Thin-Layer Chromatography and Chemometric Analysis in the Fingerprinting of Selected *Scutellaria* Species

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Summary

Seven different *Scutellaria* species were analyzed using the extraction procedure (Soxhlet apparatus, dichloromethane, and methanol as solvents) and thin-layer chromatography method. Selected standards of flavonoids and phenolic acids (caffeic acid, chlorogenic acid, ferulic acid, baicalein, wogonin, baicalin, chrysin, quercetin, scutellarin, hesperetin, hesperidin, apigenin, luteolin, rutin, and kaempferol) were separated using silica gel thin-layer chromatography (TLC) plates with the mobile phase consisting of ethyl acetate–toluene–formic acid (5:4.9:0.1, *v*/*v*) for dichloromethane and methanolic extracts. Dichloromethane extracts were also developed using cyanopropyl-bonded silica gel with the following mobile phases: propan-2-ol–*n*-heptane–formic acid (5:4.9:0.1, *v*/*v*) and methanol–water–formic acid (6:3.9:0.1, *v*/*v*), and after drying, they were sprayed using the anisaldehyde reagent. In the case of methanolic extracts, the same non-aqueous eluent was used and the aqueous eluent consisting of methanol–water–formic acid (4:5.9:0.1, *v*/*v*). The presence of selected standards in *Scutellaria* species was confirmed. The similarities between the obtained fingerprint chromatograms were performed using chemometric methods, the similarity coefficients (Pearson's correlation coefficient, determination coefficient, and congruence coefficient), distance indices (Euclidean distance, Manhattan distance, and Chebyshev's distance), and multi-scale structural similarity (MS-SSIM).

1 Introduction

The type of *Scutellaria* (*Lamiaceae* family) includes about three hundred species of plants, dispersed in the temperate climate zone and in the mountainous regions of the tropics including Europe, North America, and East Asia [1]. In Poland, it grows in the valleys of large rivers, and the species of the Polish flora are *Scutellaria hastifolia* L., *Scutellaria galericulata* L., *Scutellaria altissima* L., and *Scutellaria minor* Huds. Other species come from cropping. The current research has confirmed that the extracts or monomeric compounds, such as the flavones (baicalin, baicalein, wogonin, wogonoside, oroxylin, and oroxyloside) of *Scutellaria* possess important medical properties [1, 2].

The main group of the active compounds of *Scutellaria baicalensis* Georgi is flavones which exhibit the anti-inflammatory, anti-HIV, anti-SARS coronavirus, and anti-tumor (baicalin, baicalein) as well as anti-respiratory syncytial virus and anti-tumor effects (for wogonin) [3].

The verbascoside occurring in *S. altissima* has shown anti-inflammatory, antibacterial, antioxidant, and antitumor activity [4, 5]. The secondary metabolites from *Scutellaria alpina* L. (baicalin, wogonoside, luteolin, and verbascoside) have been also studied recently for their different biological activities [6]. The extract of *Scutellaria barbata* D. Donis known in traditional Chinese medicine has long been used for inflammation, hepatitis, osteomyelitis, gynecological, lung, and rectal tumors in China and Korea [7]. *Scutellaria albida* L. grown widely in the area from North Italy to the Balkan Peninsula and Crimea exhibits antispasmodic, diaphoretic, and febrifuge properties. It is used in folk medicine [8]. The two new clerodane diterpenoids (jodrellin A and B), which are potent insect antifeedants, were isolated from *Scutellaria woronowii* (Juz.) [9]. Similarly, the two diterpenoids have been isolated from the extract of the aerial part of *Scutellaria rubicunda* Hornem and their insect antifeedant activity was confirmed [10].

The fingerprint thin-layer chromatography (TLC) or high performance thin-layer chromatography (HPTLC) techniques, in one-dimensional (1D) or two-dimensional (2D) forms, play an important role in the preliminary identification of secondary metabolites in plant extracts [11–14]. These simple and rapid techniques are used due to the short time of analysis and low cost of experiments, and the need for only a minimum sample clean-up procedure.

To retrieve more information from the obtained chromatographic results, chemometrics as the application of mathematical and statistical techniques may be used and this method is

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commonly applied for the comprehensive identification and assessment of the fingerprint TLC analysis of potential herbal medicines [15–17].

In our work, optimization and selection of the best of the chromatographic systems were performed to obtain one-dimensional TLC (or HPTLC) fingerprint chromatograms of selected *Scutellaria* species. The plant materials (*S. baicalensis, S. rubicunda, S. alpina, S. altissima,* and *S. woronowii*) were collected from the Botanical Garden (Lublin, Poland), and *S. albida, S. barbata,* and *S. altissima*_2 were harvested from the plantation near Puławy (Poland). All plants were collected in August 2015. All chromatograms were photographed, and the images were processed using the ImageJ program (1.49s version). The different measures of the similarity indices (Pearson's correlation coefficient, determination coefficient, and congruence coefficient), distance indices (Euclidean distance, Manhattan distance, and Chebyshev's distance), and MS-SSIM were used for the evaluation of fingerprint images, and then, principal component analysis (PCA) was performed to confirm the similarity between the selected *Scutellaria* species. PCA is a useful statistical technique that has found application in fields such as face recognition and image compression, and is a common technique for finding patterns in data of high dimension.

The aim of this paper was the fingerprint analysis of extracts from different species of the genus *Scutellaria* to estimate the identity of various plant materials. Seven species were compared using thin-layer chromatography, and then, chemometric and statistical PCA analysis was performed. Our experiments give information about the composition as well as the antioxidative activity of the plant extracts and are helpful in the construction of fingerprints of the examined herbs and varieties to facilitate their identification and assist in *Scutellaria* chemotaxonomy. There are no data in the literature where this problem was investigated.

2 Experimental

2.1 Laboratory Equipment, Apparatus, Reagents, and Standards

Soxhlet apparatus (Archem, Wrocław, Poland), TLCAS 30S applicator (Desaga, Wiesloch, Germany), digital camera Panasonic Lumix DMC-FZ72 16.1 Mpx, TLC sprayer (Desaga), DSII chromatographic chambers (Chromdes, Lublin, Poland), CAMAG Cabinet UV Lamp (CAMAG, Muttenz, Switzerland), and rotary vacuum evaporator (Heidolph, Laboxact, KNF, Germany) were used in all experiments. HPTLC silica gel plates, CN F_{254S}, TLC silica gel 60 F_{254} (Merck, Darmstadt, Germany) were applied. The following solvents: methanol (HPLC gradient grade), propan-2-ol, toluene, ethyl acetate, *n*-heptane, dichloromethane, acetic acid, formic acid, sulfuric acid (all pure per analysis) (POCH, Gliwice, Poland), methoxybenzaldehyde (Sigma-Aldrich, St. Louis, MO, USA), Naturstoff reagent (2-aminoethyl diphenyl borate), polyethylene glycol 4000 (Merck, Darmstadt, Germany), and anisaldehyde (Hadron Scientific, Kielce, Poland) were used.

The standards: caffeic acid, chlorogenic acid, ferulic acid, baicalein, wogonin, baicalin, chrysin, quercetin, scutellarin, hesperetin, hesperidin, apigenin, luteolin, rutin, kaempferol were purchased from Sigma-Aldrich-Fluka (St. Louis, MO, USA).

2.2 Extraction Procedure

Selected *Scutellaria* species **(Table 1)** were dried in the shade and wind. The identities of the plant species were confirmed by a co-worker of the Botanical Garden of the Maria Curie-Skłodowska University, Lublin, Poland. Voucher specimen was placed in the Botanical Garden of the Maria Curie-Skłodowska University. The dried material was powdered using the hand mill, and about 10 g of it was weighed. Then, it was placed in a paper case and extracted using the Soxhlet apparatus for 8 h, applying dichloromethane as the solvent. After drying of the paper case, the same material was extracted for 12 h using methanol as the solvent.

Table 1

Names and the numbers of extracts.

No. of extracts	Name of plant
I	*Scutellaria baicalensis* Georgi
II	*Scutellaria rubicunda* Hornem.
III	*Scutellaria albida* L.
IV	*Scutellaria alpina* L.
V	*Scutellaria barbata* L.
VI	*Scutellaria altissima* L._2
VII	*Scutellaria altissima* L.
VIII	*Scutellaria woronowii* Juz.

The obtained extracts were separately evaporated using rotary vacuum evaporator at the temperature of 70°C. Dry residues were dissolved in methanol and poured into 25-mL graduated flasks. The extracts were stored in dark place in the refrigerator at 4°C.

2.3 Preparation of Standards

Ten milligrams each of fifteen standards (caffeic acid, chlorogenic acid, ferulic acid, baicalein, wogonin, baicalin, chrysin, quercetin, scutellarin, hesperetin, hesperidin, apigenin, luteolin, rutin, and kaempferol) were weighed and dissolved in 1 mL of methanol to obtain about 0.1% solutions.

2.4 TLC and HPTLC Methods

Dichloromethane and methanolic extracts (10 µL) were applied in 6-mm bands on two separate silica gel TLC plates (10 × 10 cm), 5 mm from the edge of the plate, using the Desaga HPTLC applicator AS 30. The chromatograms were developed in DSII chromatographic chamber using the mobile phase ethyl acetate–toluene–formic acid (5:4.9:0.1, *v*/*v*) on a distance of 9.5 cm. Before development, the plates were conditioned in the eluent's vapor for 30 min. Next, after drying, the identification of the

standards in the individual extracts was performed using an UV lamp with 254 nm. The plates with dichloromethane extracts were sprayed using anisaldehyde reagent (10 mL glacial acetic acid was mixed with 85 mL methanol, followed by 0.5 mL anisaldehyde and 1 mL 96% [*m/m*] concentrated sulfuric acid, in that order) for identification of phenols. Next, the plates were dried in a dryer (105°C) for 10 min and visualized in visible light, and the plates with methanol extracts were sprayed using the Naturstoff reagents – 5% methanolic solution of polyethylene glycol (PEG) and 1% methanolic solution of 2-(diphenylboryoxy)-ethylamine – for the identification of flavonoids, and visualized using an UV lamp with 366 nm. Next, the plates were photographed by Panasonic Lumix DMC-FZ72 16.1 Mpx digital camera.

Moreover, the eight samples of dichloromethane and eight samples of methanolic extracts were developed applying HPTLC technique by use of cyanopropyl-bonded plates (5 cm × 10 cm). Seven microliters of extracts were applied using the HPTLC applicator AS 30S. Both types of extracts were developed using non-aqueous and aqueous mobile phases. In the case of non-aqueous eluents, the plates were conditioned for 30 min using the mobile phase vapors before development. For dichloromethane extracts, the following mobile phases were used: propan-2-ol–*n*-heptane–formic acid (5:4.9:0.1, *v/v*) and methanol–water–formic acid (6:3.9:0.1, *v/v*). After drying, the plates with dichloromethane extracts were sprayed using the anisaldehyde reagent. Next, they were dried in an oven (105°C) for 10 min, and, after cooling, they were visualized in visible light.

For methanolic extracts, the non-aqueous eluent (propan-2-ol–*n*-heptane–formic acid; 5:4.9:0.1, *v/v*) was used, and the aqueous eluent consisted of methanol–water–formic acid (4:5.9:0.1, *v/v*). The dried plates were sprayed using the Naturstoff reagent and visualized using an UV lamp at 366 nm. All obtained chromatograms were photographed.

2.5 Similarity and Distance Indices

The following similarity and distance indices were used in our work:

- Pearson's correlation coefficient (R) determines the level of linear dependence between the variables ($-1 < R < 1$). The high absolute value of R confirms the strong relationships between samples.

$$r_{(P,Q)} = \frac{\sum_{i=1}^{n}\left(P_i - \overline{P}\right)\cdot\left(Q_i - \overline{Q}\right)}{\sqrt{\sum_{i=1}^{n}\left(P_i - \overline{P}\right)^2 \cdot \sum_{i=1}^{n}\left(Q_i - \overline{Q}\right)^2}}$$

- The determination coefficient (R^2) determines what percentage of one variable explains the variability of the second variable, $0 < R^2 < 1$. The great similarity of samples is when $R^2 \rightarrow$c1.

$$R^2_{(P,Q)} = \frac{\sum_{i=1}^{n}\left(P_i - \overline{P}\right)^2 \cdot \left(Q_i - \overline{Q}\right)^2}{\sum_{i=1}^{n}\left(P_i - \overline{P}\right)^2 \cdot \sum_{i=1}^{n}\left(Q_i - \overline{Q}\right)^2} R^2_{(P,Q)} = r^2_{(P,Q)}$$

- The congruence coefficient (cosine measure) is the cosine of angle between the vectors in n-dimensional space. When the value is equal to 1, the strong similarity between the samples is confirmed.

$$\cos\theta_{(P,Q)} = \frac{\sum_{i=1}^{n} P_i \cdot Q_i}{\sqrt{\sum_{i=1}^{n} P_i^2 \cdot \sum_{i=1}^{n} Q_i^2}}$$

- The Euclidean distance is the distance between two points in n-dimensional space equal to the length of the segment connecting these points.

$$d_{Euc} = \sqrt{\sum_{i=1}^{n}\left(P_i - Q_i\right)^2}$$

- The Manhattan distance (City Block) is the sum of absolute differences of coordinate pairs of both vectors.

$$d_{CB} = \sum_{i=1}^{n}\left|P_i - Q_i\right|$$

- Chebyshev's distance is the longest linear segment along one of the directions; it determines the greatest difference of coordinates.

$$d_{Cheb} = \max_i\left|P_i - Q_i\right|$$

- MS-SSIM was used to calculate the structural multidimensional parameter of similarity for the quantitative measurement of the quality of recognition in the optical character recognition (OCR) process. It is based on the picture of the analysis in various scales. It was used as the plugin for ImageJ program [18].

3 Results and Discussion

3.1 TLC Results

Some experiments were performed on silica gel chromatographic plates using various mobile phases, and the most selective eluent system was selected to obtain the most efficient separation of standards (ethyl acetate–toluene–formic acid; 5:4.9:0.1, *v/v*). The values of their retardation factor (R_F) are presented in **Table 2.**

The presence of some standards in *Scutellaria* species are presented in **Table 3.**

In the case of cyanopropyl-bonded silica gel, the optimization of mobile phases was performed, and the most selective eluent systems were used. Two different mobile phases were applied (propan-2-ol–*n*-heptane–formic acid [5:4.9:0.1, *v/v*] and methanol–water–formic acid [4:5.9:0.1, *v/v*]). The exemplary HPTLC chromatogram of the analyzed extracts is presented in **Figure 1.** The presence of some standards in the studied *Scutellaria* extracts is presented in **Table 4.**

Table 2

The R_F values of standards for silica gel and CN silica gel.

No.	Standard	R_F value		
		Silica gel	CN silica gel	
		Ethyl acetate–toluene–formic acid; 5:4.9:0.1, *v/v*	Propan-2-ol–*n*-heptane–formic acid; 5:4.9:0.1, *v/v*	Methanol–water–formic acid; 4:5.9:0.1, *v/v*
1	Caffeic acid	0.34	0.53	0.58
2	Chlorogenic acid	0.01	0.36	0.69
3	Ferulic acid	0.40	0.49	0.07
4	Baicalein	0.42	0.44	0.18
5	Wogonin	0.60	0.49	0.58
6	Baicalin	0.01	0.36	0.38
7	Chrysin	0.60	0.51	0.11
8	Quercetin	0.37	0.40	0.18
9	Scutellarin	0.01	0.36	0.44
10	Hesperetin	0.53	0.40	0.20
11	Hesperidin	0.01	0.16	0.53
12	Apigenin	0.40	0.51	0.53
13	Luteolin	0.30	0.51	0.27
14	Rutin	0.01	0.31	0.60
15	Kaempferol	0.52	0.49	0.29

Table 3

Comparison of the presence of standards in various *Scutellaria* extracts detected by UV 245 nm, UV 366 nm, and Naturstoff reagent on silica gel TLC plates. The numbers are as in Table 1.

	Dichloromethane extracts								Methanolic extracts							
	I	II	III	IV	V	VI	VII	VII	I	II	III	IV	V	VI	VII	VII
Caffeic acid	+	+	+	+	+	+	+	+	+	+	+		+	+		+
Chlorogenic acid																
Ferulic acid		+		+			+		+	+		+			+	
Baicalein	+			+?			+			+	+	+	+		+	+
Wogonin																
Baicalin																
Chrysin							+		+	+	+	+		+	+	+
Quercetin										+	+		+	+	+	+
Scutellarin																
Hesperetin	+	+		+	+		+	+		+	+		+	+	+	+
Hesperidin																
Apigenin		+	+	+		+	+	+	+	+	+	+	+	+	+	+
Luteolin										+	+	+	+	+	+	+
Rutin																
Kaempferol			+			+			+	+	+			+		+

Table 4

Comparison of the presence of standards in various *Scutellaria* extracts detected by UV 245 nm, UV 366 nm, and Naturstoff reagent on cyanopropyl-bonded plates. The numbers are as in Table 1.

	Dichloromethane extracts								Methanolic extracts							
	I	II	III	IV	V	VI	VII	VII	I	II	III	IV	V	VI	VII	VII
Caffeic acid	+	+	+	+	+	+	+	+	+	+	+		+	+		+
Chlorogenic acid																
Ferulic acid		+		+			+		+	+		+			+	
Baicalein	+			+?			+			+	+	+	+		+	+
Wogonin																
Baicalin																
Chrysin							+		+	+	+	+		+	+	+
Quercetin										+	+		+	+	+	+
Scutellarin																
Hesperetin	+	+		+	+		+	+		+	+		+	+	+	+
Hesperidin																
Apigenin		+	+	+		+	+	+	+	+	+	+	+	+	+	+
Luteolin										+	+	+	+	+	+	+
Rutin																
Kaempferol			+			+			+	+	+			+		+

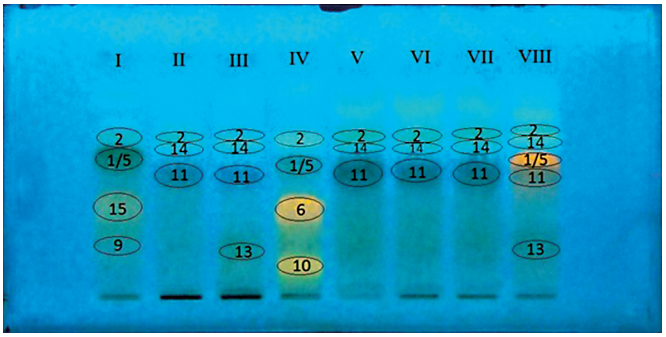

Figure 1

The photography of methanolic extracts of *Scutellaria* on cyano-bonded plate developed with 40% methanol–59% water–1% formic acid. Detection under UV lamp at 254 nm. Arabic numbers are the same as in Table 2, and Roman numbers as in Table 1.

3.2 Chemometric Processing for TLC Data

3.2.1 Silica Gel and Cyanopropyl-Silica Gel Stationary Phases

The obtained TLC results (images of the developed chromatographic plates) were elaborated using the ImageJ program. At the beginning, the images of the silica gel chromatographic plates were modified using macro to align it to the size 1000 × 1000 pixels. Next, every second image was inverted to obtain the light images on a dark background. The "eliminate maxima" and "Gaussian blur filters" were used to eliminate impurities. Then, the areas containing inequality edge were cut off: 5 mm from the top, 5 mm from the left, 2 mm from the bottom, and 12 mm from the right, and as a result, pictures of 830 × 930 pixels were obtained.

The presence of irregularities of all images was observed. Due to different values of the zero level, the baselines were subtracted. Then, from the prepared images, the tracks (corresponding to each extract) were cut out from the left to the right and they were clustered as piles. The piles were pretreated; it means they were rotated by 90 degrees, aligned to the size of 90 × 930 pixels, and smoothed using the Savitzsky–Golay algorithm. In the next step, the similarity indices (the Pearson's correlation coefficient, R; the determination coefficient, R^2; the congruence coefficient – the cosine of vectors; Euclidean, Manhattan, and Chebyshev distances) were calculated, and then the correlation coefficients matrix was generated to determine the trac where alignment should be performed. Additionally, the multi-scale structural similarity (MS-SSIM) measures (luminance, contrast, structure, and MS-SSIM factors) were calculated using the plugin for the ImageJ program. The obtained data were registered in csv (data separated by commas) form, and next, they were aligned using the SpecAlign program with parameter 10. Again, the selected similarity, distance, and MS-SSIM measures were calculated. The picture montages before and after the alignment process were performed.

The obtained images of cyanopropyl silica gel chromatograms were modified using the corresponding macro to obtain the appropriate size. The "eliminate maxima" and "Gaussian blur filters" were used to eliminate impurities.

Next, the tracks corresponding to the individual extracts were cut (from the left to the right) and presented as files. The pre-

Table 5

Measures of similarity for cyanopropyl silica gel.

Type of extract	No. of extract	*R*		R^2		Cosine		Distance measures						Other measure	
		Best correlating	Value of *R*	Best correlating	Value of R^2	Best correlating	Value of cosine	Best correlating	Euclidean	Best correlating	Manhattan	Best correlating	Chebyshev	Best correlating	MS-SSIM
Propan-2-ol–*n*-heptane–formic acid; 5:4.9:0.1, *v*/*v*															
Dichloromethane	1	4	0.8826	4	0.7790	4	0.9336	4	0.7276	4	12.63	4	45.00	2	0.3939
	2	8	0.8629	8	0.7445	8	0.9281	7	0.8256	7	10.36	8	64.67	7	0.4702
	3	7	0.8526	7	0.7269	7	0.9246	7	0.9923	7	15.14	6	64.00	4	0.4397
	4	1	0.8826	1	0.7790	1	0.9336	1	0.7276	1	12.63	1	45.00	3	0.4397
	5	7	0.9290	7	0.8631	7	0.9605	7	0.6074	7	10.22	7	40.00	2	0.4102
	6	7	0.9186	7	0.8438	7	0.9558	7	0.6099	7	9.84	7	42.33	5	0.3768
	7	8	0.9408	8	0.8851	8	0.9687	8	0.5693	8	8.31	5	40.00	2	0.4702
	8	7	0.9408	7	0.8851	7	0.9687	7	0.5693	7	8.31	7	59.00	7	0.4658
Methanolic	1	6	0.9390	6	0.8818	6	0.9992	2	0.2118	2	4.55	2	15.67	4	0.4151
	2	3	0.9886	3	0.9774	3	0.9998	3	0.0867	3	1.85	3	13.00	3	0.6710
	3	2	0.9886	2	0.9774	2	0.9998	2	0.0867	2	1.85	2	13.00	2	0.6710
	4	1	0.7912	1	0.6261	1	0.9974	1	0.4277	1	9.36	1	44.33	1	0.4151
	5	7	0.9896	7	0.9794	7	0.9996	7	0.1250	7	3.31	7	18.33	6	0.6512
	6	7	0.9899	7	0.9799	7	0.9998	3	0.1320	2	3.24	1	21.33	2	0.6568
	7	6	0.9899	6	0.9799	6	0.9998	5	0.1250	5	3.31	3	18.33	6	0.6499
	8	2	0.8681	2	0.7535	2	0.9984	2	0.2487	2	6.04	5	36.00	2	0.5392
Methanol–water–formic acid; 6:3.9:0.1, *v*/*v*															
Dichloromethane	1	4	0.9368	4	0.8776	4	0.9496	4	0.5521	4	8.4294	4	72.67	2	0.2331
	2	5	0.8621	5	0.7433	5	0.9134	5	0.7286	5	11.0595	5	55.67	7	0.5024
	3	8	0.9341	8	0.8726	8	0.9562	8	0.6408	8	9.7869	7	50.67	7	0.5774
	4	1	0.9368	1	0.8776	1	0.9496	1	0.5521	1	8.4294	1	72.67	5	0.3134
	5	7	0.9693	7	0.9395	7	0.9799	7	0.5043	7	6.4046	2	55.67	7	0.5955
	6	7	0.8753	7	0.7661	7	0.9157	7	0.8122	7	9.9857	7	77.33	8	0.3774
	7	8	0.9874	8	0.9750	8	0.9915	8	0.2979	8	4.6518	8	33.33	8	0.6326
	8	7	0.9874	7	0.9750	7	0.9915	7	0.2979	7	4.6518	7	33.33	7	0.6326
Methanol–water–formic acid; 4:5.9:0.1, *v*/*v*															
Methanolic	1	6	0.7759	6	0.6021	4	0.9947	2	0.4797	2	7.7233	2	68.00	4	0.3798
	2	3	0.9686	3	0.9382	3	0.9991	3	0.1836	3	4.506	3	31.67	3	0.5542
	3	2	0.9686	2	0.9382	2	0.9991	2	0.1836	2	4.506	2	31.67	2	0.5542
	4	3	0.8378	3	0.7019	3	0.9958	3	0.4803	2	11.2733	8	39.00	3	0.4688
	5	7	0.9694	7	0.9397	7	0.9989	7	0.1921	7	3.682	7	35.67	6	0.6730
	6	7	0.9909	7	0.9818	7	0.9997	7	0.1333	7	3.3103	7	19.00	5	0.6730
	7	6	0.9909	6	0.9818	6	0.9997	6	0.1333	6	3.3103	6	19.00	6	0.6603
	8	3	0.8948	3	0.8006	3	0.9971	3	0.3299	3	7.0087	6	38.00	3	0.4914

treatment process was performed: they were rotated 90 degrees, expanded to 90 pixels in vertical and smoothed by the Savitzsky-Golay algorithm. Similar to the silica gel, the same similarity indices and the MS-SSIM parameter were calculated and the standard track was selected.

Some of the data were recorded in csv form, then the alignment process was performed using SpecAlign with parameter 20 and again the similarity, distance measures and MS-SSIM were calculated. The images before and after alignment process were presented.

It is impossible to perform unequivocal confirmation of the similarity by TLC and the used chemometric methods, but a preliminary estimation may be carried out. In our study, the similarity between silica gel and CN-silica gel chromatograms of the *Scutellaria* species was verified using various similarity and distance measures. The best correlating samples (numbers 1–8 mean samples of *Scutellaria* species) are presented in **Table 5.** The extensive data obtained for silica gel are not presented due to worse results – these are shown in abbreviated form in the section dealing with PCA. Generally, in most cases, among the data of the cyanopropyl-bonded silica gel, high values of R ($0.77 < R < 0.99$), R^2 ($0.70 < R^2 < 0.98$) and cosine ($0.91 <$ cosine < 0.99) were obtained (Table 5).

The similarity between samples 6 (*S. altissima*_2) and 7 *(S. altissima)* was confirmed for both dichloromethane and methanolic extracts using the above similarity indices. The values of Euclidean, Manhattan, and Chebyshev distances confirm the similarity of samples 6 and 7 for dichloromethane extracts (for aqueous and non-aqueous eluents) and for methanolic extract (for aqueous mobile phase). Samples 7 *(S. altissima)* and 8 *(S. woronowii)* are also similar; it was confirmed for dichloromethane extracts and non-aqueous eluent by R, R^2, cosine, Euclidean, and Manhattan parameters and in the case of aqueous eluent by all similarity and distance measures. For methanolic (aqueous and non-aqueous eluents) and for dichloromethane extracts (non-aqueous eluent), all similarity and distance measures confirm the similarity between samples 5 *(S. barbata)* and 7 *(S. altissima).* The similarity between *S. baicalensis* (1) and *S. alpina* (4) was confirmed for dichloromethane extracts by all similarity and distance measures. Both similarity and distance parameters confirmed the similarity between *S. albida* (3) and *S. rubicunda* (2) for methanolic extracts with aqueous and non-aqueous mobile phases.

3.2.2 PCA

After chemometric processing, the following matrices were obtained: 930 rows and 8 columns (for silica gel stationary phase), and 538 rows and 8 columns (for cyanopropyl-bonded silica gel). The analysis in both cases was performed using the PCA Mollegro program; and a set of 8 eigenvalues, eigenvectors matrix, and results matrix were obtained. Based on the eigenvalues, the percent of variation (determined by the principal component) was calculated.

The charts were created based on the eigenvectors matrix, where the number of columns and rows corresponded with the number of the studied extracts. The similarity between the extracts (corresponding with the appropriate rows and numbers) was illustrated as close proximity of points.

The obtained PCA results are graphically presented in **Figure 2** (for silica gel) and **Figures 3 and 4** for cyano-bonded silica gel. The proximity of points shows the similarity between the samples. Figure 2 presents the PC2 vs. PC1 graphs for silica gel before and after the alignment process. The two graphs are similar, and one point corresponding with sample 4 *(S. alpina)*

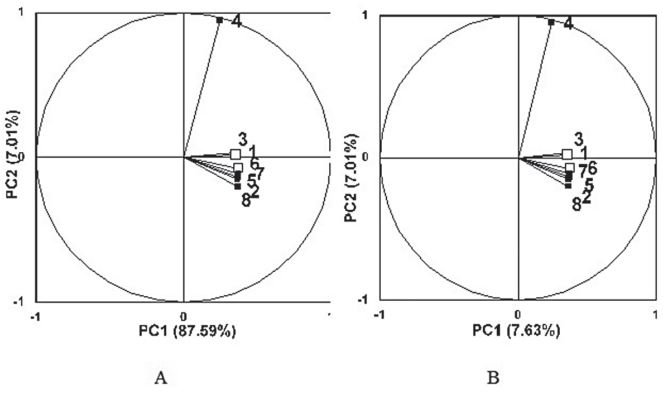

Figure 2

The PCA graphs for methanolic extracts with silica gel and ethyl acetate–toluene–formic acid (5:4.9:0.1, *v*/*v*): A, before alignment; B, after alignment. The numbers are as in Table 1.

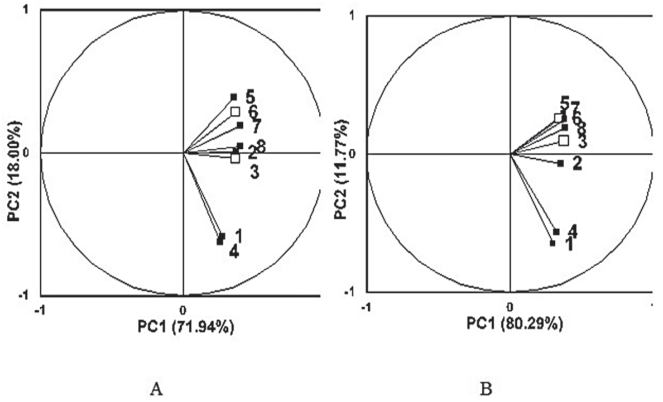

Figure 3

The PCA graphs for dichloromethane extracts with CN-silica gel: A, propan-2-ol–*n*-heptane–formic acid (5:4.9:0.1, *v*/*v*); B, methanol–water–formic acid (6:3.9:0.1, *v*/*v*). The numbers are as in Table 1.

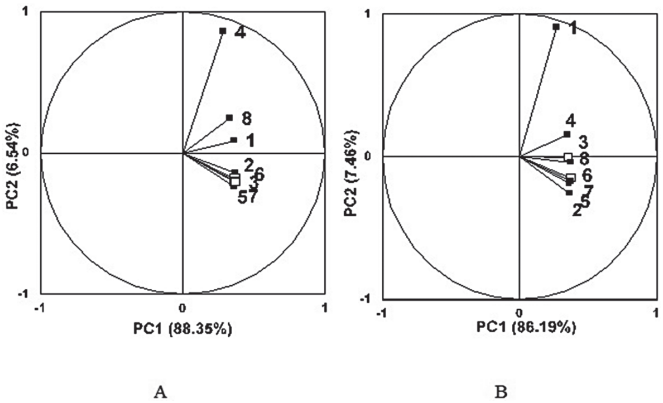

Figure 4

The PCA graphs for methanolic extracts with CN-silica gel: A, propan-2-ol–*n*-heptane–formic acid (5:4.9:0.1, *v*/*v*); B, methanol–water–formic acid (4:5.9:0.1, *v*/*v*). The numbers are as in Table 1.

clearly stands out. Other points (corresponding with the other extracts) are close to each other. Clear grouping of individual vectors is not shown; within the cluster, only two pairs of vectors are closely similar: 3 with 1 and 6 with 7.

In the case of cyanopropyl-bonded silica gel, the vectors are more scattered and, therefore, the similarity between the studied plants is easily noticed. In Figure 3, where the dichloromethane extracts were compared on aqueous and non-aqueous eluents, the individual vectors may be divided into three groups, especially for the non-aqueous mobile phase: the first – samples 5, 6, and 7; the second – 2, 3, and 8; and the third – 1 and 4. For the aqueous eluent, samples 1 and 4 also differ from the others, but among other samples, point 2 is located further. In the case of methanolic extracts (Figure 4), the results are different. For the non-aqueous eluent (Figure 4A), point 4 differs significantly from the others; samples 1 and 8 are close and the other points corresponding with samples 2, 3, 5, 6, and 7 form the cluster. In the case of the aqueous mobile phase, sample 1 differs from the others. Among the other points, the following clusters were observed: 2, 5, 6, and 7; next, 3 and 8. Sample 4 is located further (Figure 4B).

4 Conclusion

The dichloromethane and methanolic extracts of seven *Scutellaria* species were analyzed using the TLC method. The presence of selected standards of flavonoids and phenolic acids was confirmed in the studied plant extracts. Generally, the higher numbers of standards were noted in methanolic extracts rather than in dichloromethane for both stationary phases (silica gel and cyanopropyl-bonded silica gel) and aqueous and non-aqueous eluents.

The similarity of the selected *Scutellaria* species was confirmed using the similarity and distance measures. Better results were obtained for cyanopropyl-bonded silica gel where the higher values of the similarity measures were noted (in most cases, R, R^2, and cosine were higher than 0.9). The similarity between the samples was also confirmed by the distance parameters (Euclidean, Manhattan and Chebyshev distances) and MS-SSIM. The greatest accordance between the extracts was obtained for *S. altissima*_2 (sample 6) and *S. altissima* (sample 7) and confirmed by the similarity and distance measures for dichloromethane and methanolic extracts. In most cases, the similarity of samples 7 *(S. altissima)* and 8 *(S. woronowii)* was also confirmed. For methanolic (aqueous and non-aqueous eluents) and for dichloromethane extracts (non-aqueous eluent), all similarity and distance measures confirmed the similarity between *S. barbata* and *S. altissima.* The similarity of *S. baicalensis* and *S. alpina* was confirmed for dichloromethane extracts by all similarity and distance measures. For methanolic extracts (aqueous and non-aqueous mobile phases), the similarity and distance parameters confirmed the similarity of *S. albida* and *S. rubicunda.*

In most cases, the similarity of the selected *Scutellaria* species was confirmed using PCA analysis.

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