Validated Binary High-Performance Thin-Layer Chromatographic Fingerprints of Polyphenolics for Distinguishing Different *Salvia* Species

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Abstract

***Salvia* (sage) is the largest plant genus in the family Lamiaceae, embracing ca. 900 species. There is a growing interest in investigating chemical contents of different *Salvia* species, as some of them have been reported to exhibit a wide spectrum of biological activity. In this paper, conjugated (i.e., binary) chromatographic fingerprints have been introduced for the twenty *Salvia* species that are grown and cultivated in Poland. Apart from videoscans traditionally used for a comparison of the high-performance thin-layer chromatography fingerprints, digital scanning profiles and images obtained with use of the image processing program have also been employed. It is the first time that binary chromatographic fingerprints are used for the investigation of chemical contents of the *Salvia* species. The proposed procedure is rapid when compared with the similar ones presented in the literature, and moreover, it is easy to handle. The proposed method offers a possibility to discern the investigated species. It can be applied not only for chemotaxonomic purposes but also for finding new plant species that can be used as medical herbs (as it has been proposed, with *S. triloba* having a similar profile to *S. officinalis*). Validation of the method reveals that it is specific, reproducible, precise, and robust.**

Introduction

Salvia (sage) genus embraces ca. 900 species, is widespread throughout the world, and includes several ornamental, culinary, and medical species (1). Different species from this genus are commonly used in traditional medicine due to a wide spectrum of biological activity (e.g., antioxidant, antiplatelet, antitumor, antiviral, etc.). Over the years, classification of different *Salvia* species has been very confusing. Many of them are similar to each other, and lots of them produce the varieties that have been given different specific names (2).

In recent years, much attention has been paid to biologically active, water-soluble components, mainly polyphenolic flavonoids, phenolic acids, and simple coumarins, as *Salvia* preparations are used predominantly as water decoctions. The majority of phenolic acids in the *Salvia* species are the caffeic acid derivatives, whose depsides (e.g., rosmarinic and chlorogenic acid) are believed to be the major biologically active constituents (1). According to the literature, rosmarinic acid is the major phenolic compound responsible for antioxidant activity of *Salvia* samples (3). When it comes to flavonoids, flavones (apigenin, luteolin derivatives), flavonols (kaempferol and quercetin methyl esters), and their glycosides are the most widespread among the *Salvia* species (1). Investigation of chemical composition of the *Salvia* extracts can probably help to better understand the biological potential and the taxonomic relationships among the investigated species.

Chromatographic fingerprint profiling is a very convenient and effective method for quality assessment of herbal materials (4). By definition, chromatographic fingerprint is a chromatogram that represents chemical characteristics of herbal medicine (5). Several chromatographic techniques have been employed to construct fingerprints, such as high-performance liquid chromatography (HPLC) (6–9), high-performance thin-layer chromatography (HPTLC) (4,10–13), gas chromatography (GC) (14), capillary electrophoresis (CE) (15,16), high-speed counter-current chromatography (HSCCC) (15,17), etc. Even, if HPLC is the fingerprinting method of the first choice due to its specificity, good separation power, and an ability to derive a plethora of chemical information (11), HPTLC offers a number of unique features that can outperform the other separation techniques used for the fingerprinting (18). Identification is one of the leading targets of HPTLC, and it is the only chromatographic method that allows presentation of the results in the picture form. The great advantage of HPTLC is the speed of method development also and its flexibility (12).

As plant extracts usually are very complex, it is hardly possible to find one chromatographic system enabling a satisfactory resolution of the sample's constituents. In a vast majority of published methods, chromatographic fingerprints are based on a single chromatogram. However, with complex natural samples

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such an approach can prove inadequate (5). Several approaches have been proposed to fingerprint construction with very complex natural samples. Certain authors recommend a combination of analytical methods with different separation principles as with the development of *Caulophyllum robustum* fingerprint (19). Other authors advertise the construction of "multiple chromatographic fingerprints" as a beneficial solution (5). Hyphenated chromatographic techniques (HPLC–mass spectrometry, LC×LC, HPLC–nuclear magnetic resonance) also produce the information-rich fingerprints that are applicable to herb analysis (20,21). In paper (22), application of special modes of the chromatogram development has been proposed for complex samples. All the proposed techniques can provide the reliable results, yet in their case method development is not a trivial task. The application of hyphenated techniques and the combination of analytical methods demands usage of sophisticated equipment, whereas construction of multiple fingerprints and the use of special development modes can effectively be performed by means of HPTLC.

Several examples of *Salvia* fingerprints are present in the literature (15,17,20,23,24). Most of them focus on development of chromatographic profiles of *Salvia miltiorrhiza*, a traditional Chinese medicinal herb used for treating coronary heart disease, hepatitis, or insomnia. However, no chromatographic method has yet been established for a comparative analysis of the different *Salvia* species, especially those characteristic of Europe. Besides, all the existing methods focus on defining the acceptance criteria for one species only. Thus, for the chemotaxonomic research and also for the comparative studies, development of a new method is an urgent task.

Validation of qualitative methods is usually based on specificity, which has been commonly accepted as an only relevant aspect of an identification method. Recently, the trend started growing in favor of the adoption of the concepts of precision (repeatability, intermediate precision, and reproducibility), stability, and robustness as the separate validation points for the identification methods (25).

In this study, we present the development of the binary HPTLC chromatographic fingerprints with polar and semipolar constituents for a variety of *Salvia* species. The main focus is on the presence of polyphenolics as major constituents, justifying common use of the different sage preparations. The method was validated according to the latest directives referring to the qualitative HPTLC methods (25). It is for the first time that the conjugated and comprehensively validated HPTLC fingerprint is used for authentication of species. Modeling of activity (e.g., extraction of information or the chemometric aspects of the fingerprint data analysis) remains outside the scope of this paper.

Experimental

Apparatus and reagents

The following standards, caffeic acid, chlorogenic acid, *trans-p*-coumaric acid, ferulic acid, protocatechuic acid, and rosmarinic acid were obtained from ChromaDex (Santa Ana, CA), and the remaining standards were purchased from Aldrich (St. Louis, MO).

n-Hexane, methanol (96%), toluene, ethyl acetate, and formic acid used as the extracting solvents or the mobile phase components were manufactured by Merck (Darmstadt, Germany). Acetic acid and sulfuric acid were obtained from Polish Reagents (Gliwice, Poland). All these solvents were of analytical purity grade. $AlCl_3$ was obtained from Fluka (Buchs, Switzerland).

TLC was performed on glass-backed silica gel HPTLC 60F254s plates purchased from Merck (10 cm × 20 cm and 10 cm × 10 cm).

Solutions of all test substances and plant extracts were applied to the chromatographic plates band-wise by means of a Camag automatic TLC sampler (Muttenz, Switzerland) and developed in the horizontal DS chambers (Chromdes, Lublin, Poland). Location of the bands was carried out in visible and UV (λ = 254 and 366 nm) light after derivatization. Chromatograms were documented with use of the Camag TLC Reprostar 3 device with Videostore computer program and scanned with the Camag TLC scanner 3 densitometer equipped with the CATS 4 computer program. Additionally, videoscans and densitograms were processed with use of Image J image processing program, which is available on-line and elaborated by the National Institutes of Health.

Table I. The Used Polyphenolic Standards

Substance	Symbol	RF of solvent system 1	Color after spraying with H_2SO_4 (λ = 366 nm)
Rutin	R	0.0	Brown
Kaempferol	K	0.09	Purple
Luteolin	L	0.09	Purple
o-Coumaric acid	*o*-C	0.22	Pale orange
Caffeic acid	Cf	0.11	Dark blue
Gallic acid	Ga	0.06	Dark purple
Rosmarinic acid	Rm	0.04	Dark purple
Protocatechuic acid	Pc	0.11	Purple
Ferulic acid	F	0.22	Brown
Chlorogenic acid	Ch	0.0	Purple
Aesculetin	Ae	0.10	Blue
Scopoletin	Sc	0.20	Blue
Vanillic acid	V	ND*	–
Sinapinic acid	S	0.40	Purple
trans-p-Coumaric acid	*tp*-C	0.42	Orange
Gentisic acid	G	0.45	Brown
7-Hydroxycoumarin	hCo	0.47	Blue
Cinnamic acid	Ci	ND*	–
3,5-Dihydroxybenzoic acid	B	0.25	Dark green
Coumarin	Co	ND*	–
Acacetin	A	ND*	–
Naringenin	N	0.44	Purple
Hiperosid	H	0.0	Brown
p-Coumaric acid	*p*-C	0.49	Pink/brown†
m-Coumaric acid	*m*-C	0.41	Dark green
Quercetin	Q	0.0	Brown
Herniarin	He	0.55	Blue
Hesperitin	Hs	0.46	Purple
Apigenin	Ap	ND*	–

* Not detected at λ = 366 nm. † After some time.

Standard substance solutions and plant extracts

All reference substances were dissolved in methanol to prepare the 1.0 mg/mL solutions. The list of the used standards is given in Table I.

Extracts from the twenty dried *Salvia* species, namely: *S. lavandulifolia,* 1; *S. sclarea,* 2; *S. tesquicola,* 3; *S. staminea,* 4; *S. amplexicaulis,* 5; *S. cadmica,* 6; *S. pratensis,* 7; *S. canariensis,* 8; *S. nemorosa,* 9; *S. jurisicii,* 10; *S. stepposa,* 11; *S. hians,* 12; *S. officinalis,* 13; *S. forskaohlei,* 14; *S. azurea,* 15; *S. verticillata,* 16; *S. triloba,* 17; *S. deserta,* 18; *S. glutinosa,* 19; *S. atropatana* 20 (Table II); were obtained from the plants collected in the Pharmacognosy Garden (Lublin, Poland) in the course of the vegetation periods of 2007 and 2008. Botanical material was authenticated and voucher specimens were deposited at the Department of Pharmacognosy, Medical University of Lublin, Poland. This material was oven-dried at a temperature not exceeding 40°C for 40 h and remained frozen until the day of analysis.

All samples were milled into a powder and oven-dried at 35°C until a constant weight was obtained. Accelerated solvent extraction (ASE) was performed with the ASE extractor (Dionex, Sunnyvale, CA). Firstly, 5 g of each sample was extracted with *n*-hexane and then with methanol. *n*-Hexane extracts were discarded, as they contained mainly chlorophylls and apolar, water-insoluble constituents. The optimized extraction conditions were: temperature, 40°C (*n*-hexane) and 100°C (methanol); pressure, 68 atm (*n*-hexane) and 65 atm (methanol); initial heating time, 10 min (*n*-hexane) and 2 min (methanol); heating time after introduction of solvent, 5 min (both solvents); static extraction time, 5 min (both solvents); solvent volume, 40 mL (both solvents); the number of cycles, 2 (both solvents). The obtained methanol extracts were evaporated to dryness in a rotary evaporator the under reduced pressure, and the residue was dissolved in 5 mL of methanol. All sample solutions were then placed in an ultrasonic bath (model RK 255H Sonorex Super; Bandelin, Berlin, Germany) for 15 min. Finally, the samples were concentrated to a volume of 1 mL each, and filtered through a membrane filter Anotop (0.02 µm, cat. # 11320; Merck). Samples prepared in such a way underwent the chromatographic analysis.

Table II. Extracts from the 20 Dried *Salvia* Species

Name	Identification Number used in Figures
S. lavandulifolia	1
S. sclarea	2
S. tesquicola	3
S. staminea	4
S. amplexicaulis	5
S. cadmica	6
S. pratensis	7
S. canariensis	8
S. nemorosa	9
S. jurisicii	10
S. stepposa	11
S. hians	12
S. officinalis	13
S. forskaohlei	14
S. azurea	15
S. verticillata	16
S. triloba	17
S. deserta	18
S. glutinosa	19
S. atropatana	20

Chromatography

Standard and sample solutions were applied to the 10 cm × 20 cm silica HPTLC plates band-wise (band length 12 mm, 1 µL/s delivery speed, track distance 1 mm, distance from the left edge 10 mm and from the low edge 10 mm). With the 10 cm × 10 cm-plates, the following application parameters were set: band length 5 mm, 1 µL/s delivery speed, track distance 6 mm, distance from the left edge 5 mm and from the low edge 10 mm. The 5-µL aliquots of standard solutions and 10-µL aliquots of the investigated *Salvia* samples were applied onto the plates. The plates were dried in a hood for 10 min before development. The plates were developed in the horizontal DS chambers without chamber pre-saturation step at ambient temperature 20 ± 1°C. The following mobile phases were used for the less polar constituents: solvent system 1, 1a, 1b, 1c; for polar and highly polar substances, the following eluents were applied: 2, 2a, 2b, and 2c (mobile phases' composition can be found in Table III). The plates were developed to the distance of 90 mm. The plates were dried at room temperature for 15 min prior to derivatization.

Derivatization and documentation

Sulfuric acid reagent was prepared by mixing 10 mL concentration sulfuric acid with 90 mL methanol. Both reagents were cooled in a refrigerator for 30 min prior to mixing. The $AlCl_3$ derivatizing agent was prepared by dissolving 1 g aluminum chloride in 99 g methanol.

For detection of semi-polar constituents, the plates developed with mobile phase 1 were sprayed with 10% concentration H_2SO_4 in methanol and heated at 105°C for 10 min. The plates developed with eluent 2 were sprayed with the $AlCl_3$ reagent and documented. The plates were sprayed with use of the automatic spraying device (Merck).

Plate images were taken in visible and UV (λ = 254 and 366 nm) light, and scanned at λ = 254 and 366 nm with the slit parameters 1 mm × 0.1 mm. Bands in the analyzed samples were identified by matching their RF values and UV-Vis spectra with those obtained for the standards. Peak purity was checked by recording the UV-vis spectra at the centre and the peak flanks.

Table III. Mobile Phases Used in the Experiment

Symbol	Mobile phase (quantitative composition, v/v)
1	Toluene–ethyl acetate–formic acid (70:30:1)
1a	Toluene–ethyl acetate–formic acid (68:32:1)
1b	Toluene–ethyl acetate–formic acid (72:28:1)
1c	Toluene–ethyl acetate–formic acid (70:30:0.8)
2	Ethyl acetate–water–formic acid–acetic acid (100:26:11:11)
2a	Ethyl acetate–water–formic acid–acetic acid (98:28:11:11)
2b	Ethyl acetate–water–formic acid–acetic acid (102:24:11:11)
2c	Ethyl acetate–water–formic acid–acetic acid (100:26:10:12)

Method validation

Method was validated for its specificity, precision (repeatability and intermediate precision), stability, and robustness, according to the recognized AOAC guidelines (25). Precision was expressed as precision of the positions (the RF values) of the separated zones. Checking repeatability, three individual portions of a given *Salvia* sample were prepared according to the earlier described method. Three aliquots of each sample were applied onto the three plates. The plates were subsequently chromatographed using the same chamber but with a fresh portion of mobile phase each time. The intermediate precision test was performed in an analogous way, i.e., a number of the selected *Salvia* species were chromatographed on the three different days, one sample per one species. Variability of RF values for several "marker zones" across each plate and variability of the average RF values of those markers on the three plates was evaluated.

Stability on the plate was determined by comparing fingerprints obtained for the samples applied to the plates by 1, 2, and 3 h prior to the development with the fingerprint obtained for the sample applied just before the development.

Stability of the sample in solution was determined by multiple measurements of one and the same sample solution at different storage times, i.e., after 0, 6, 24, and 48 h. In order to check sample stability during the chromatographic process, a simple 2D test was performed, i.e., selected *Salvia* samples were chromatographed with the same eluent in both directions. To this effect, solvent systems 1 and 2 were checked.

The influence of the mobile phase composition, heating temperature, and drying time on the final results was also checked: In this part of the experiment, mobile phases 1a, 1b, 1c, 2a, 2b, and 2c were tested. The plates were sprayed with 10% concentration H_2SO_4 solution in methanol and then heated at 95, 100, 102, 105, 107, and 110°C. To check the influence of the drying time, the plates were derivatized with use of sulfuric acid reagent 15, 20, 30, and 45 min after the removal from a chromatographic chamber.

Results and Discussion

It is essential for the fingerprint analysis to work with an authenticated botanical reference material. To this effect, all *Salvia* species analyzed in our experiment were grown in the same conditions and harvested on the same day, and they have undergone an identical drying procedure.

Application of solvent system 1 enabled separation of less and semi-polar compounds. However, certain higher polar substances remained at the start, e.g., rutin, chlorogenic acid, or quercetin (Table I), as it can be seen from Figures 1–2 (See Page 3A). For the separation of these compounds, solvent system 2 was used. Then the less polar constituents were squeezed near the front line of the chromatogram with an inadequate separation (Figure 2, See Page 3A). Use of the two chromatographic systems generates comprehensive information on a botanical drug. The band of rosmarinic acid appeared as a "boundary" between the less and the more polar fraction on the HPTLC images (Figure 3, See Page 4A), and these images were linked to give conjugated fingerprints of the *Salvia* species. The applied chromatographic systems caused separation of several polyphenolic compounds present in different *Salvia* samples. However, some overlapping bands can be seen (Figure 1–2, See Page 3A). For fingerprint construction, complete resolution of all the substances present in the extract is not needed. For accurate quantitative data, further adjustments of the presented systems should be performed. Multiple fingerprints have been generated from the same plate by multiple detections, different derivatizing agents, detection under visible or UV light, etc., as presented in Figure 3, See Page 4A.

Applying commonly used $AlCl_3$ solution caused this reagent, several blue spots were visualized on the plate developed with solvent system 1. However, with several species (e.g., *S. hians, S. verticillata, S. pratensis, S. azurea,* etc.), either no spots at all or very pale ones appeared. The use of sulfuric acid as a visualizing reagent produced chromatograms with a greater amount of chromatographic bands, thus generating more informative fingerprints (Figure 1, See Page 3A). The $AlCl_3$ solution turned out a better choice for the fingerprints generated with use of solvent system 2, which was aimed at the more polar compounds. Several spots visible in UV light (λ = 366 nm) appeared on the chromatograms due to the presence of polar flavonoid glycosides, aglycones, and more polar phenolic acids (Figure 2, See Page 3A).

All the investigated samples were checked for the presence of 29 polyphenolics by comparing the RF values, colors, and UV-Vis spectra for the bands of the standards with those originating from the samples. However, for the fingerprint comparison, bands with an unknown identity were also taken into the account. For fingerprint construction, the identity of all the components does not have to be known. The comparison of the samples is performed on the basis of the obtained HPTLC images.

Several zones have been identified in all analyzed *Salvia* species, which can be regarded as the markers. The presence of two phenolic acids, very important from the medicinal point of view (i.e., caffeic and rosmarinic acid) was confirmed in all the samples. In chromatographic system 1, rosmarinic acid shows RF = 0.04 (blue zone before derivatization and dark purple one after derivatization with the sulfuric acid reagent). Its presence is even better confirmed upon application of solvent system 2. Rosmarinic acid appears as a blue band (RF = 0.77) visualized by the $AlCl_3$ reagent. In chromatographic system 1, caffeic acid can be identified as a blue band (RF = 0.11). There are also other common zones, obtained with use of eluent system 1, that is characteristic of all the investigated *Salvia* species. One is a pink band of *p*-coumaric acid (UV, λ = 366 nm), which appears soon after derivatization with RF = 0.49. It can also be detected in visible light as an intense purple zone that turns brown some time after derivatization with the sulfuric acid reagent. Two *Salvia* species, *S. sclarea* and *S. canariensis*, are characterized by relatively low amounts of this substance when compared with the other investigated plant species. Another characteristic pink zone (turning orange after certain period of time; UV, λ = 366

nm) can be identified in all fingerprints obtained with chromatographic system 1 (RF = 0.26), although it is less distinct with *S. canariensis*. There is also one orange band at RF = 0.58 noticeable in all the investigated samples. In the case of fingerprints obtained after application of solvent system 2, the following bands can be considered markers for all the investigated species: blue spot of rosmarinic acid, RF = 0.77 ($AlCl_3$ reagent, UV light, λ = 366 nm), and a band at RF = 0.61, corresponding to rutin. Chlorogenic acid was identified in several *Salvia* species, such as in *S. pratensis, S. amplexicaulis*, and *S. nemorosa*. Rutin and chlorogenic acid were identified only upon using solvent system 2, and with chromatographic system 1 they always remained at the start line.

The digital scanning profiles and those obtained with application of the Image J processing program are used in parallel with the traditional ways of presenting fingerprints. Binary chromatographic fingerprints are used for a comparison of the constituent contents in the extracts obtained from the dried *Salvia* samples and to define similarities and differences in the chemical profiles of the investigated plant species. Digital scanning profiles allow detection of the constituents that are difficult to see in the videoscans and also to assess the amounts of compounds (Figure 4, See Page 3A). This option has proved very useful with the plates developed in solvent system 2 and derivatized with the $AlCl_3$ solution. Bands not clearly seen in the videoscans were well presented in the densitograms. The "image calculator" function of the Image J program was applied to compare the fingerprints, exposing differences that otherwise can easily pass unnoticed (Figure 5).

Salvia officinalis is the only pharmacopoeial species in this study present in the traditional Polish medicine. Fingerprint analysis can prove useful in targeting species that have similar chemical profiles and hence are good candidates for herbal remedies. A comparison of fingerprints obtained from solvent systems 1 and 2 shows that *S. triloba* has very similar chemical profiles to those of *S. officinalis* (Figure 5B). Both species characterize with the presence of dark bands in the less polar fraction (under the orange band of *p*-coumaric acid with the RF values of 0.38 and 0.44, and one above *p*-coumaric acid with an RF = 0.57). The aforementioned dark bands can also be seen in the fingerprint of *S. canariensis*, although this species characterizes with a relatively low amount of *p*-coumaric acid. Despite very similar fingerprints, a closer looks enables differentiation between *S. officinalis* and *S. triloba*. This can be done comparing the more polar fraction. Here an additional yellow zone is also present in the lower RF range in the case of *S. triloba*. Other species produce distinctive fingerprints of their own, so *S. officinalis* can easily be distinguished from them.

For a better description of chromatographic profiles, we propose to divide the whole chromatogram obtained with solvent system 1 into three parts, using two marker compounds (RF = 0.26 and RF = 0.49) as the border zones, as presented in Figure 5. The amount, position, color, and intensity of all bands present in each of the three parts were compared for all the investigated samples. Densitograms and images constructed with use of Image J program were also utilized for the comparative studies. A comparison of each part of each fingerprint revealed that all investigated *Salvia* species characterize with an identical or at least a very similar profile of the first part (zone between the application position and the marker band at RF = 0.26). This part of the fingerprint can be regarded as characteristic for all the investigated *Salvia* samples. The other two zones of the chromatogram are more specific for the individual species and can be used for differentiation thereof.

The following *Salvia* species produce comparable overall fingerprints, obtained with chromatographic system 1: *S. tesquicola, S. amplexicaulis, S. cadmica, S. nemorosa, S. jurisicii, S. stepposa, S. glutinosa,* and *S. atropatana*. Similarity can also be observed for the same species in the case of the fingerprints of more polar constituents. All the aforementioned species are characterized by the presence of zones (UV light, λ = 366 nm) with the lower RF values that are missing in the chromatographic profiles of the other investigated plant samples (solvent system 2). This group of *Salvia* species can further be divided into the smaller subgroups, based on the obtained fingerprints. *S. tesquicola, S. amplexicaulis,* and *S. cadmica* can easily be differentiated from the rest, due to the presence of a blue band of herniarin (RF = 0.55) (marked in Figure 5A). However, with *S. cadmica*, a zone at RF = 0.45 is missing, which is clearly seen with the other two species. Remaining species, although producing similar fingerprints, can also be differentiated from one another. For the sake of an example, *S. jurisicii* is characterized by the presence of a pale orange zone (RF = 0.32) that is not observed in the other profiles.

Based on fingerprints, *S. officinalis, S. triloba,* and *S. canariensis* can be classified into a separate group of species, characterizing with similar chromatographic profiles. The color and sequence of zones character-

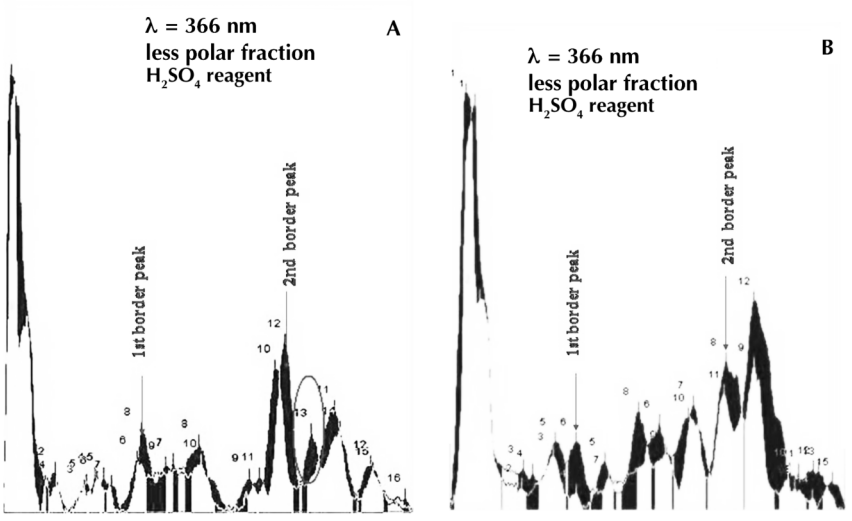

Figure 5. Results of using the image processing program for finding similarities and differences among the selected Salvia species. Labeling of densitograms: (A) black, S. amplexicaulis, grey, S. stepposa, (B) black, S. officinalis, grey, S. triloba. (Note: Figures 1, 2, 3, 4, and 6 appear on page 3A.)

istic for these species has already been described in this study. The following *Salvia* species can be assigned to the third group: *S. sclarea, S. staminea, S. pratensis, S. hians, S. forskaohlei, S. azurea, S. verticillata,* and *S. deserta*. All three parts of their respective fingerprints obtained with solvent system 1 contain lesser bands than those of the other species. The same can be said about the respective chromatographic profiles originating from solvent system 2. However, this subgroup is considerably more heterogeneous when compared with the other two.

Salvia lavandulifolia produces a fingerprint that is completely different from the other chromatographic profiles, and hence, it cannot be ascribed to any of the aforementioned groups.

A comparison of the contents of several phenolic acids shows that *S. officinalis* produces the highest yields of polyphenolics among all the analyzed species (Figure 6B, See Page 3A). Other species characterized with the relatively high total peak areas include *S. lavandulifolia, S. triloba, S. nemorosa, S. jurisicii, S. azurea,* and *S. atropatana*. *Salvia sclarea, S. deserta,* and *S. staminea* produce the lowest total peak area values. *Salvia lavandulifolia, S. tesquicola, S. canariensis, S. nemorosa, S. stepposa,* and *S. hians* contain the greatest amount of caffeic acid, whereas *S. lavandulifolia, S. jurisicii, S. azurea, S. nemorosa,* and *S. hians* are characterized by the highest *p*-coumaric acid contents (Figure 4, See Page 3A).

From Figures 1–2 (See Page 3A), the proposed method is specific for individual *Salvia* species. All the analyzed species produce distinctive fingerprints, thus they can easily be differentiated. Fingerprints obtained in the repeatability and intermediate precision tests have been identical in terms of the amount of peaks, appearing in the individual chromatograms. The variability of the RF values tested for several randomly chosen bands does not exceed 0.02 for repeatability and 0.04 for intermediate precision, meeting the cGMP regulations (25). The obtained results indicate that all the peaks present in HPTLC profiles can be assigned as "common peaks", which represent the characteristics of the particular *Salvia* species. The further use of these chromatographic profiles for the comparison between different species is therefore justified (Figure 5).

Stability of the samples in the course of the chromatographic process was evaluated by means of a 2D experiment. All the spots were lying on the diagonal after checking solvent systems 1 and 2; thus the analyzed samples proved stable during the process. The samples proved stable in the solution also.

As the fingerprints were visualized with use of derivatization, stability of the results was also evaluated. Images taken 5 and 10 min after the completion of derivatization did not differ from those obtained immediately after derivatization. However, after 30 min, several bands have changed their color from pink to orange, and certain bands (e.g., caffeic acid) were even better detected, as can be seen from Figure 6 (See Page 3A). After 24 h, the fingerprints did not change, and all the separated zones were clearly visible without any further change in the band color. Thus one can expect reliable results when images are taken immediately after completion of derivatization and then they are compared after certain amount of time.

In the course of method development, the impact of several experimental parameters was tested, such as the mobile phase composition, heating temperature, and drying time. All the scrutinized changes exert minor effect on fingerprints, except for the heating temperature. When the temperature was lower than 100°C, the obtained zones were fainter and some of them were not seen at all. Thus, it is important to maintain the temperature during the method development within the range of 105 ± 2°C. As far as the changes in mobile phases' composition are considered, only minor shifts in RF values (± 0.02) were observed, which did not lead to any significant differences in the chromatographic profiles.

The method was validated in terms of specificity, precision (repeatability and intermediate precision), stability, and robustness. Detailed description of the performed tests is given in the experimental section.

When compared to HPLC fingerprinting methods, HPTLC fingerprint application is characterized definitely with worse resolution and reproducibility. However the obtained results show that TLC fingerprints are a good choice for the parallel comparison of several samples at a relatively short time and are more cost-effective. One of the greatest advantages of the proposed solution is probably the provision of colorful images.

Conclusion

A new chromatographic procedure has been proposed for developing fingerprints with twenty different *Salvia* species. Use of the digital scanning profiles and images obtained with the image processing program makes the comparative studies performed in the course of the fingerprint development easy. The proposed method turned out to be useful in defining chemical similarities and differences of the investigated species. It can be applied to chemotaxonomic research focused on defining relationships within the *Salvia* genus. Presented method is easy to operate and is speedy, as the results for 20 samples (including sample application, chromatogram development, derivatization, and documentation) can be obtained within ca. 1 h. The validation of the method clearly indicates that it is a reliable chromatographic procedure.

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