while CBD, non-psychoactive, is characterized by many therapeutic medicinal properties, such as anti-inflammatory and, antioxidant effects, to name but a few^{1,2,3,4}. CBD purification from hemp extracts could be challenging

due to large number of other components such as terpenes, waxes, cannabinoids, etc.. For some medical applications, the complete removal of Δ^9 -THC is required in order to obtain highly pure CBD. In this work, a set of six analytical columns with different modifications has been tested under normal phase (NP) conditions in order to have an overview of the retention behaviour of the main cannabinoids present in a crude CBD extract. This study has provided the best separation conditions for the crude mixture (in terms of mobile and stationary phases) in an analytical scale. A useful information for purification purposes.

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RESULTS

The CBD sample (original and decarboxylated) was analysed with the previously described reverse phase (RP) method⁵. The chromatograms are shown in **Fig. 1**.

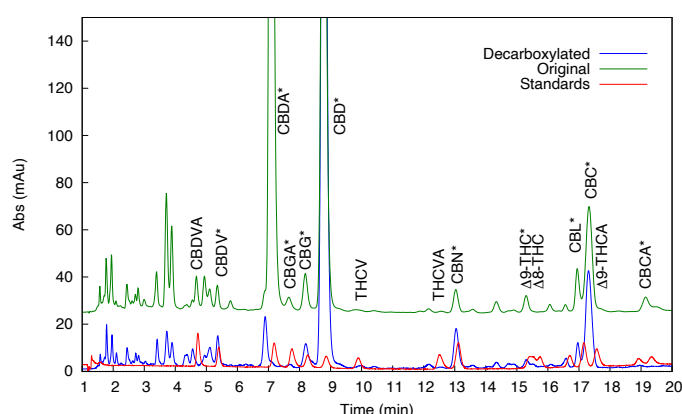


Fig. 1 Overlay chromatograms of the original CBD sample (green), the decarboxylated CBD sample (blue) and 16 cannabinoids standard mixture. Positive identified substance peaks are highlighted with * in the original sample. The concentration of the two samples is 12 mg/ml; 2 µl injection, 228 nm; C18P, 3 µm, 150 x 4.6 mm ID, analytical RP method. The slight shift in retention for the last eluting compounds present in the two samples with respect to the standard mixture depends on the different solvent in which they are dissolved (samples: heptane, standards: methanol).

Most impurities eluted within the first five minutes. An overlay of the chromatograms of the sample and the mixture of 16 cannabinoids, as well as a subsequent retention time comparison led to the identification of ten cannabinoids: CBDV, CBDA, CBGA, CBG, CBD, CBN, Δ⁹-THC, CBL, CBC and CBGA. A five-point calibration curve for the major cannabinoids (CBD, CBDA, Δ⁹-THC, THCA, CBL, CBC, CBN, CBG) was measured and the resulting coefficients were above $R > 0.999$ for all cannabinoids (**Fig. 2**).

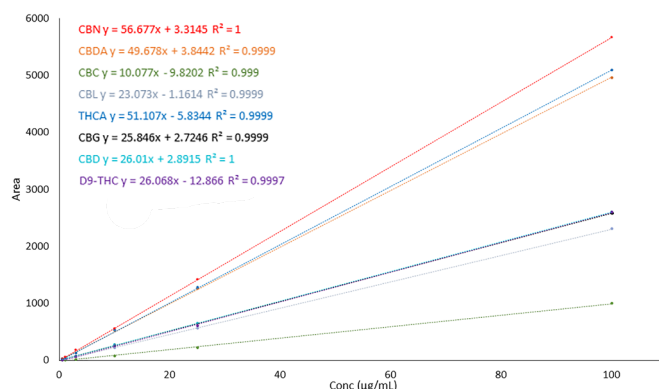


Fig. 2 Calibration curves and linearity for the cannabinoids CBD, CBG, CBDA, CBN, Δ⁹-THC, THCA, CBC and CBL for analytical RP method at 228 nm.

The calibration was used for the analysis of the two CBD samples. The area %, calculated at 228 nm, was used to quantify the purity of the identified cannabinoids. The results show that, in the original sample, CBDA and CBD are the most abundant cannabinoids with approximately 40.9% and 30.7% (area), while in the decarboxylated sample the CBD purity is 65% (area). The calculated cannabinoid contents (expressed as % w/w) in the two samples are shown in **Tab. 1**.

Tab. 1 Composition (% w/w) of the original and the decarboxylated samples and comparison with manufacturer results

	CBDA%	CBG%	CBD%	CBN%	THC%	CBL%	CBC%
Manufacturer	5.50	0.23	6.18	0.07	0.15	-	0.55
Our results	4.36	0.28	6.25	0.08	0.18	0.32	0.57
Decarboxylated	-	0.24	7.90	0.15	0.10	0.18	0.50

After the analysis of the two samples, a column and mobile phase screening has been performed in order to find the best separation conditions under normal phase elution mode. Six columns (NH₂, Diol, CN, C18, Phenyl and Si) and four mobile phase compositions (90:10 % v/v of heptane/isopropanol, heptane/ethanol, hexane/isopropanol and hexane/ethanol) have been applied.

RESULTS

C18 and Phenyl columns were not used for the complete study since no separation was observed under these experimental conditions (Tab. 2).

Tab. 2 Comparison between separation efficiency of the six columns: Bad separation (-), further optimisation needed (+-), good separation (+), very good separation (++), n/a: no separation.

90:10 %	NH ₂	CN	Diol	C18	Phenyl	Si
Heptane/IPA	+	+	+-	n/a	n/a	-
Heptane/EtOH	+-	++	++	n/a	n/a	+-
Hexane/IPA	++	+	+-	n/a	n/a	-
Hexane/EtOH	+-	++	++	n/a	n/a	+-

Fig. 3 and Fig. 4 show, as an example, the separation of the decarboxylated sample on NH₂, Diol, CN and Si columns with 90:10 % v/v heptane/isopropanol and 90:10 % v/v heptane/ethanol mobile phases. The identification of the peaks was obtained through the comparison of the chromatograms of the CBD sample and six cannabinoid standards, namely CBD, CBG, CBN, Δ^9 -THC, CBC and CBL. If all the six cannabinoids are considered, the best separation with heptane/isopropanol as mobile phase was achieved with CN and NH₂ columns (Fig. 4).

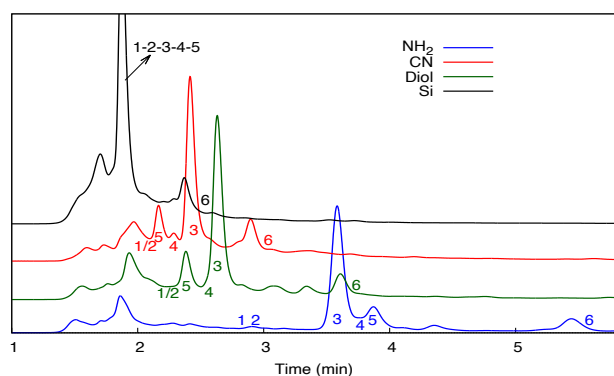


Fig. 3 Comparison between chromatograms of decarboxylated CBD sample obtained with NH₂ (blue), Diol (green), CN (red) and Si (black) columns under NP conditions. Mobile phase = 90:10 % v/v heptane/isopropanol, flow rate = 1 ml/min, injection volume = 3 μ l, injected concentration = 12 g/l. 1: CBL, 2: Δ^9 -THC, 3: CBD, 4: CBN, 5: CBC, 6: CBG.

Conversely, ethanol as polar modifier leads to better resolution for CN, Diol and Si columns, but worse for NH₂ column if compared to isopropanol (Fig. 4).

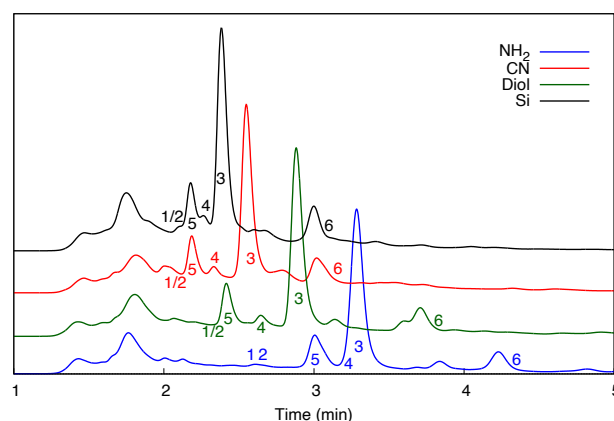


Fig. 4 Comparison between chromatograms of decarboxylated CBD sample obtained with NH₂ (blue), Diol (green), CN (red) and Si (black) columns under normal phase (NP) conditions. Mobile phase = 90:10 % v/v heptane/ethanol, flow rate = 1 ml/min, injection volume = 3 μ l, injected concentration = 12 g/l. 1: CBL, 2: Δ^9 -THC, 3: CBD, 4: CBN, 5: CBC, 6: CBG.

Nevertheless, independently from the composition of the mobile phase, all the columns considered, except for the Si column, are able to separate Δ^9 -THC and CBD easily. With all columns, hexane leads to a slightly higher resolution and slightly smaller retention if compared to heptane, as shown in Fig. 5 for NH₂ column.

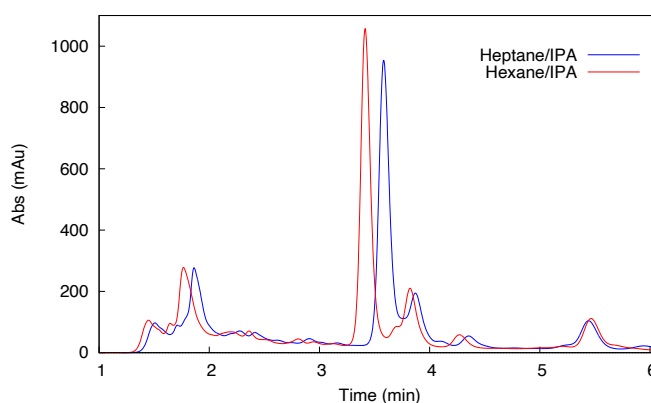


Fig. 5 Chromatograms obtained on NH₂ column with 90:10 % v/v heptane/isopropanol (blue) and hexane/isopropanol (red) mobile phases.

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However, heptane/isopropanol mixture was chosen as mobile phase for this separation problem. On the one hand, heptane shows lower toxicity and hazard if compared to hexane. On the other hand, isopropanol leads to a reversed elution order of CBD, CBN and CBC on the NH_2 column (peaks 3, 4 and 5) with respect to ethanol with clear advantages for purification purposes. After the screening, Si and Diol columns were excluded due to their poor resolution for target substances and

late eluting compounds (> 20 min) for the latter (data not shown), while CN and NH_2 columns were selected for further optimisation of the separation. In **Fig. 6** and **Fig. 7** chromatograms of the standards and the sample obtained with optimized mobile phase conditions are shown for NH_2 and CN columns, respectively. The composition of the mobile phase was 92:8 % v/v for NH_2 column and 95:5 % v/v for CN column.

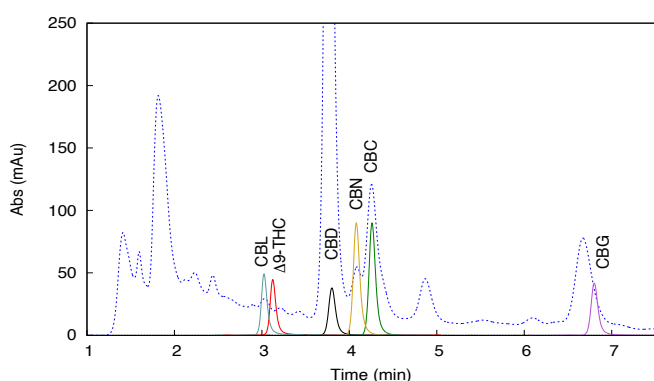


Fig. 6 Detailed view overlay of decarboxylated CBD sample and single standard chromatograms obtained with NH_2 150 x 4.0 mm, 5 μm column with 92:8 % v/v heptane/isopropanol mobile phase (NP method). CBD sample (dashed line) 12 mg/ml, standards (full line) 10 $\mu\text{g}/\text{ml}$.

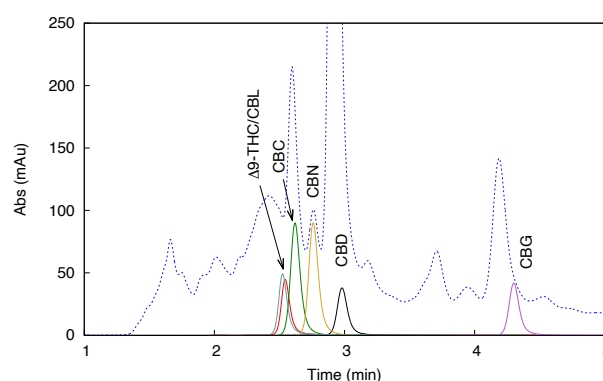


Fig. 7 Detailed view overlay of decarboxylated CBD sample and single standard chromatograms obtained with CN 150 x 4.0 mm, 5 μm column with 95:5 % v/v heptane/isopropanol mobile phase (NP method). CBD sample (dashed line) 12 mg/ml, standards (full line) 10 $\mu\text{g}/\text{ml}$.

SAMPLE PREPARATIONS

A CBD-rich extract, obtained with supercritical CO₂ extraction method, was kindly donated by MH Medical Hemp GmbH. The sample mainly contained CBDA and CBD, 4% and 6% (w/w), respectively (see **Tab. 1**). A sample decarboxylation was performed to convert acidic cannabinoids into neutral cannabinoids. Different reactors, temperatures and amounts of sample were tested. The decarboxylation kinetic showed, as expected, a high dependence on the heating temperature. On the one hand, low temperature (ex. 80 °C) leads to slower kinetics and smaller CBD degradation. On the other hand, too high temperature (ex. 150 °C) leads to fast decarboxylation but larger CBD degradation. Therefore, a temperature of 120 °C was chosen. The decarboxylation kinetic of CBDA measured in the oven at 120 °C with open reactors is shown in **Fig. 8** for different amounts of sample. For the analytical methods, a defined amount of sample was diluted in heptane and filtered through 0.2 µm PTFE syringe filter prior to injection. Cannabinoid calibration standards were prepared from 1 mg/ml cannabinoid standard solution (**Tab. 3**) and diluted with methanol to

indicated concentrations. The standards were prepared in two mix solution, mix 1 (CBD, CBDA) and mix 2 (Δ⁹-THC, THCA, CBL, CBC, CBN, CBG). A five-point calibration curve with 0.5 µg/ml, 3 µg/ml, 10 µg/ml, 25 µg/ml, and 100 µg/ml of each of the eight cannabinoids was established.

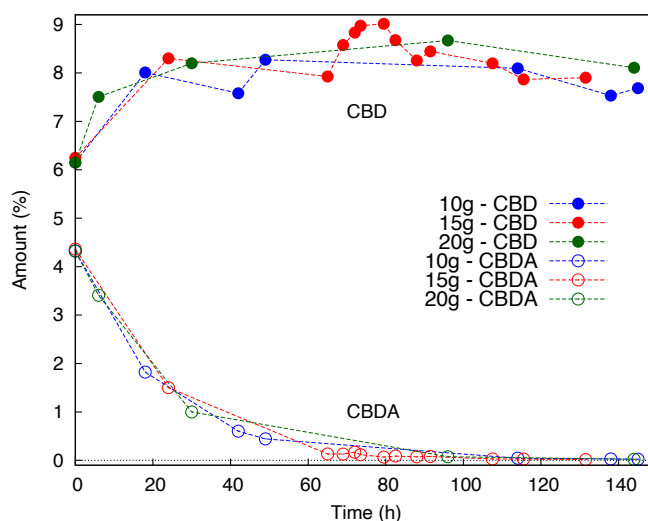


Fig. 8 Kinetic study obtained at 120 °C in open reactors of the amount (% w/w) of CBD (full points) and CBDA (empty points) as a function of time and the heated amount of sample (10 g: blue, 15 g: red and 20 g: green).

CONCLUSION

In this study the separation capacity of a set of six analytical columns, namely NH₂, CN, Diol, Si, C18 and Phenyl, has been evaluated on a variety of mobile phase compositions for a crude CBD extract under normal phase conditions. On the one hand, the results showed that CN, NH₂ and Diol columns were able to easily separate Δ⁹-THC from CBD under all the experimental conditions. On the other hand, it has been demonstrated that a mobile phase made of heptane/isopropanol can lead to a reversion of the elution order of CBD-CBN-CBC cannabinoids on the CN and NH₂ columns with clear advantages for purification purposes.

REFERENCES

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MATERIALS AND METHODS

Tab. 3 Material

Standard	CAS
Δ^9 -tetrahydrocannabinol	1972-08-3
Cannabidiol	13956-29-1
Cannabidiolic acid	1244 -58 -2
Cannabigerol	25654-31-3
Δ^9 -tetrahydrocannabinolic acid	23978-85-0
Cannabichromene	20675 - 51- 8
Cannabinol	521-35-7
Cannabicyclol	21366-63-2
CBD sample	Hemp extract batch 40, product sample

Tab. 4 Method parameters for analytical RP

Column Eurospher II	100-3 C18P 150 x 4.6 mm, 3 μ m
Column temperature	25 °C
Injection volume	2 μ l
Injection mode	Partial loop
Detection	UV 228 nm
Data rate	10 Hz

Tab. 5 Pump parameters for analytical RP

	Eluent A: H ₂ O + H ₃ PO ₄ (85%) pH 2.2	Eluent B: ACN	
Flow rate	1 ml/min		gradient
Pump program	25%	75%	0 - 7 min
	10%	90%	7 - 17 min
	10%	90%	17 - 19 min
	25%	75%	19 - 25 min

Tab. 6 System configuration for analytical RP

Instrument	Description	Article No.
Pump	AZURA P6.1L	APH35EA
Autosampler	AZURA AS 6.1L	AAA10AA
Detector	AZURA DAD 2.1L	ADC01
	Measuring cell: Pressure proof 10 mm, 10 μ l	AMC38
Thermostat	AZURA CT 2.1	ATC00
Column	Eurospher II 100-3 C18P	15EE182E2G
Software	ClarityChrom 8.2.3	A1670

Tab. 7 Method parameters for analytical NP

Column Eurospher II	100-5 150 x 4.6 mm, 5 μ m
Column temperature	25 °C
Injection volume	3 μ l
Injection mode	Partial loop
Detection	UV 228 nm
Data rate	10 Hz

Tab. 8 Pump parameters for analytical NP

	Eluent A: Heptane - Hexane	Eluent B: Ethanol - Isopropanol
Flow rate	1 ml/min	isocratic
Pump program	90%	10%

Tab. 9 System configuration for analytical NP

Instrument	Description	Article No.
Pump	AZURA P6.1L	APH35ED
Autosampler	AZURA AS 6.1L	AAA10AA
Detector	AZURA DAD 2.1L	ADC01
	Measuring cell: Pressure proof 10 mm, 10 μ l	AMC38
Thermostat	AZURA CT 2.1	ATC00
Multi-position valve	VU 4.1L	AVS36AE
Column	Eurospher II 100-5 CN	15WE200E2J
	Eurospher II 100-5 Phenyl	15WE050E2J
	Eurospher II 100-5 Diol	15WE410E2J
	Eurospher II 100-5 Si	15WE000E2J
	Eurospher II 100-5 NH ₂	15WE190E2J
	Eurospher II 100-5 C18	15WE181E2J
Software	ClarityChrom 8.2.3	A1670