3-1-2021

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Recommended Citation

Al Bakain, R. Z., Al-Degs, Y. S., Cizdziel, J. V., & Elsohly, M. A. (2020). Comprehensive chromatographic profiling of cannabis from 23 USA States marketed for medical purposes. Acta Chromatographica, 33(1), 78–90. https://doi.org/10.1556/1326.2020.00767

AKADÉMIAI KIADÓ

Acta Chromatographica

33 (2021) 1, 78–90

DOI:
10.1556/1326.2020.00767

ORIGINAL RESEARCH PAPER

Comprehensive chromatographic profiling of cannabis from 23 USA States marketed for medical purposes

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Received: January 24, 2020 • Accepted: February 29, 2020
Published online: June 5, 2020

ABSTRACT

In this research, cannabis varieties represent 23 USA States were assayed by GC-FID to generate their complex chemical profiles informative for plants clustering. Results showed that 45 cannabinoids and terpenoids were quantified in all plant samples, where 8 cannabinoids and 18 terpenoids were identified. Among organics, Δ^9-THC, CBN (cannabinoids) and Fenchol (terpenoid) not only showed the highest levels overall contents, but also were the most important compounds for cannabis clustering. Among States, Washington, Oregon, California and Hawaii have the highest cannabis content. GC-FID data were subjected to PCA and HCA to find (1) the variations among cannabis chemical profiles as a result of growing environment, (2) to reveal the compounds that were responsible for grouping cultivars between clusters and (3) finally, to facilitate the future profile prediction and States clustering of unknown cannabis based on the chemical profile. The 23 cannabis USA States were grouped into three clusters based on only Δ^9-THC, CBN, C1 and Fenchol content. Cannabis classification based on GC-profile will meet the practical needs of cannabis applications in clinical research, industrial production, patients' self-production, and contribute to the standardization of commercially-available cannabis cultivars in USA.

KEYWORDS

cannabinoids, terpenoids, chemical profiles, GC-FID, USA states, PCA, HCA Clustering, Δ^9-THC

INTRODUCTION

The plant cannabis (*Cannabis sativa L*) is the most widely consumed and popular medicinal botanical drug product in the world due to its high usage and its diverse pharmacological properties [1]. Chemically, cannabis is a complex species containing large number of active constituents [1–3]. Herbal cannabis (known as marijuana), cannabis resin (hashish), and extracts of cannabis resin (hashish oil) are still the most illicit drugs in the world. About 8,000 tons of cannabis are intake in USA per year [1]. In many countries, cannabis is popular including Canada and North America [1, 2, 4]. In 2017, many USA states have legalized the medical use of cannabis, where, 38 licensed producers in Canada are authorized to produce and sell dried marijuana [4]. There has been a major increase of domestic production worldwide not only in USA and Canada, but also in Colombia, Mexico, Jamaica, and Thailand [1].

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Cannabinoids and terpenoids are the active ingredients in cannabis [4, 5]. Both classes of compounds are known of their variables biological activities [6]. Terpenoids are of great interest because of their production by the plants reflects the immediate environment and they are responsible for cannabis' distinctive odor [7], whereas, cannabinoids tend to reveal genetic relationships [8].

Today, most nations worldwide regard cannabis as an illegal drug of abuse. Despite the abuse potential of cannabis and its illegal status at the federal level in the USA, research into its chemistry and pharmacology has demonstrated that it also has medicinal properties. Cannabis has a long history of human use as a medicinal plant, intoxicant, and ritual drug [9, 10]. Clinical trials into cannabis, pure cannabinoids, and synthetic analogs have demonstrated some effectiveness as analgesics for chronic neuropathic pain, appetite stimulants for cancer and AIDS patients, multiple sclerosis, pain, inflammation, depression, anticancer, palliative, epilepsy and infection [11–16]. The increased medical interest in these substances has prompted the development of various cannabis based medicines such as the oral Δ^9-THC (delta-9-tetrahydrocannabinol) preparation Marinol®, a synthetic analog of Δ^8-THC (delta-8-tetrahydrocannabinol) and an oral mucosal spray containing 1:1 ratio of Δ^8-THC and CBD (cannabidiol) [17, 18].

There are three classification systems for cannabis. The first, is by species based on physical appearance, THC (tetrahydrocannabinol) content, and geographical origins since environmental factors and marijuana cultivated sources can induce different cannabis profiles [1, 8, 19–22]. The second classification is based on the ratio of two major cannabinoids THC and CBD which is decided by their corresponding allelic loci [23, 24]. The third is based on both cannabinoids and terpenoids for drug standardization and clinical research purposes [24]. Novotny et al. reported that data relative to the use of GC analysis of marijuana samples of different origin indicated that the chromatograms appeared to be different, so correlation between chromatographic data and geographical origin of marijuana might be possible [25]. Hazekamp et al. [26] reported the impact of changing the environmental conditions on the chemical composition and variability of terpenoids and cannabinoids in different cannabis varieties.

A wide variety of analytical techniques have been used for chemical profiling (i.e., fingerprinting) of cannabis. Thin Layer Chromatography [22], fingerprinting with HPLC [26–28], GC coupled with mass spectrometry [1] and ^{1}HNMR have been used to fingerprint cannabis aqueous extracts and tinctures [29] as well as to chemically differentiate cannabis cultivars [30]. SFC also has been used to analyze cannabis [13, 31–34]. However, GC is the most commonly used instrument for analyzing cannabinoids and terpenoids [1, 13, 19, 20, 35]. GC has been used to differentiate cannabis from different countries, including Mexico, Colombia, Jamaica, Thailand, and the USA [1].

The current approaches for cannabis classification may be inadequate because they analyze cannabis from botanical perspectives based on only two cannabinoids; THC and CBD. Moreover, there is currently no available comprehensive chemical profiling for all USA states medical-type cannabis samples which is necessary to explore the similarities/differences if any among plants samples of different States. Therefore, this study was carried out.

In this study, a comprehensive work was carried out to identify the compounds most important in distinguishing cannabis varieties and to find the variation on cannabis chemical profiles as a result of growing plants in different environments and in growth time from 23 USA States that have enacted Medical Marijuana laws, including: Alaska, Arizona, California, Colorado, Delaware, Florida, Hawaii, Illinois, Maine, Maryland, Massachusetts, Michigan, Montana, Nevada, New York, Ohio, Oregon, Pennsylvania, Vermont, Washington, West Virginia, Wisconsin and Mississippi. GC-FID was applied for the chemical analysis. Cannabis plants samples obtained from each of the 23 USA States were collected, extracted and analyzed using GC-FID. The plant samples were analyzed to detect all possible cannabinoids and terpenoids from different cannabis seeds and origins which are necessary for cannabis fingerprinting. The method was validated and evaluated for selectivity and precision (i.e., repeatability). The advanced multivariate tools including Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) are efficient towards sample clustering [36–41]. Hence, these tools were performed in this study to; (1) identify the compounds most important in distinguishing cannabis varieties, (2) find the variation on cannabis chemical profiles as a result of growing plants in different States and with different in growth times, (3) confirm whether the cultivars (i.e., States) in the cluster analysis would also be grouped together, (4) reveal the compounds that were responsible for grouping cultivars between clusters and (5) develop a database that can predict the origins and type of unknown cannabis grown in the USA. To our knowledge, this study had never been carried out before.

MATERIAL AND METHOD

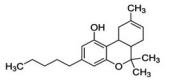
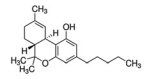
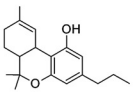
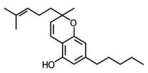
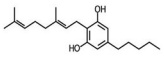
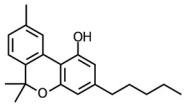
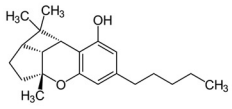
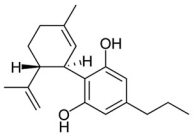
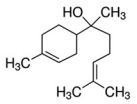
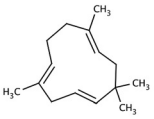
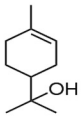
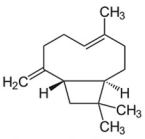
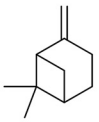
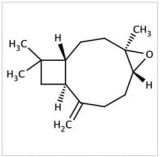
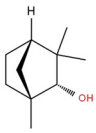
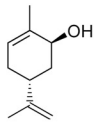
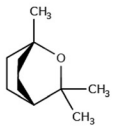
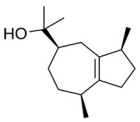
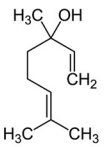
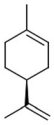
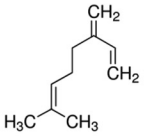
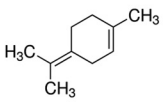
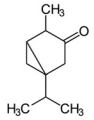
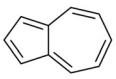
Cannabis plants

Cannabis samples (leaves and inflorescences) were collected from the supply of materials provided from seized samples by The Drug Enforcement Administration (DEA). The samples were obtained in tightly closed plastic bags and stored in a dry cool storage facility in the Coy Waller Complex at the University of Mississippi prior to analysis. The samples were selected from 23 States that have enacted Medical Marijuana laws, including: Alaska, Arizona, California, Colorado, Delaware, Florida, Hawaii, Illinois, Maine, Maryland, Massachusetts, Michigan, Montana, Nevada, New York, Ohio, Oregon, Pennsylvania, Vermont, Washington, West Virginia, Wisconsin and Mississippi.

Reagents and solutions

Twenty six standards of cannabinoids and terpenoids were provided from Sigma-Aldrich® (St. Louis, USA). Structural formula of organics are summarized in Table 1.

Table 1. Structural formula of assayed cannabinoids and terpenoids

Cannabinoids		
 Δ^8-THC/Δ^8-Tetrahydrocannabinol	 Δ^9-THC /Δ^9-Tetrahydrocannabinol	 THCV/ Tetrahydrocannabivarin
 CBC/Cannabichromene	 CBG/Cannabigerol	 CBN/Cannabinol
 CBL/ Cannabicyclol		 CBDV/ Cannabidivarin
Terpenoids		
 α-bisabolol	 α -humulene	 α – pinene
 α –terpinol	 β – caryphyllene	 β –pinene
 Caryophylleneoxide	 Fenchol	 Carveol
 Cineol	 Sabinene	 Guaiol
 Linalool	 Limonene	 Myrcene
 Terpinolene	 Thujone	 Azulene

Phenanthrene (99% purity) used as the internal standard was supplied from Sigma-Aldrich®. All chemicals and solvents used for extraction and other preparations were of HPLC ultra-grade: acetone and ethyl acetate (≥99.7%), hexane (≥98.5%), ethanol (>98%), and methanol (≥99.8%) were purchased from Sigma-Aldrich®. Chloroform (≥99.8%) was provided from Fischer Scientific (New Jersey, USA). Ultrapure water (18 MΩ cm^{-1}) generated by Milli-Q Plus water purification system (Millipore, Billerica, MA) was used to prepare aqueous solutions and dilutions.

Extraction of cannabinoids and terpenoids prior to GC analysis

Dried cannabis samples were grinded to get a homogenous mixture of leaf particles. A 100 mg portion was transferred to a test tube and 3.0 mL of extraction solution (methanol-chloroform 9:1 *v/v* spiked with 0.2 mg mL^{-1} phenanthrene) was added. Phenanthrene was used as both a retention time marker (R_t between the terpenoids and cannabinoids) and as a reference to calculate peak ratio of solute. The extraction tube was then placed in an ultrasonic water bath for 15 min to allow soluble cannabinoids and terpenes to dissolve in the extraction solution. The samples were then centrifuged for 30 s at 2,000 rpm. Finally, the extract was filtered using Acrodisc syringe filter (PAU-Gelman Lab, 0.45 μm, 25 mm diameter) and collected in a screw-capped amber vial. Samples were stored in a freezer (−10 °C) prior to analysis time. Duplicate extractions and injections were made for each cannabis sample.

Chromatographic analysis of samples

The chromatographic profiles of cannabis were all recorded in the splitless mode using an Agilent GC 6890 series system equipped with a 7683B autosampler. The GC column was an Agilent, DB-5, 30 m length, 0.25 mm internal diameter, film thickness 0.25 μm, (J&W Scientific Inc., Folsom, California, USA). The detailed experimental conditions applied in this study are available in our previous study [40].

Common standard stock solutions of cannabinoids and terpenoids were prepared at the concentration of 100 μg mL^{-1} in pure MeOH. Each solution was injected individually in two identical injections to determine the retention time of each component, and then the average was registered.

Chromatographic profiles of extracted samples

Selectivity was determined by injecting solvent blank to confirm that there were no false signal peaks at the targeted retention time. Intraday reproducibility was determined by injecting an aliquot of reference sample of β-pinene (100 μg mL^{-1}) five times from the same vial in a single day ($n = 5$). The peak area ratio was calculated for each solute (Peak ratio = Peak area of solute/Peak area of IS) and further used to fingerprint the cannabinoids and terpenoids. The results reported as the average of two identical injections.

In the current investigation, 45 solutes were directly assayed by GC-FID compared to only 23 in our published work [40]. Hence, more comprehensive profile was adopted in this investigative. The identity of the new detected 22 solutes was identified from their retention times compared to the other common cannabinoids/terpenoids [1]. Hence, solutes detected after 30 min are related to cannabinoids-family [1].

Numerical analysis

Numerical analysis by PCA and HCA was carried out using Chemoface 1.61 software [42] which had been run under Matlab® (Mathworks, 8.6, USA). The size of chromatographic data used numerical analysis is 23 × 45 (23 samples × 45 organic solutes). Initially, the chromatographic data was loaded in Chemoface prior to PCA and HCA analysis.

RESULTS AND DISCUSSION

Quantitative analysis of cannabis varieties

For the comparison and the acquisition of a large number of complex chemical profiles, it is necessary to extract the maximum amount of plant contents. Therefore, a mixture of methanol: chloroform (9:1 v/v) was used to extract the highest amount of cannabinoids and terpenoids. Over the 23 USA cannabis samples, 45 active ingredients were detected. Among these ingredients, the identity of 26 compounds (eight cannabinoids and eighteen terpenoids) was established by comparing their retention times with authentic standards. The sesquiterpenoids symbolized as (T_n) and cannabinoids (C_n) have not been identified since no reference compounds were available for confirmation. The detected cannabinoids and terpenoids have variable proportions but comparable to those reported for Canadian cannabis [4]. All solutes were separated in 63 min. The sharp and intense GC peak positioned at ≈31.30 min was for phenanthrene (IS), and used for quantitative evaluation of the chemical profile contents (peak area ratio for each solute = Peak area of solute/Peak area of IS). The relative amount of separated solutes was reported as peak ratio rather concentration as reported in our previous work [40]. Moreover, numerical analysis by PCA and HCA was also based on peak ratios of solutes which is a common procedure. The position of phenanthrene encountered very small variations in both intensity and retention time during all injections. The proposed GC method was stable and convenient to quantify all the 45 organics.

In general, cannabinoid solutes such as CBN, Δ^9-THC, CBG, and CBC have higher peak intensity and eluted at longer retention times compared to terpenoids, which would be attributed to the higher polarity of cannabinoids. Interday reproducibility was determined by injecting the same reference sample 12 times using fresh aliquots on each day ($n = 12$). The intra and inter-day precisions (%RSD) were 0.37% and 0.32%, respectively. These low RSD values indicate that the method was precise in terms of repeatability,

reproducibility, and intermediate precision. Instrumental precision (RSD), defined as the variation in peak area of the IS to all solutes was found to be 1.22%. The retention time (min) and chemical profiles of cannabis samples (expressed as the solute/internal standard peak area ratios) were obtained from duplicate measurements by GC-FID as provided in Table 2.

As indicated in Table 2, forty five common compounds were detected in each sample, where, 8 cannabinoids and 18 terpenoids were identified based on standard comparison. Usually, the detected cannabinoids were related to six different classes: Δ^8-Tetrahydrocannabinol (Δ^8-THC), Δ^9-Tetrahydrocannabinol (Δ^9-THC and THCV), Cannabichromene (CBC), Cannabigerol (CBG), Cannabicyclol (CBL), and Cannabinol (CBN) [7]. The other unidentified cannabinoids and terpenoids were symbolized as C_n and T_n, respectively, were *n* represents a number. In the current investigation, 45 solutes were directly assayed by GC-FID compared to only 23 in our published work [40]. Hence, more comprehensive profile was adopted in this investigative. The identity of the new 22 detected solutes were identified from their retention times compared to the other common cannabinoids/terpenoids, hence, solutes detected after 30 min are related to cannabinoids-family [1].

The unidentified C_n and T_n have been determined as terpenoids and cannabinoids based on comparison studies [1, 43]. It is known that cannabinoids would be available in neutral and acidic forms and quantification of both forms will require silylation/methylation of the acidic ones before GC analysis [12]. Hence, the provided data in this work gave the total contents of neutral and acidic forms of cannabinoids as no silylation of the acidic groups was carried out. Moreover, most cannabinoids are available in their neutral form, for example, ten isolated forms are known for Δ^9-Tetrahydrocannabinol and only two of these are in acidic form.

Among all cannabinoids, the total content in all states of Δ^9-THC and CBN were remarkably higher than the rest of all other ingredients; 78,520 and 16,450, respectively. It is known that the psychoactive nature of cannabis is highly related to the level of Δ^9-THC. In the same time, the high content of CBN indicated the long storage time of samples as CBN is generated from Δ^9-THC with time. CBG, the first biogenic cannabinoid formed in the plant, was also available in acceptable amounts of 4,323. CBC content (5,774) found to be as intermediate level between Δ^9-THC and CBG. It occurs mainly as cannabichromenic acid (CBCA, 2-COOH–CBC, CBC–COOH). Geranylpyrophosphate and olivetolic acid combine to produce cannabigerolic acid (CBGA; the sole intermediate for all other phytocannabinoids), which is cyclized by the enzyme CBCA synthase to form CBCA. Over time, or when heated above 200° F, CBCA is decarboxylated, producing CBC. Beside cannabinoids, terpenoids impart the scent of cannabis plants where most of the peaks are present in the terpenes region (retention time ≈ < 30 min).

The marked compound/s in the 23 states is/are the following: limonene, fenchol, linalool, Guaiol, and CBC are typical fingerprints for cannabis Oregon State. Regarding Nevada, it was marked for its high content of Fenchol, CBL, T3 and T7. Washington State can be distinguished easily due to its high content of CBL, C1 and Fenchol in comparison to other states, where it gives notable highest level overall states for C1 with a peak ratio of 6,386. T2 compound was characteristics for cannabis from Vermont State. THCV and CBC contents showed the highest level in Illinois State. Considering the contents obtained for Δ^9-THC, all states detected it with a very large content, but Hawaii (6,352), Oregon (5,459) and California (8,035), respectively, have the highest peak ratios among all States. Concerning CBN rates as the second highest content after Δ^9-THC, where its content considered high in all states with comparable values. The highest content among all 45 organics over all states was for California State (11,192), Oregon (11,511) and Washington (11,095) that has the highest notable content of cannabinoids, especially Δ^9-THC and CBN.

For the rest of organics, comparable ratios for cannabis in the States have been observed. Finally, cineol, T11 and α-terpinol were not detected in all samples, and even if detected, their levels were very low. As shown, specific cannabinoids such as Δ^9-THC, CBN, CBG, C1 and CBC are dominants in all States. This is referred to the strong influence of geographical position, maturity, age and storage conditions and the fluctuation of cannabinoids content between states with age that is numerous [1, 44, 45].

Regarding the terpenoids family, Fenchol was the most abundant one. Terpenoids were grouped to: monoterpenoids, sesquiterpenoids, and triterpenoids. Although the number of identified terpenoids was relatively high (18 solutes), the content of cannabinoids was higher (Table 2). For terpenoids, two main classes were identified: a) Sesquiterpene including (α-bisabolol/α-humulene/β-caryphyllene/caryophylleneoxide/guaiol), and b) Monoterpene including (α-pinene/β-pinene/α-terpineol/fenchol/linalool/myrcene/terpinolene/limonene/sabinene/carveol/cineol). The content of Fenchol was relatively high in the majority of collected samples. The explanation is that the quantities of Fenchol within cannabis plants, as well as other plants, vary significantly refers to: the growing conditions, including groundwater mineral content, soil/growing medium mineral content, soil condition, light, temperature, age of the plant, maturity of the plant, storage conditions and air pollution. Therefore, terpene's levels vary dramatically not just from one growing region to another, but from plant to plant within the same growing area [8, 46].

Based on the content of Δ^9-THC, cannabis can be divided into three chemical phenotypes [47]: (i) drug type, in which the major compound Δ^9-THC is about 1–20%; (ii) intermediate type, in which Δ^9-THC are the leading cannabinoids and their concentration range is 0.3–1.0%; and (iii) fiber type mainly contains Δ^9-THC is in the concentration <0.3%. Another method to distinguish between drug-type and fiber type cannabis has been defined by the UNODC [48] with a simple mathematic equation. According to this criterion, about 86% of the cannabis samples analyzed containing detectable amount of Δ^9-THC belongs

Table 2. Chemical profiles of cannabis samples obtained from the 23 USA States (C^n and T^n are symbols for unidentified cannabinoids and terpenoids, respectively).* The value represents the Peak ratio

Solute	Retention time (min)	Alaska	West Virginia	Wisconsin	Washington	Vermont	Pennsylvania	Oregon	Montana	Ohio	Mississippi
α-Pinene	**9.95**	88*	21	15	13	43	31	68	35	58	54
Sabinene	**10.67**	33	42	27	48	33	24	29	23	20	32
β-Pinene	**11.08**	41	11	26	23	18	18	37	21	20	29
Myrcene	**11.12**	48	11	19	23	18	14	32	20	20	16
Limonene	**12.30**	20	16	13	10	11	12	407	14	13	10
Cineol	**12.45**	11	N.D	N.D	N.D	N.D	N.D	12	6	7	N.D
Terpinolene	**13.78**	26	51	25	22	23	27	32	25	27	26
Linalool	**14.12**	30	56	25	31	22	26	422	30	26	37
Thujone	**14.93**	14	13	11	29	18	27	15		17	24
T1	**15.15**	8	8	7	29	12	11	8	6	9	8
α-Terpineol	**16.98**	11	N.D	N.D	N.D	8	N.D	16	8	8	5
Carveol	**17.39**	18	36	9	17	26	20	17	13	14	36
Azulene	**20.74**	17	10	34	6	N.D	13	21	10	18	9
β-Caryphyllene	**22.48**	97	96	101	49	150	105	153	155	187	56
T2	**22.91**	55	191	141	7	556	132	71	68	122	6
T3	**23.02**	34	31	29	95	37	28	43	21	16	6
α-Humulene	**23.72**	27	69	181	23	48	30	54	48	65	20
T4	**24.12**	13	13	11	27	9	8	8		15	4
T5	**24.43**	9	19	23	8	44	13	43	25	24	17
T6	**25.69**	41	50	33	9	17	87	53	10	35	48
T7	**25.81**	60	50	43	11	52	109	10	10	12	31
Caryophylleneoxide	**26.76**	46	24	32	23	40	43	432	19	38	13
Guaiol	**26.91**	24	20	24	19	34	19	37	27	40	15
T8	**27.36**	107	152	30	61	103	39	165	21	30	7
T9	**27.71**	81	171	33	28	47	37	49	36	20	18
α-Bisabolol	**28.94**	60	52	80	35	40	35	63	56	78	40
Fenchol	**28.22**	115	52	89	614	45	34	1,027	61	62	40
T10	**29.55**	7	8	7	6	10		7	8	7	
Phenanthrene (IS)	**31.30**										
T11	**32.63**	7	6	6	8	8	N.D	N.D	N.D	N.D	N.D
T12	**34.09**	12	6	13	7		7	13	14		7
T13	**36.95**	20	20	15	23	21	10	28	33	11	18
CBDV	**40.84**	20	22	17	6		34	27	28	42	19
THCV	**41.42**	53	28	80	55	23	26	51	47	50	20
CBL	**42.87**	12	11	17	216	10	12	86	14	17	8
CBC	**44.41**	163	225	228	53	138	172	740	270	402	101
Δ^8-THC	**46.65**	17	56	16	14	12	9	33	78	18	10
Δ^9-THC	**47.54**	4,695	1,183	2,383	2,191	2,241	1,442	5,459	3,646	3,950	1,950
C1	**48.71**	72	47	65	6,386	37	59	90	76	58	59
CBG	**49.70**	343	95	154	140	124	139	221	203	240	96
CBN	**50.09**	1,083	386	670	616	424	523	1,135	955	856	502
C2	**51.82**	22	30	60	29	11	36	49	28	48	22
C3	**52.89**	13	10	15	22	7	18	8	25	20	11
C4	**54.22**	63	65	70	21	85	41	52	128	71	37
C5	**55.83**	63	72	150	33	66	66	137	99	48	38
C6	**58.08**	13	8	13	8	8	11	50	56	21	10

to the drug type. However, those classification methods are not accurate because an assumption was made that the acidic cannabinoids were completely converted to neutral cannabinoids during the decarboxylation process, for example, THCA acid decarboxylates as a result of high temperature during gas chromatography analysis to produce THC.

As a promising field combining computer science and analytical chemistry, chemometrics has increasingly found application in natural products chemistry, and has been used extensively for analytical data mining, graphical visualization, and class discrimination and prediction [37–41]. An important step in this research is data analysis where mathematical algorithms were used to extract useful

Table 2. Continued

Nevada	New York	Massachusetts	Michigan	Maryland	Maine	Illinois	Florida	Colorado	Arizona	Hawaii	Delaware	California
77	47	38	42	91	46	69	67	14	31	69	32	181
24	17	27	20	14	15	18	16	21	23	19	23	16
46	18	25	18	31	17	24	24	17	11	40	23	100
33	17	29	18	33	18	25	26	17	11	40	23	100
32	18	14	15	18	17	13	28	16	10	25	12	32
7	10	5	6	4	4	5	6	5	2	8	3	8
17	15	25	26	29	22	26	27	38	11	34	28	36
31	23	36	27	29	21	28	26	45	21	36	46	34
103	19	8	8	21	5	19	17	7	7	16	7	14
34	10	4	8	18	4	4	7	10	27	7	27	10
8	9	5	6	7	6	6	6	3	3	7	4	9
16	17	29	18	8	29	21	8	31	17	12	27	17
26	38	7	31	14	10	7	20	17	10	39	10	10
150	175	168	136	116	91	142	181	136	51	223	132	247
28	508	186	146	63	40	82	214	119	14	220	200	234
468	8	5	11	7	19	17	7	24	8	7	10	48
60	55	55	49	38	27	46	56	32	18	73	39	64
21	11	6	8	4	7	8	15	4	5	62	7	17
92	33	19	20	18	9	17	27	17	31	13	20	31
345	276	56	15	43	41	7	98	54	31	69	20	86
533	59	60	4	46	5	11	98	75	14	92	30	110
23	24	31	31	31	24	30	29	38	21	38	49	33
40	45	19	25	25	17	19	22	25	13	42	24	27
92	96	111	27	24	33	7	34	182	68	52	177	31
115	37	90	21	80	85	19	51	58	47	69	10	45
66	34	42	67	49	25	33	49	45	42	67	35	83
394	33	28	66	49	24	32	49	36	30	67	45	86
12	6	5	8	6	12	7	17	5	5	13	7	7
9	3	6	6	7	6	24	7	5	31	11	10	24
	26	13	5	10	7	7	10	10	10	13	17	10
10	13	14	13	11	13	31	14	11	14	16	20	24
65	45	39	54	45	91	71	17	173	81	36	33	28
46	15	37	44	57	32	235	61	41	28	104	32	128
298	16	12	14	13	13	11	10	12	15	29	17	40
137	183	222	176	131	354	648	201	160	159	426	203	283
303	13	10	19	17	17	18	14	11	10	21	13	18
4,503	1,663	2,206	4,216	3,949	2,446	3,709	4,985	2,243	2,388	6,352	2,684	8,035
149	79	53	42	51	64	20	64	58	27	98	40	45
165	81	247	236	130	119	351	139	170	125	276	199	330
883	978	223	865	922	603	531	713	564	900	1,138	485	494
74	92	17	85	58	37	14	81	54	27	13	27	24
27	14	9	9	14	18	31	7	10	5	13	13	21
65	69	50	12	58	98	20	37	48	34	69	43	52
144	101	30	27	87	91	12	34	54	31	89	20	45
26	22	18	15	20	91	4	35	22	7	49	13	21

information from huge data sets obtained from GC-FID as will be shown in the following section.

Data analysis and classification of states based on cannabinoids and terpenoids contents

Although Table 2 and Fig. 1 give comprehensive profile of all cannabinoids and terpenoids levels in each state sample, some compounds are more important to the clustering. In order to clearly differentiate among cannabis States and to specify the compounds responsible for clustering the groups, the GC-FID scan data were subjected to HCA and PCA analysis. PCA and HCA are unsupervised clustering techniques commonly employed to reduce the complexity of multivariate data sets without losing important information, observe variance in data sets, and visualize data clustering. In our study, 45 cannabinoids and terpenoids are the original variables (45 dimensions) in PCA. By calculating the

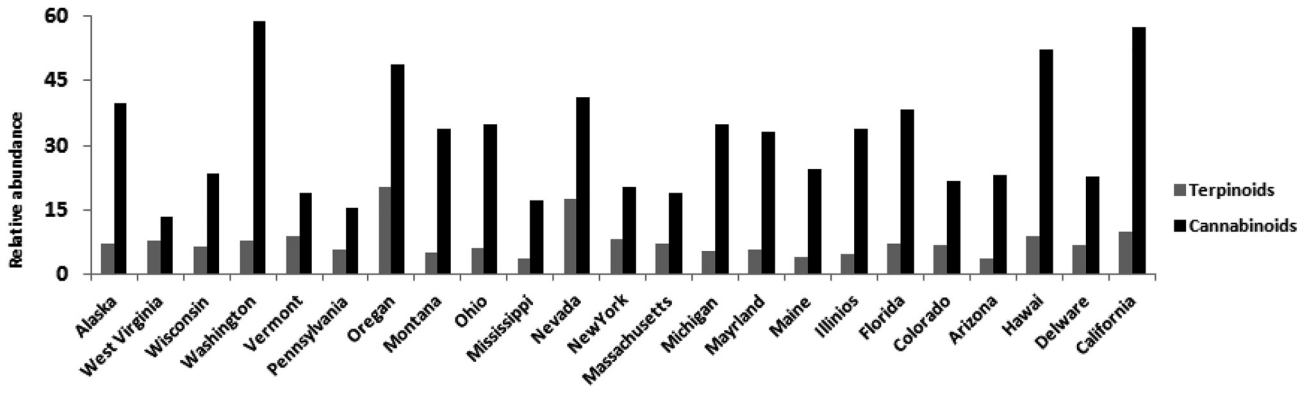


Fig. 1. Total cannabinoids and terpenoids contents in each state based on the dendrogram and a full chemical profile

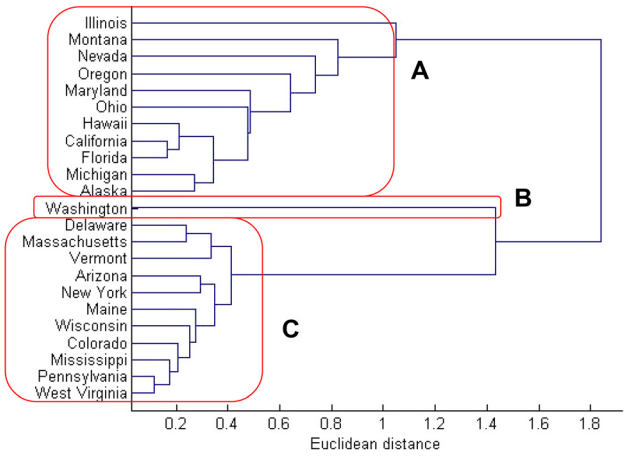


Fig. 2. Dendrogram obtained from the whole chromatographic data

covariance matrix between these 45 dimensions, PCA can generate 45 PCs that are orthogonal to each other and can explain 100% of the total variance of the orthogonal data. In this work, the first two PCs explain 89.73% of the total variance. Each PC is correlated with the original 45 variables. The chromatographic data was preprocessed using mean-center methodology for better interpretably of PCA and HCA outputs [49, 50]. All detected organics were rather necessary for states clustering. Accordingly, the number of variables used in clustering was 45 (detected compounds) × 23 (USA States cannabis samples). The resulted HCA clustering of states is provided in Fig. 2.

HCA results are shown in Fig. 2. This dendrogram was obtained by calculating the Euclidean distance among samples and grouping them by the complete linkage method. There are three main groups that are clearly discriminated; Group A includes 11 States; Alaska, California, Florida, Hawaii, Illinois, Maryland, Michigan, Montana, Nevada, Ohio, and Oregon. Group B contains only Washington. Where, group C clustered 11 States; Arizona, Colorado, Delaware, Maine, Massachusetts, New York, Pennsylvania, Vermont, West Virginia, Wisconsin and Mississippi. All states are clustered while shown no mixing in different USA States.

The results of PCA projection of the data of GC into the plan of the first two principles components are carrying an accumulative average of 89.73% of the total variance. Hence, loading, score and bi-plots can be viewed using two factors only.

PCA was applied to the matrix of 23 × 45 (23 USA States × 45 detected cannabis). The results of PCA projection of the data from the first two principle components are carrying 89.73% of the total variance as shown in Fig. 3A.

As indicated in Fig. 3A, the score plot indicated three main different clusters collecting different number of states-this clustering corresponds with cannabinoids and terpenoids content. For example, cluster A has 11 states as mentioned in HCA analysis which is related to the similar

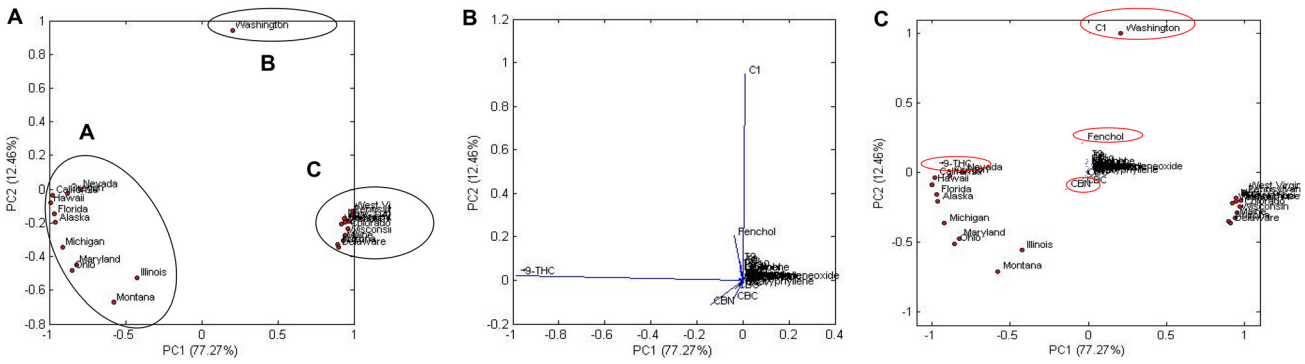


Fig. 3. PCA outputs, (A) score plot, (B) loading plot, and (C) bi-plot obtained for cannabinoids and terpenoids components

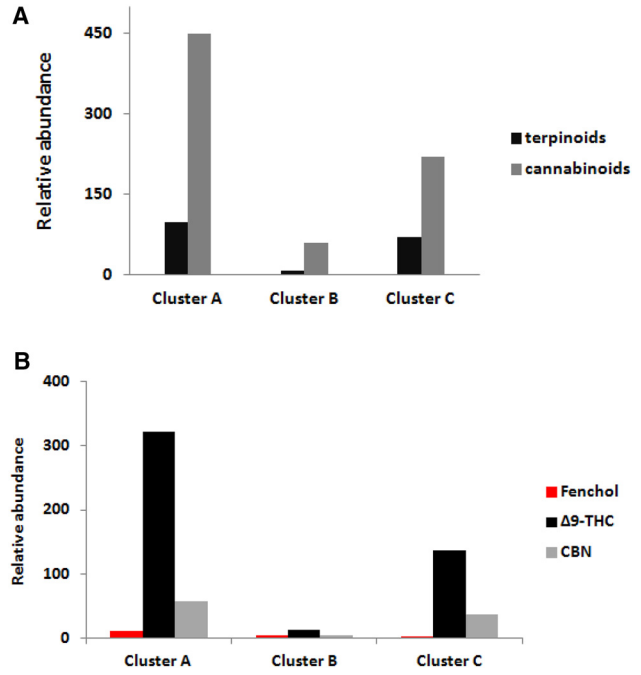


Fig. 4. Total cannabinoids and terpenoids contents in each cluster based on the dendrogram and a full profile for the: A) 42 cannabis without Fenchol, CBN and Δ^9-THC, B) for Fenchol, CBN and Δ^9-THC

and/or comparable contents of cannabis samples. Cluster B has only Washington; accordingly, cannabis samples obtained from Washington is significantly different from the rest of samples from other states, due to distinct contents of C1 than the other states.

PC1 describes 77.27% of the variance of the data set, and as shown in Fig. 3A, PC1 has high positive loading for states of group C which includes 11 states and also positive loading on all cannabinoids and terpenoids except Δ^9-THC, and negative loading for group A which contains 11 states (Fig. 3A) and one content which is Δ^9-THC (Fig. 3B). On the other hand, PC2, accounting for 12.46% of the original information has a significance contribution from Washington State only and C1 component which makes PC2 a "cannabinoid" distinct item. Again, together, these 2 PCs account for 89.73% of the total variance in data.

Figure 3B, loading plot for PC1 and PC2, gives an intuitive explanation whereby the longer the radial separation of the compound from the center, the more important the compound is in distinguishing states. The mathematical explanation is that the radial equals the square sum of the compound's correlations with PC1 and PC2. From the loading plot, it can be seen that the Δ^9-THC was responsible for isolating 11 states Hawaii, California, Florida, Michigan, Alaska, Illinois, Montana, Nevada, Oregon, Maryland and Ohio who all have high content of Δ^9-THC > 3000 and (Table 2). The position of Washington highly depends on C1, since it showed the second highest content overall components of cannabis (6,386).

In conclusion, if States are separated along PC1, they contain a distinct amount of Δ^9-THC, CBN and Fenchol. If cultivars are separated along PC2, they contain different amount of cannabinoids C1.

In PCA loading plot Fig. 3B. that has been illustrated to show the most significant solutes for states clustering. Δ^9-THC and C1 were not correlated with other cannabinoids and terpenoids and more significant for samples clustering. While with low distinct for clustering; Fenchol and CBN showed lower impact on states separation. This result is supported by the results obtained above for the marker solutes and indeed with the our recently published outcomes

Table 3. The average levels of the total 45 compounds in each cluster of Fig. 2

No.	Compound name	Cluster A	Cluster B	Cluster C
1.	α-Pinene	844	13	373
2.	Sabinene	232	48	287
3.	β-Pinene	403	23	214
4.	Myrcene	394	23	193
5.	Limonene	616	10	148
6.	Cineol	79	0	29
7.	Terpinolene	304	22	290
8.	Linalool	718	31	360
9.	Thujone	244	29	145
10.	T1	119	29	129
11.	α-Terpinol	91	0	43
12.	Carveol	162	17	277
13.	Azulene	214	6	158
14.	β-Caryphyllene	1,645	49	1,261
15.	T2	1,211	7	1,767
16.	T3	679	95	205
17.	α-Humulene	579	23	575
18.	T4	171	27	84
19.	T5	320	8	244
20.	T6	802	9	713
21.	T7	987	11	527
22.	Caryophylleneoxide	330	19	255
23.	Guaiol	749	23	338
24.	T8	590	61	998
25.	T9	586	28	632
26.	α-Bisabolol	669	35	470
27.	Fenchol	2,007	614	455
28.	T10	100	6	65
29.	T11	95	8	81
30.	T12	94	7	117
31.	T13	210	23	168
32.	CBDV	433	6	556
33.	THCV	876	55	362
34.	CBL	544	216	145
35.	CBC	3,578	53	2,143
36.	Δ8-THC	557	14	176
37.	Δ^9-THC	53,500	2,191	22,829
38.	C1	768	6,386	587
39.	CBG	2,634	140	1,549
40.	CBN	9,575	616	6,259
41.	C2	495	29	412
42.	C3	189	22	131
43.	C4	627	21	639
44.	C5	783	33	719
45.	C6	310	8	224

for samples clustering, but when only 26 standards were injected as master solutes for clustering [40]. The other 41 cannabis contents were positioned close together and this indicating their limited usage for cannabis states classification. It was interesting to notice the limited performance of some important cannabinoids and terpenoids (CBC and CBG, pinene, etc. . .) for cannabis states clustering compared to Fenchol. In summary, Fenchol, CBN, C1 and Δ^9-THC seems to be the most significant contents for cannabis clustering with comprehensive chemical profiles provided or/and only common contents included (Fig. 4) (Table 3).

As depicted in Fig. 3C, Δ^9-THC, CBN and Fenchol were of high efficiency to separate large number of states from the rest. On the other hand, C1 was dominant to separate Washington away from the rest of cannabis samples obtained from other states. Compared to terpenoids, number of cannabinoids for states clustering is more significant due to their: a) therapeutic uses including pain management and neurological disorders [4, 5, 7, 11], and b) large abundance in cannabis [7]. As shown in Table 2, Δ^9-THC and CBN were available in large excess compared to the rest of compounds, 78,520, 16,450, respectively. Where, Δ^9-THC and CBN have notably large difference in contents than other components. In fact, Δ^9-THC is a common constituent with levels varying even within the same sample depending on the composition of the sample (i.e., leaves vs. bud, vs. mixture and the ratio of small leaves to large leaves). Hence, the variation in THC content among samples is expected. In the same time, CBN is a degradation product of THC and reflects the age of the sample, seeds source and storage environments. Therefore, with the obtained separation among the states, this data set could be used as a database set to simple and fast classification future of unknown sample of cannabis from any of the studied states.

Since Δ^9-THC, CBN from cannabinoids, and Fenchol from terpenoids group showed the highest content over all components, the impact of excluding these 3 contents on states clustering is studied in the next section.

Examination of chemical profiles for distinguished peaks characteristics at specific states: Impact of Δ^9-THC, CBN and Fenchol

To determine if certain distinct chemical "marker" compounds presence in cannabis plants from one State, but absent in plants from another State, will affect the clustering and cannabis classification, data were re-arranged and PCA was run again with excluding the highest three contents from the chemical profiles; Δ^9-THC, CBN from cannabinoids, and Fenchol from terpenoids. PCA analysis carried out 95.98% of the total variance. PCA outputs showed that excluding Δ^9-THC, CBN and Fenchol: 1) has improved the separation of Nevada, Oregon and Illinois from the rest of states as shown in the score plot in Fig. 5A. Interestingly, this

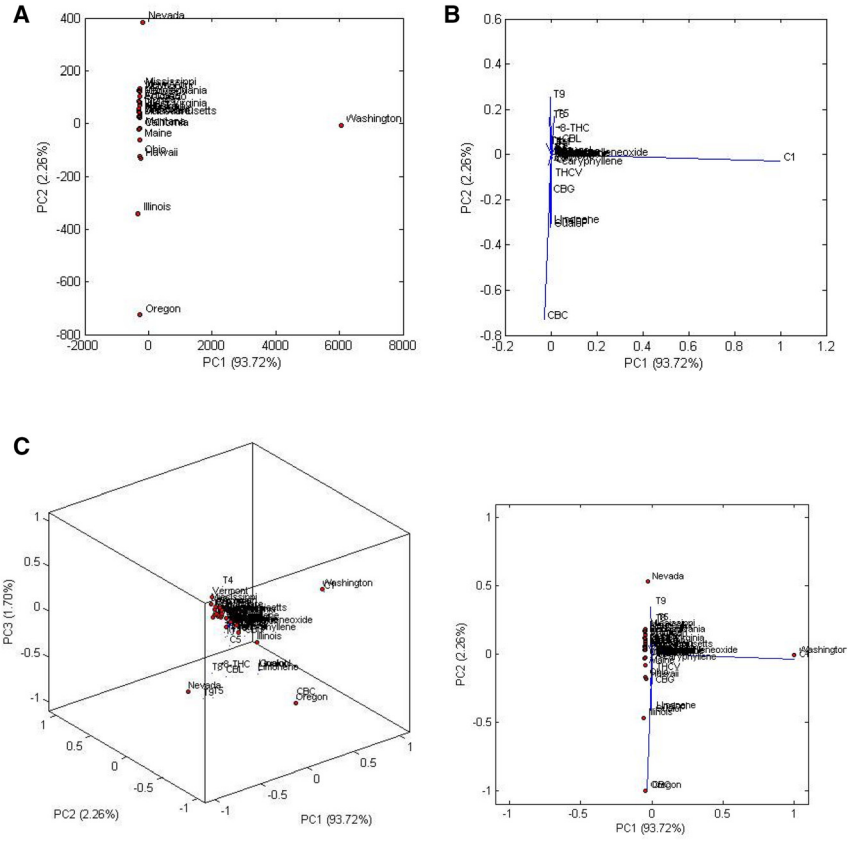


Fig. 5. PCA outputs, (A) score plot, (B) loading plot, and (C) bi-plot obtained for the 42 cannabinoids and terpenoids components while excluding Δ^9-THC, CBN, and Fenchol

states-origin cannabis when Δ^9-THC, CBN and Fenchol were included was clustered in one cluster which is A (Fig. 2) and (Compare Figs 3A and 5A) with Alaska, Montana, Maryland, Ohio, Hawaii, California, Florida and Michigan, and this would indicate the importance of Δ^9-THC, CBN, and Fenchol for these states. 2). In addition, removing Δ^9-THC, CBN, and Fenchol merge cluster A and cluster B (Fig. 2) in only one group of 19 states (i.e., all 11 States of group C with 8 States of group A), which means that Δ^9-THC, CBN and Fenchol are the main responsible for States and cannabis separation. Finally, 3). only Washington State cannabis-origin has not been change in position, which means that this state depends on C1 content and not other compounds since it was not affected upon excluding as shown in PCA (Compare Fig. 3A with Fig. 5A).

As shown in Table 2, Δ^9-THC, CBN, C1 and CBC cannabinoids and Fenchol as terpenoids were available in large excess compared to the rest of compounds, 78,520, 16,450, 7,741, 5,774, and 3,076, respectively. Figure 5B indicated that after excluding Δ^9-THC, CBN, and Fenchol, CBC and C1 were the most significant variables needed for samples clustering. This result supports the data obtained in Table 1 which showed that after Δ^9-THC and CBN, compounds C1 and CBC becomes the second important and largest contents. On the other hand, CBG, CBL and THCV have same influence and both not highly correlated with C1 (angle 90°). The rest of variables (cannabis contents) were accumulated in the center indicating their limited applicability for samples clustering. In fact, this result confirms the reality and the rigidity of the outputs obtained in Table 2 and in Fig. 3 that Δ^9-THC and CBN which have the highest contents; have the largest impact on cannabis clustering, and if excluded; CBC and C1 will be the responsible for cannabis states clustering (Fig. 5). To validate this result, bi-plot (Fig. 5C), has been demonstrated in 2PCs and 3PCs plots for clarification issue only since 2 PCs only has very overlapped data and difficult to be read. This figure indicated that CBC was necessary to separate Oregon while, C1 was necessary to isolate Washington from the rest of states. At this stage, it is clear that sample clustering is highly sensitive to the included cannabinoids, but with low distinct to terpenoids.

CONCLUSIONS

GC-FID was adopted to record the chemical profiling of 45 terpenoids and cannabinoids in 23 USA-cannabis samples. The obtained profiles were further used to cluster cannabis samples with the aid of PCA and HCA. The clustering results would uncover the geographic origin of grown cannabis specimen. Using HCA and PCA, the 23 USA cannabis plants were classified; group A consists of 11 states and also group C, where group B has only Washington State that showed totally different cannabis contents. Multivariate analysis showed also which contents are critical in discriminating cultivars since samples were grouped into 3 clusters; cluster A is THC dominant, cluster B is C1 dominant, and finally cluster C is Fenchol and CBN dominant. The results were different from cluster analysis using THC, CBN and Fenchol content only, which supports the hypothesis that classifications based exclusively on limited numbers of content may be insufficient when considering all medically relevant compounds in cannabis.

Conflicts of interest: The authors declare that there are no conflicts of interest regarding the publication of this paper.

ACKNOWLEDGMENT

Dr. Ramia Al Bakain gratefully acknowledges the financial support for her Fulbright post doctorate year 2017/2018 at The University of Mississippi, USA, provided by (1) The Binational Fulbright Commission in Jordan, and (2) the University of Jordan. Indeed, great appreciation goes to Dr. Omar Marzouk for his experimental help and technical support. Many thanks also go to Dr. Mohamed Radwan, Elsayed Ibrahim, Avery Claire Jones, and Chandrani Gon Majumdar for their assistance.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1556/1326.2020.00767.