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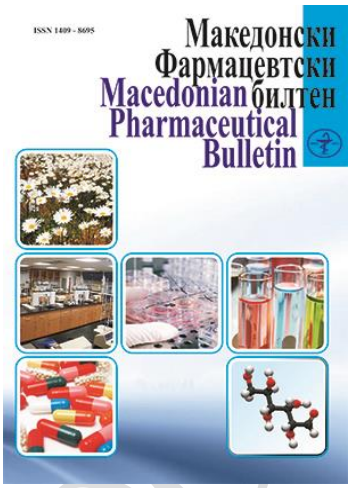

Title: DAB method transfer for potency of cannabinoids in dry cannabis flower

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DAB method transfer for potency of cannabinoids in dry cannabis flower

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Abstract

Cannabis is considered as heterogeneous matrix that contains complex profile of secondary metabolites, where more than 100 are classified as phytocannabinoids, present with an uneven distribution. In order to ensure its quality, application of suitable sample preparation techniques as well as time- and cost-efficient analytical methods is required. The DAB method depicts the obligatory procedure for potency testing of cannabinoids in cannabis flower in the EU. In this study we have performed method transfer in order to confirm the applicability of the method in determination of varying quantities of cannabioniods in different cannabis strains, focusing on samples with low content of CBD or THC or samples where one of these two cannabiniods is not detected. The HPLC-DAD method was validated and used for routine control of the content and consistency of medical cannabis. Limits of detection, limits of quantitation, accuracy, precision, and intermediate precision were found to be highly satisfactory.

Keywords: HPLC assay, CBD, THC, CBN, CBDA, THCA, method validation, DAB, quality control

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Introduction

Cannabis sativa L. from the family Cannabaceae, although is widely cultivated, trafficked, and consumed, yet can be considered as the most controversial plant in the world (Stefkov et al., 2022). A wide variety of chemical constituents, i.e., more than 750 compounds, have been identified in cannabis plant, among them more than 100 are classified as phytocannabinoids. Cannabidiol (CBD) and tetrahydrocannabinol (Δ9-THC) - wich posses psychoactive properties, are the most prominent and predominant constituents in cannabis flower (Pourseyed Lazarjani et al., 2020; Stefkov et al., 2022). Cultivation and production of medical cannabis is an emerging industry globally, and Republic of North Macedonia is the only country in the region that has legalised growth of cannabis for medical purposes (Stoilkovska Gjorgievska et al., 2023). In the past years several Pharmacopoeias, including the German pharmacopoeia (DAB), Swiss pharmacopoeia (Ph.Helv.), and the American Herbal Pharmacopoeia (AHP) have published monographs for "Cannabis inflorescence" as herbal substance obtained from the female plants of *C. sativa* L. According to DAB, the content of phytocannabinoids (presented as total Δ9-THC and CBD) specified on the label, should be within the limits of 90.0-110.0% (DAB, 2018). On the other hand according to AHP, these limit are in the range from 80 to 120% (Sarma et al., 2020). Authorities have classified generally three chemotypes of *C. sativa*, depending on the content of Δ9-THC and CBD: CBD-predominant type, also known as fiber type or "hemp" type (CBD/Δ9-THC = 15.0-25.0); intermediate chemotype (CBD/Δ9-THC = 0.5-3.0) and Δ9-THC-predominant type or drug-type (CBD/Δ9-THC = 0.00-0.005), due to legal issues (Stefkov et al., 2022).

In the past years, testing of cannabis has been sporadic and there is no universally agreed standard to which manufacturers or testing laboratories must comply. However, recently quality control of medicinal cannabis include quantification of cannabinoids, microbiological tests and mycotoxin monitoring, residual solvents determination, determination of pesticides, foreign matter, content of water, terpenes and others parametars relevant for reflection of the quality of the product (Mandrioli et al., 2019).

There are significant number of analytical methods to determine the phytocannabinoids potency in the flowers, described in the literature, however many of these do not provide sufficient validation data to establish the method performance. Therefore, users transfering the method for routine use, necessaraly need to perform rigorous validation procedures, in order to obtain data on

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method performance and to ensure the validity of analytical results (De Backer et al., 2009). In the German Pharmacopoeia 2020 (DAB, 2020) the Federal Institute for Drugs and Medical Devices (BfArM) published a revised monograph for cannabis flower, where this method depicts the obligatory procedure for potency testing of cannabinoids in cannabis flower in the EU. Until now, there is no equivalent official monograph in the European Pharmacopeia. According to ISO/IEC 17025:2018, the well-recognised test methods (compendial methods) which are validated in accordance to the accepted scientific practice and recommendations on analytical validation, when used in routine analysis does not undergo validation during their transfer, as long as system suitability criteria are fulfilled (ISO 17025, 2019). But, due to the lack of literatute data for detection limit of (DL) and quantifiqation limit (QL), when transferring the DAB method for determination of cannabiniods in the laboratory for routine analysis of cannabis flower, it is necessary to demonstrate the suitability of the method by performing formal validation procedures. Therefore, the aim of our study was to perform method transfer, in order to confirm the applicability of the method in determination of variyng quantities of cannabioniods in different chemotypes of cannabis, especially for the samples with low content of CBD or THC or samples where one of these two cannabiniods is not detected.

Materials and methods

Samples, standards, solvents and reagents

Dried cannabis flowers from THC reach strain (Meringue) were purchased from local licensed producer and was used for sample preparation and testing. Cannabidiol, Certified Reference Materials (CRMs) solution with concentration of 1 mg/mL in methanol (purity 99.42%), cannabinol, CRMs solution with concentration of 1 mg/mL in methanol (purity 96.32%), (-)-△9-tetrahydrocannabinol, CRMs solution with concentration of 1 mg/mL in methanol (purity 96.21%), △9-tetrahydrocannabinolic acid A, CRMs solution with concentration of 1 mg/mL in acetonitrile (purity 98.64%) and cannabidiolic acid, CRMs solution with concentration of 1 mg/mL in acetonitrile (purity 99.45%) were purchased from Cayman Chemical (USA). 85% *o*-phosphoric acid, reagent grade and acetonitrile HPLC grade were purchased from Carlo Erba. Ethanol 96%, Ph.Eur. grade was purchased from Alkaloid AD Skopje.

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HPLC method for cannabinoid potency determination

For determination of cannabinoid content, the methods for loss on drying and assay of cannabinoids, described in the monogrpah for *Cannabis flos* from German pharmacopoeia, were applied (DAB, 2018).

Loss on drying (LOD)

Loss on drying for each sample was determined by accurately weighing 1 g of each sample and further drying for 24 h at 40°C in an oven under vaccuo (RVT-220, Heraeus). The sample was weighted again. The obtained percentage was involved in the equation for accurate calculation of cannabinoid content (DAB, 2018).

Sample preparation and calibration standards

500 mg dry cannabis flower was used and extracted with 96% ethanol. The final concentration of plant material was 1 mg/mL. The obtained extract was filtered throught 0.45 µm regenerate cellulose membrane filter before injection in HPLC system.

Chromatographic conditions used

The chromatographic analyses were carried out on Agilent 1200 Model HPLC (Agilent Technologies, USA). Separation was achieved by using InfinityLab Poroshell 120 EC-C18 chromatographic column (150 mm x 3 mm ID, 2.7 µm, Agilent Technologies, USA). Mobile phase consisted of aqueous solution of *o*-phosphoric acid (8.64 g/L) as solvent A and acetonitrile as solvent B. Gradient mode of elution was applied: 0 - 16 minute from 36% to 18% A linear gradient, 16 - 17 minute 18% to 36% A linear gradient and from 17 to 30 minute re-equilibration of column with flow rate adapted from 1 mL/min (according to DAB monograph) to 0.7 mL/min and injection volume of 10 µL. Column compartment temperature was maintained at 40°C throughout analysis and DAD measurements were carried out at 225 nm wavelength for neutral cannabinoids (CBD, CBN and △9-THC) and 306 nm wavelength for acidic cannabinoid forms (CBDA and △9-THCA-A).

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Validation of the method

The transferred method for assay of cannabinoids content was validated according to the ICH guideline Q2(R2) and guidline for validation/verification of analytical procedures, PA/PH/OMCL (13) 82 R5 (ICH guidelines, 2005; OMCL Network/EDQM of the Council of Europe, 2020).

System suitability

Solution for system suitability check was prepared according to the monograph in the German pharmacopoeia (DAB, 2018) by diluting 1.0 mL of Δ8-THC and Δ9-THC stock standard solutions with 10.0 mL of methanol (standard mixture of concentration 10 µg/mL).

Aditionally, Δ9-THCA standard solution with concentration 50 µg/mL was used for system precision.

Specificity

Methanol and ethanol, used as a solvents for preparation of standard and sample solutions are used as a blanc at the beginning and at the end of each chromatographic sequence in order to evaluate the absence of interference with the peaks of cannabinoids from the analysed samples and spiked-samples.

Linearity

Five standard solutions of CBD and THC in concentration range of 0.50-75 µg/mL and six standard solutions in the concentration range of 0.10-10 µg/mL for CBN; 0.5-100 µg/mL for CBDA and 0.5-250 µg/mL for THCA were prepared. Linearity of the method, detection limit (DL) and quantification limit (QL) were determined using regression analysis.

The DL was calculated using the formula $(3.3 \times \sigma)/S$, where: σ represents the residual standard deviation of the calibration curve and S represents the slope of the calibration curve. The QL was calculated according to the following formula: $QL = (10 \times \sigma)/S$, where σ represents the residual standard deviation of the calibration curve and S represents the slope of the calibration curve (ICH gudline, 2005).

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Accuracy

Accuracy of the method for assay was evaluated from the recovery values obtained by analysis conducted on spiked samples (dry cannabis flower from variety Meringue) with three different quantities (1 µL, 5 µL and 10 µL) of standard stock solution (100 µg/mL) containing mixture of five cannabinoids (CBDA, CBD, CBN, THC and THCA). The obtained data were analyzed statistically (using Microsoft Excel 2013), and compared against the acceptance criteria given in the ICH guideline Q2(R2) (ICH guideline, 2005).

Precision

The repeatability of the method (RSD %) was estimated from the potency values of the five cannabionoids (CBDA, CBD, CBN, THC and THCA) obtained by analysing six independed sample preparations. Intermediate precision of the method was demonstrated by performing the repeatability experiment by a second analyst on different day.

Results and discussion

The method for determination of cannabinoids content, described in German Pharmacopoeia, transferred in the Center for Natural Products, Faculty of Pharmacy, Ss. Cyril and Methodius University - Skopje, an accredited laboratory, was validated according to ICH guidelines, and used for assay of cannabioniods in different chemotypes of cannabis. Method validation parameters included: system suitability, linearity, specificity, accuracy and precision (repeatability and intermediate precision), detection limit and quantification limit.

System suitability testing

The column efficiency was confirmed with the results for the number of theoretical plates, retention factor and peak symmetry. Additionally, the values obtained for resolution between the peaks of Δ9-THC and Δ8-THC in the chromatograms (Fig. 1, Rs=1.85), after application of the solution for system suitability check, indicate good separation of the components (Rs≥1.2). The repeatability of the system is satisfactory (RSD≤1%). Results from system suitability testing are summarized in Table 1.

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Fig. 1

Table 1

Specificity/Selectivity

The specificity and selectivity of the method were confirmed by comparing the chromatograms obtained after application of the solvents (methanol and ethanol), standard solution (standard mix containing 2 µg/mL CBDA, CBD, CBN, THC and THCA), system suitability solution and sample solution (Fig. 2). Chromatograms show a good separation between the components, without interference from the solvents. Peak purity (similarity/treshhold) ratios were within the calculated treshold limit (1.000) for all peaks, indicating that there are no interfering peaks.

Fig. 2

Linearity

The obtained values for the coefficient of correlation (R^2) from the regression analysis confirmed the linearity of the method for each cannabinoid in the defined concentration range, with acceptable precision (RSD≤1%) of the peak area (n=3). The results are given in Table 2.
The detection limit (DL) and quantification limit (QL) for each cannabinoid were calculated using the values of the standard deviation and the slope from the regression analysis (Table 2). The obtained values are comparable with the results from similar methods described in the literature (Mandrioli et al., 2019), with exception to THC where we have determined lower values for DL and QL, confirming the suitable sensitivity of the transferred method.

Table 2

Accuracy

The Recovery values (%) of CBDA, CBD, CBN, THC and THCA were determined at three different concentrations levels using the calibration curve method (Table 3). The percentage recovery of the spiked analysis, including confidence interval (for a 95.0 level of confidence) are within the range of 90-110%, for CBDA, CBD, Δ9-THC and Δ9-THCA, and within the range of

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80-120% for CBN, with coefficient of correlation, $R^2 \geq 0.999$. Taking into account that the limit for CBN is ≤1.0 %, the obtained result for all components are considered accurate, demonstrating high extraction efficiency of these cannabinoids and confirming the suitable transfer of the method.

Table 3

Precision

Method precision (repeatability and intermediate precision) was confirmed by the results for the RSD (≤10%) for all five cannabinoids (Table 3).

The DAB method was applied for determination of the cannabionids content in three different strains of Cannabis inflorescences (flos). Sample prepration included extraction in 96% ethanol, filtering and injection into an HPLC 1200 system. The obttained results (Table 4), confirm that the transferred method is sensitive (CBDA and CBN are detected in a quantity bellow QL). This method is suitable for testing of all cannabis chemotypes (fiber type, intermediate chemotype and drug-type) enabyling distinction of samples with THC content below 0.2%, that are classified as hemp type.

Table 4

Conclusion

The method described in the monogrpah for *Cannabis flos* from German pharmacopoeia, for determination of the content of five cannabinoids, was successfully transferred, which was confirmed by the results from performed validation study in THC dominant cannabis strain. The method performance for discriminatation of cannabis samples with low content of CBD and THC or samples where one of these cannabiniods are not detected was confirmed, by the obtained values for DL and QL. Finally, the results that were obtained from method validation study, i.e precission and accuracy can be further used for estimation of measurement uncertanity, which will reduce the risk of misinterpretation of the results and will assure the quality and reliability of the quantitative results.

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Awknoleggement

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Резиме

Трансфер на HPLC метод за тестирање на потенција на канабиноиди во суво соцветие од канабис (DAB - Германска фармакопеја)

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Клучни зборови: HPLC анализа, CBD, THC, CBN, CBDA и THCA, валидација на метод, DAB, контрола на квалитетот

Канабисот се смета за хетероген матрикс која содржи комплексен профил на секундарни метаболити, каде што повеќе од 100 компоненти се класифицирани како фитоканабиноиди, присутни со нерамномерна дистрибуција во однос на содржината. За да се обезбеди квалитетот на канабисот, потребна е примена на соодветни техники за подготовка на примерок, како и временски и економични аналитички методи. Во DAB монографијата тестирањето на потенцијата на канабиноидите во цветот на канабис е вклучена како задолжителен параметар за следење во ЕУ. Во оваа студија извршен е трансфер на методот, со цел да се потврди применливоста на методот при одредување на различни количини на канабиноиди во различни сорти на канабис, фокусирајќи се претежно на примероци со мала содржина на CBD или THC или примероци каде што еден од овие канабиноиди не е детектиран. HPLC-DAD методот е потврден и е користен за рутинска контрола на содржината и конзистентноста на медицинскиот канабис. Добиените вредности за лимитот

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на детекција, лимитот на квантификација, точноста и прецизноста се покажаа како задоволителни.

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Table 1. Results from system suitability testing

System suitability parameters / Component	System precision (RT, n=6) RSD (%)	System precision (peak area, n=6) RSD (%)	USP tailing factor (T)	Theoretical plates (N)	Retention Factor (k')
CBDA	0.12	0.86	1.10	22070	4.50
CBD	0.14	0.06	1.03	22545	5.47
CBN	0.04	0.13	1.06	42009	8.05
THC	0.06	0.20	1.04	52987	9.84
THCA	0.07	0.07	1.12	71983	12.29

*RT = retention time (min)

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Table 2. Results for linearity (regression coefficients values and R^2), detection limit (LD), quantification limit (QL) and signal to noise (S/N)

Component	Concentration range (μg/mL)	Linear equation	R^2 value	DL (μg/mL)	QL (μg/mL)	S/N
CBDA	0.50 - 100	y = 10745x + 0.21585	0.9999	0.58	1.76	106.14
CBD	0.50 - 75	y = 36349.6901x + 5.2604	1.0000	0.53	1.61	189.60
CBN	0.10 - 10	y = 91483.6899x - 0.2818	0.9999	0.09	0.28	89.59
Δ9-THC	0.50 - 75	y = 34660.4402x - 3.4756	0.9998	0.13	0.40	164.58
Δ9-THCA	0.50 - 250	y = 12646.3867x + 3.0213	1.0000	0.25	0.76	287.18

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Table 3. Results for accuracy and precision

Amount of standard solution added		Components									
		CBDA		CBD		CBN		THC		THCA	
		Accuracy (%) (n=3)	RSD (%) (n=3)	Accuracy (%) (n=3)	RSD (%) (n=3)	Accuracy (%) (n=3)	RSD (%) (n=3)	Accuracy (%) (n=3)	RSD (%) (n=3)	Accuracy (%) (n=3)	RSD (%) (n=3)
1 µL		96.83±0.36	0.15	91.21±0.58	0.26	101.91±0.93	0.37	97.89±1.49	0.61	101.91±1.06	0.42
5 µL		101.28±0.86	0.34	104.02±0.39	0.15	104.46±0.70	0.27	101.13±0.03	0.01	102.01±0.56	0.22
10 µL		100.47±2.23	0.89	100.60±2.51	1.01	90.07±1.71	0.76	100.97±0.25	0.10	101.76±0.63	0.25
Precision (%RSD≤10%)	Repeatability	4.96		/		1.51		1.79		2.48	
	RSD (%) (analyst 1)	4.23		/		4.07		1.35		2.36	
	RSD (%) (analyst 2)	3.03		/		2.54		2.35		1.46	

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Table 4. Results from analysis of CBD or THC predominant strains applying the DAB method for cannabis flos

Paramethar	Referent values (%w/w) according to DAB monograph	Result (%w/w)			QL (%)
		CBD dominant strain	THC dominant strain		
		CBD Charlotte's Angel	Meringue	Jack Kush	
Loss on drying	≤ 10.00	5.29	5.36	7.89	
CBDA potency	/	17.95	0.04	0.02	0.18
CBD potency	/	0.52	BLD	BLD	0.16
CBN potency	≤ 1.00	BLD*	0.02	0.01	0.03
Δ9-THC potency	/	0.07	1.84	0.45	0.04
Δ9-THCA potency	/	0.68	20.03	6.99	0.08
Total CBD [CBD + (CBDA x 0.877)]	/	16.26	0.04	0.02	/
Total Δ9-THC [Δ9-THC+ (Δ9-THCA x 0.877)]	/	0.66	19.41	6.58	

*BLD - bellow limit of detection

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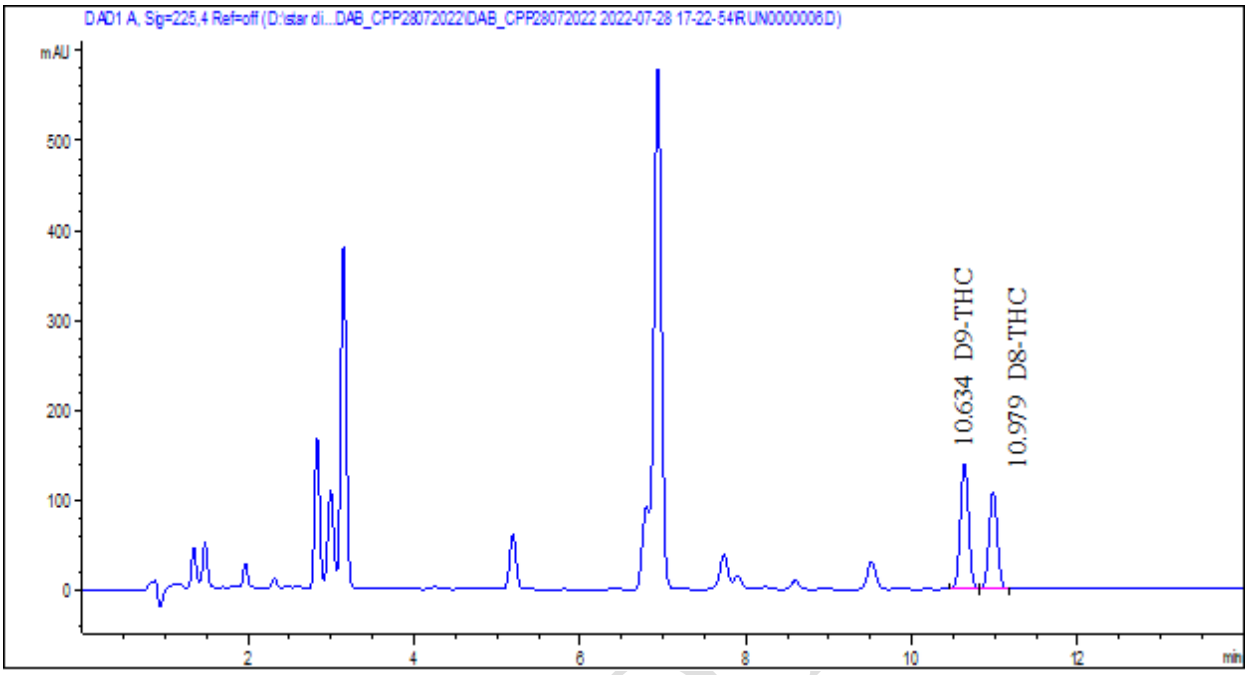

Fig. 1. Chromatogram obtained from resolution solution.

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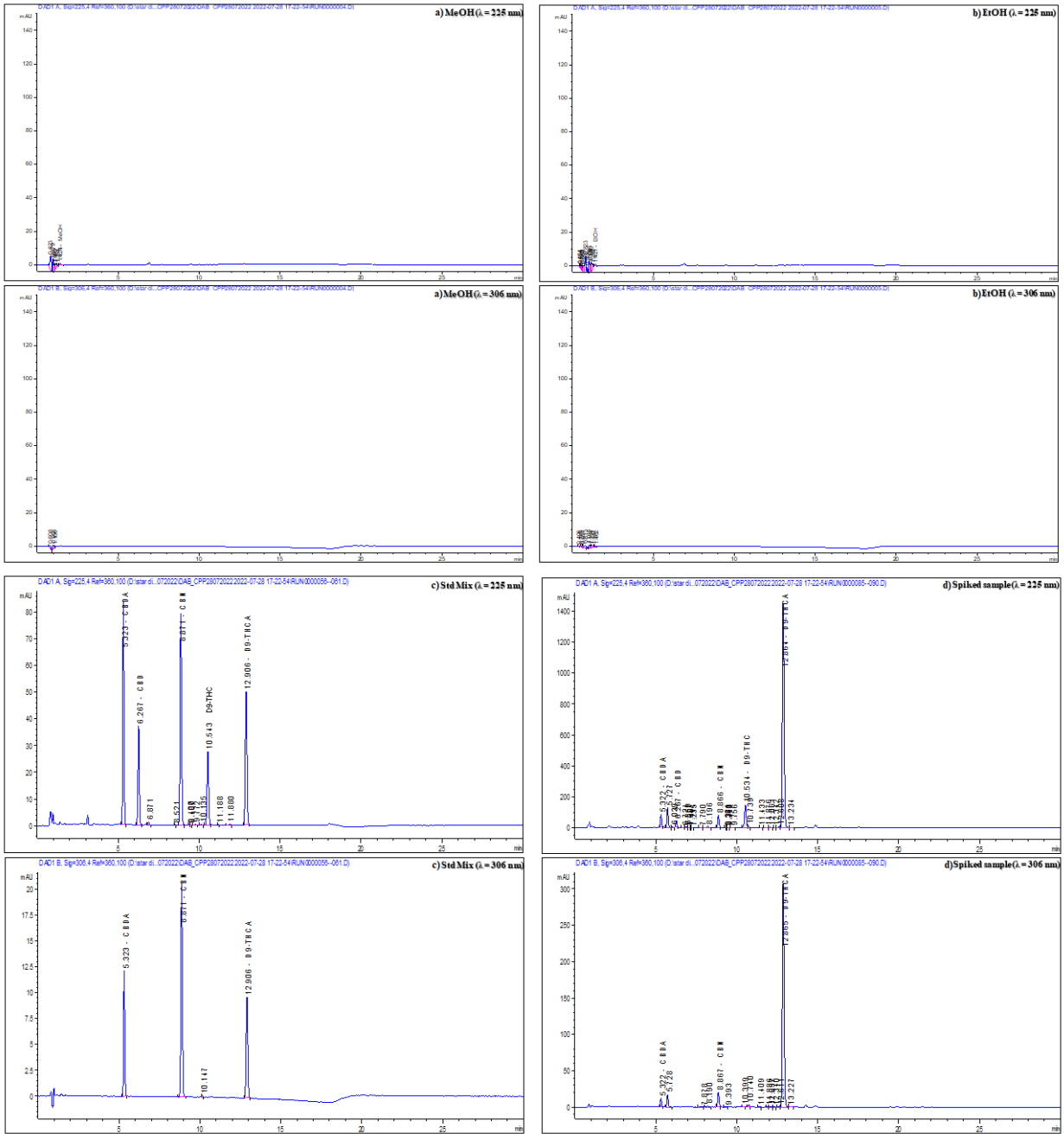

Fig. 2. Chromatograms of two diluents (blank) (a and b), standard mixture of five cannabinoids (c) and spiked sample (d), monitored on two different wavelengths (for CBD, CBN and Δ9-THC and 306 nm for CBDA and Δ9-THCA).

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