(C)an(n)alyze: determination of 16 cannabinoids inside flowers, oils and seeds

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SUMMARY

Research within cannabinoids for pharmaceutical purposes increases, likewise does the number of substances to be tested. Therefore, compared to the German pharmacopoeia (Deutsches Arzneibuch; DAB), an increase of 10 cannabinoids was performed for qualification and quantification with this work. The aim of the method development was to decrease the runtime and optimize the gradient program in comparison to the DAB method[1]. To ensure accuracy of the method, a validation was performed according to ICH Guidelines Q2 R1[2]. Parameters for the validation were selectivity, linearity, repeatability and the recovery rate. The given specification was derived from the Association of Official Analytical Chemists (AOAC) with the given standard method performance requirements for cannabis flowers and oils[3].

INTRODUCTION

Cannabis sativa L. is one of the oldest agricultural and medicinal plants, which produces a variety of compounds such as terpenoids, flavonoids and cannabinoids[4]. The interaction of cannabinoids with the body's own cannabinoid receptors, which occur in a variety of brain cells for coordination, memory processing and spatial orientation, enables new pharmacological and psychological treatment options[5]. Probably the most psychoactive cannabinoid of the 4 different isomers of Δ[9]-THC is the (-)-Δ[9]-*trans*-tetrahydrocannabinol, also known as dronabinol **(Fig. 1)**. In Germany Δ[9]-THC is controlled by the narcotics law (Betäubungsmittelschutzgesetz; BtMG) due to its psychoactive properties. Since March 2017 the regulations changed by the amendment of article 1 BtMG. The amendment of annexes II and III of the BtMG now allows cannabis such as marijuana plants and plant parts to be marketed and prescribed. Thereby, cannabis was authorised for medical purposes as ready-to-use medicinal products[6]. Production of cannabis products must be conducted and monitored in accordance with good manufacturing practice guidelines (GMP) to guarantee accurate labelling of medicinal products, food and cosmetics.
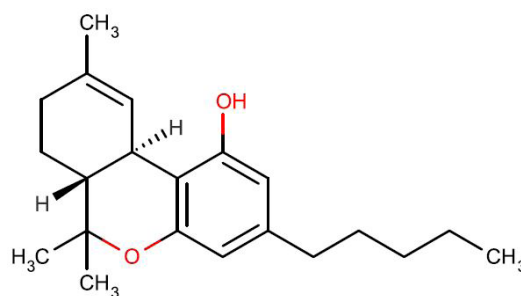

Fig. 1 Structure of (-)-Δ[9]-*trans*-tetrahydrocannabinol.

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INTRODUCTION

Therefore, the demand for standardized methods for the quantitative and qualitative determination of the ingredients especially the cannabinoids is increasing[7]. In this work an HPLC method development for the qualification and quantification of 16 different cannabinoids inside the matrices flowers, oils and seeds was carried out with the KNAUER AZURA® HPLC system. In order to check the performance of the method, a validation according to the ICH Guidelines Q2 R1 was carried out[2]. The tested cannabinoids are described in **Tab. 1**.

RESULTS

As shown in previous work, the column Eurospher II C18P 100-3, 100 x 4.6 mm was determined as the most robust one while separating 6 different cannabinoids[7]. Therefore, the development was carried out with a C18P phase. The final method was selected to provide the best compromise of a simple method with short runtime and high resolution **(Tab. 5)**. During the method development, the aqueous eluent was acidified once with phosphoric acid and once with formic acid to pH 2.2 with the result of no difference in elution behavior. In all following chromatograms 2 different absorption lines were monitored. While the blue line is detected at 228 nm, the red one is detected at 306 nm. Due to the various absorption behavior of each cannabinoid, a differentiation between the neutral and acid forms can be concluded. The resulting chromatogram with a 5 µg/mL standard mix is shown in **Fig. 2**.
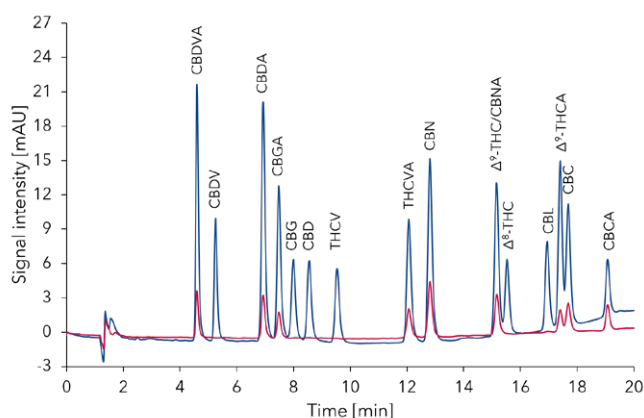
To confirm the selectivity within the given matrices flower, oil and hemp seeds, 3 different samples were measured during the method development and before validation. The chromatograms are shown in **Fig. 3** to **Fig. 5**.
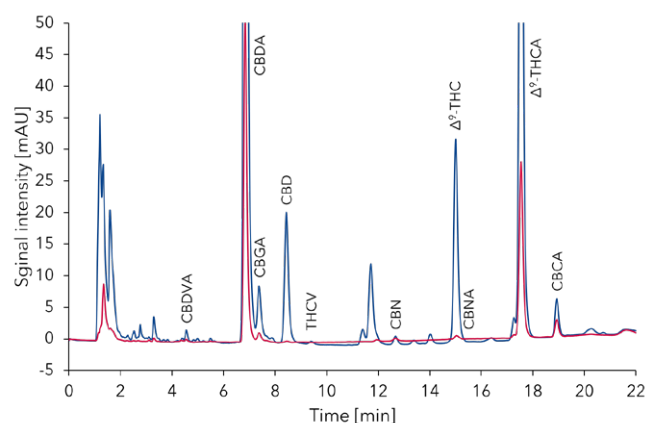

Fig. 3 Bediol sample preparation according to Mudge et al. 2017, 0.84 mg flower/mL methanol (blue: 228 nm; red: 306 nm)[9].
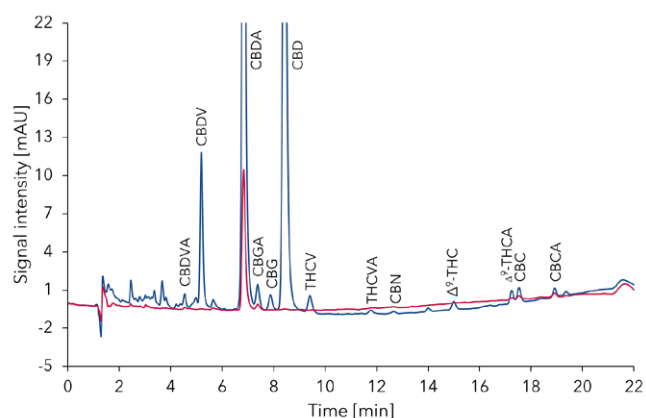

Fig. 2 Reference standard mixture of 16 cannabinoids with a concentration of 5.00 µg/mL (blue: 228 nm; red: 306 nm).

Fig. 4 CBD oil sample preparation according to Mudge et al. 2017, 0.62 mg oil/mL methanol (blue: 228 nm; red: 306 nm)[9].
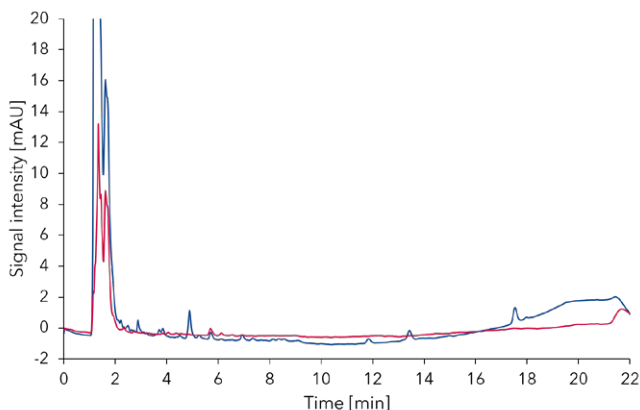
RESULTS

Up to 11 cannabinoids were found in the cannabis sample bediol and 6 of them were within the calibration range of 0.15 - 75.00 µg/mL. 8 out of 13 cannabinoids could be quantified within the oil matrix. As previously suspected the matrix „hemp seed" showed no cannabinoids within the calibration range. In all cases the quantification of Δ^9-THC is only possible in the absence of CBNA. This is confirmed by the absorption at 306 nm. Since CBNA is a degradation product of Δ^9-THCA, it only occurs in products that have been stored for a longer time. During this validation CBNA was only found in a trace amount in bediol. This amount correlates with the high amount of Δ^9-THCA inside the bediol sample as well as the long storage since the harvest in April 2018. The following results were produced within the validation. During the calibration it was figured out that linearity was given between the concentration of 0.15 - 75.00 µg/mL. All calibration curves in the range of 0.15 - 75.00 µg/mL were passing the variance homogeneity as well as the linearity after the mathematical equation of Mandel. The resulting coefficients were all

above R > 0.999 for every cannabinoid. The calibration curves are shown respectively for the cannabinoids CBD, CBDA, CBN, Δ^9-THC and Δ^9-THCA in **Fig. 6**.
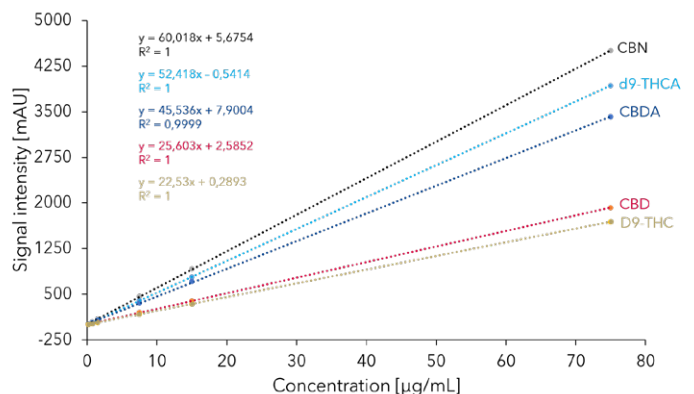

Fig. 6 Calibration curves and linearity for the cannabinoids CBD, CBDA, CBN, Δ^9-THC and Δ^9-THCA at 228 and 306 nm.

The repeatability was measured with a six-time repetition of different concentrations inside the CBD oil. This was made to ensure reproducibility over the entire calibration range. The area ratios of the different dilutions range from 0.6 - 5200 mAU*s. The results of the repeatability shown in **Tab. 2** are all below 1 % relative standard deviation (RSD) and thereby within the lowest limit of RSD < 2 % by the AOAC[3]. Even CBN with an area of 0.6 mAU shows an RSD of under 2 % and is therefore within the limits. CBL, CBNA and Δ^8-THC could not be detected inside the CBD oil, so the verification was made over a six-time repetition within the recovery rate. The recovery rate was evaluated within 2 different concentration levels close to the lowest calibration value. A blank sample was spiked with 50 µL/100 µL of each cannabinoid standard (0.1 mg/mL) filled up with 25 mL solvent and was prepared 3 times according to Mudge et al. 2017[9]. For both quantified levels (0.20 and 0.40 µg/mL) the recovery rates were in between the specification range of the AOAC from 95 - 105 %. The results are shown in **Tab. 3**.

SAMPLE PREPARATIONS

5 g of bediol were grinded with a flower grinder. The hemp seed was grinded with a kitchen mixer, after a mechanical pretreatment to open the seeds. The difference between grinded and original bediol and hemp seeds is shown in **Fig. 7**. The sample preparation was performed according to Mudge et al. 2017, where 200 mg flowers/seeds were extracted with 25 mL 80 % methanol or 50 mg oil were extracted with 10 mL methanol for 15 min inside an ultra-sonic bath, with every 5 min shaking onto a vortex device[9]. After filtrating over a 0.20 μm PTFE syringe filter the solution was measured with the HPLC system.

Fig. 7 Bediol sample: original (A) and grinded (B). Hemp seeds: orginial (C) and grinded (D).

CONCLUSION

Compared to the given pharmacopoeia from 2017 a 20 min shorter method with a less acidic eluent was developed[1]. The separation of all 16 cannabinoids was achieved with a sufficient resolution in both, the standard mixture and the 3 different sample matrices. The developed method is suitable for the qualification and quantification of 15 cannabinoids in the absence of CBNA. The validation was performed according to the given ICH Q2 R1 guideline with standard method performance requirements of the AOAC. In accordance with the prescribed laws for hemp cultivation, the lowest calibration point of 0.15 μg/mL (0.02 %) falls below the maximum specification content of 0.2 % Δ^9-THC by one order of magnitude. Additionally, in all cases the correlation coefficient of the linearity was over R > 0.999. The recovery rate and the repeatability were inside the performance requirements of the AOAC.

KNAUER does not endorse the use of its products in connection with the illegal use, cultivation or trade of cannabis products. KNAUER does not endorse the illicit use of marijuana, we merely provide an overview of the methods and systems of cannabis analysis.

MATERIALS AND METHODS

Tab. 1 Standard solutions and samples

Analyte	CAS	Purity / Concentration
Cannabichromene (CBC)	20675-51-8	0,972 mg/mL (MeOH)
Cannabichromene acid (CBCA)	185505-15-1	0,999 mg/mL (ACN)
Cannabidivarin (CBDV)	24274-48-4	0,986 mg/mL (MeOH)
Cannabidivarinic acid (CBDVA)	31932-13-5	1,009 mg/mL (ACN)
Cannabidiol (CBD)	13956-29-1	1,000 mg/mL (MeOH)
Cannabidiolic acid (CBDA)	1244-58-2	1,000 mg/mL (ACN)
Cannabigerol (CBG)	25654-31-3	0,995 mg/mL (MeOH)
Cannabigerolic acid (CBGA)	25555-57-1	0,991 mg/mL (ACN)
Cannabinol (CBN)	521-35-7	1,000 mg/mL (MeOH)
Cannabinol acid (CBNA)	2808-39-1	0,986 mg/mL (ACN)
Cannabicyclol (CBL)	21366-63-2	0,992 mg/mL (ACN)
Δ^8-Tetrahydrocannabinol (Δ^8-THC)	5957-75-5	1,000 mg/mL (MeOH)
Δ^9-Tetrahydrocannabinol (Δ^9-THC)	1972-08-3	1,001 mg/mL (MeOH)
Δ^9-Tetrahydrocannabinolic acid (Δ^9-THCA)	23978-85-0	1,000 mg/mL (ACN)
Δ^9-Tetrahydrocannabivarin (THCV)	28172-17-0	0,991 mg/mL (MeOH)
Δ^9-Tetrahydrocannabivarinic acid (THCVA)	39986-26-0	1,015 mg/mL (ACN)

Solvent	CAS	Purity / Concentration
Acetonitril	75-05-8	Gradient grade
Formic acid	64-18-6	99 %
H_3PO_4	7664-38-2	AnalaR 85 % NORMAPUR
Methanol	67-56-1	Gradient grade

Sample	Manufacturer	Batch
CBD oil (hemp seed)	Pharma Hemp (Slovenia)	DR05018142B
Hemp seeds	VEGJi (Germany)	
Bediol flower	Bedrocan (Netherlands)	18D13FB19A23

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Tab. 2 Repeatability of all cannabinoids

Analyte	Repeatability [%]				
	6.2 mg oil/mL solvent	0.62 mg oil/mL solvent	0.31 mg oil/mL solvent	Recovery rate 0.40 µg/mL	Recovery rate 0.20 µg/mL
CBC	0.71	0.79	0.65	-	-
CBCA	0.65	0.88	0.89	-	-
CBD	0.19	0.21	0.46	-	-
CBDA	0.18	0.16	0.34	-	-
CBDV	0.21	0.40	0.61	-	-
CBDVA	0.54	0.65	0.66	-	-
CBG	0.37	0.77	0.89	-	-
CBGA	0.30	0.65	0.83	-	-
CBL	-	-	-	0.95	0.82
CBN	0.41	1.49	1.25	-	-
CBNA	-	-	-	0.88	0.84
Δ^8-THC	-	-	-	0.95	0.85
Δ^9-THC	0.56	0.70	0.90	-	-
Δ^9-THCA	0.55	0.51	0.58	-	-
Δ^9-THCV	0.21	0.87	0.68	-	-
Δ^9-THCVA	0.44	0.66	0.86	-	-

Tab. 3 Recovery rate of all cannabinoids

Analyte	Recovery rate [%]	
	0.20 µg/mL	0.40 µg/mL
CBC	96.36	98.45
CBCA	95.07	103.43
CBD	102.19	98.80
CBDA	98.33	100.65
CBDV	100.11	102.49
CBDVA	100.65	100.70
CBG	103.55	95.66
CBGA	98.54	101.46
CBL	101.27	104.70
CBN	102.83	104.97
Δ^8-THC	101.94	95.02
Δ^9-THCA	96.50	98.33
Δ^9-THCV	100.75	97.34
Δ^9-THCVA	102.60	95.68

MATERIALS AND METHODS

Tab. 4 Method

Column temperature	25 °C
Injection volume	10 µL
Injection mode	Partial loop
Detection	UV 228 nm, 306 nm
Data rate	10 Hz

Tab. 5 Gradient

Eluent (A)	Water, HPLC grade, pH 2.2 with 85 % H_3PO_4		
Eluent (B)	Acetonitrile, gradient grade		
Flow rate	1.0 mL/min		
	Time [min]	**(A) %**	**(B) %**
Pump program	0	25	75
	7	25	75
	17	10	90
	19	10	90
	20	25	75
	22	25	75

Tab. 6 System configuration

Instrument	Description	Article No.
Pump	AZURA P 6.1 L LPG	APH34EA
DAD detector	AZURA MWD 2.1 L	ADB01
Flow cell	10 mm, 10 µL, PressureProof	AMC38
Autosampler	AZURA AS 6.1 L	AAA00AA
Column thermostat	AZURA CT 2.1	A05852
Column	Eurospher II 100-3 C18P, 150 x 4.6 mm	15VE182E2G
Software	ClarityChrom 8.1	A1670
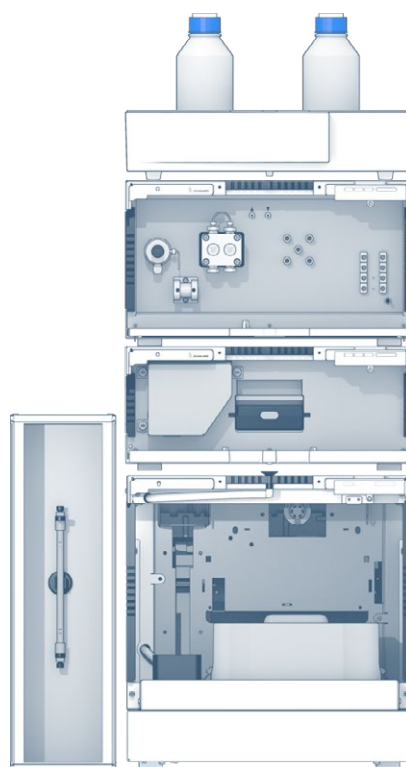
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RELATED KNAUER APPLICATIONS

VPH0069 – Analyzing cannabis flowers according to the German Pharmacopeia - monograph 2017

VPH0072 – Analyzing cannabis flowers according to the German Pharmacopeia - monograph 2018