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Development of Chromatographic and Free Radical Scavenging Activity Fingerprints by Thin-layer Chromatography for Selected *Salvia* Species

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ABSTRACT:

Introduction – Plant-derived free radical scavengers have become the subject of intensive scientific interest. Recently, the concept of coupling chromatographic fingerprints with biological fingerprinting analysis has gained much attention for the quality control of plant extracts. However, identification of free radical scavenging activity of each single compound in a complex mixture is a difficult task. Thin-layer chromatography with post-chromatographic derivatisation with the methanol solution of DPPH can be a valuable tool in such analyses.

Objective – Development of chromatographic and free radical scavenging fingerprints of nineteen *Salvia* species grown and cultivated in Poland.

Methodology – Chromatography was performed on the silica gel layers with use of two eluents, one for the resolution of the less polar compounds, and the other one for the resolution of the medium and highly polar ones. The plates were sprayed with the vanillin–sulfuric acid reagent to produce chemical fingerprints, and with DPPH solution to generate free radical scavenging fingerprints.

Results – With four *Salvia* species, it was revealed that their strong free radical scavenging properties are not only due to the presence of polar flavonoids and phenolic acids, but also due to the presence of several free radical scavengers in the less polar fraction. Because of the similarities in both the chromatographic and the free radical scavenging fingerprints, *S. triloba* can be introduced as a possible equivalent of the pharmacopoeial species, *S. officinalis*.

Conclusion – Fingerprints developed in the experiments proved useful for the analysis of complex extracts of the different *Salvia* species.

Keywords: *Salvia* spp.; fingerprint; free radical scavengers; thin-layer chromatography; polyphenolics

Introduction

Salvia, the largest genus of the *Lamiaceae* family, has been credited with a long list of medical uses, including: spasmolytic, antiseptic, astringent, antibacterial and antioxidant activities (Kivrak *et al*., 2009). Recently, an attention has been directed toward the water soluble antioxidants and free radical scavengers of the *Salvia* genus, which have proved as protective against cardiovascular diseases and certain forms of cancer (Rice-Evans *et al*., 1996; Lu and Foo, 2002). Plant phenolics (such, as flavonoids, phenylpropanoids and phenolic acids) can act as free radical scavengers, or as contributing agents to the mechanisms of anticarcinogenic or cardioprotective action (Rice-Evans *et al*., 1996; Matkowski and Piotrowska, 2006). The *Salvia* genus is a rich source of polyphenols, with over 160 of them having been identified so far (Lu and Foo, 2002). Becuase the reactive oxygen species (ROS) were found as responsible for numerous human diseases (e.g. atherosclerosis, Alzheimer's disease, inflammation, asthma, rheumatoid arthritis, etc.), antioxidants and free radical scavengers are currently the subject of an intensive research interest (Niederländer *et al*., 2008). Usually simple spectrophotometric techniques have been applied to determination of the total antioxidant activity of complex mixtures (such as, e.g., the plant extracts; Tepe *et al*., 2004; Orhan *et al*., 2007; Tepe, 2008). However, determination of the antioxidant potential and antiradical activity for each individual compound contained in a complex mixture is a very difficult task. Several techniques that combine HPLC with post-column chemical detection have been proposed to screen plant extracts for their antioxidant contents (Neungchamnong *et al*., 2005; Chang *et al*., 2008; Niederländer *et al*., 2008). A good separation power of the HPLC methods combined with fast post-column derivatisation is the greatest asset of such approaches. However, several of those have never been applied

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since being reported, apparently due to experimental problems (Niederländer *et al*., 2008). Another shortcoming of the on-line HPLC techniques is their limitation to one sample per run, which greatly extends the analysis time.

Therefore, recently, a need to develop new methods for screening a free radical scavenging potential of individual compounds contained in complex mixtures is steadily growing. Thin-layer chromatography (TLC) is often the method of choice for the screening of plant extracts for the presence of biologically active compounds. An advantage of TLC is the speed of method development, high sample throughput and flexibility. It is particularly well suited for direct biological detection, since the separation result is immobilised prior to the detection and, moreover, the open solid bed layer allows direct access to the sample (Poole, 2003). The number of selective visualising reagents that can be used for the different target compounds is also large. One more advantage is that the normal-phase TLC systems used for identification of polar bioactive compounds often outperform the reversed-phase systems, where polar compounds tend to be poorly retained (Poole, 2003). TLC has been utilised for isolation and/or identification of the free radical scavengers and antioxidants in the plant extracts and foods. In that case, the free radical DPPH (2,2-diphenyl-1-picrylhydrazyl) was used as a derivatising agent (Bhattarai *et al*., 2008; Pozharitskaya *et al*., 2008). However, with most published methods, the real potential of the TLC-antioxidant screening technique has remained not yet fully exploited or even explored (e.g. certain compounds with the known free radical scavenging properties are left unresolved). Therefore, it is an urgent task now to develop a TLC method that allows screening of the free radical scavenging potential of all the compounds present in a complex natural sample.

Usually, plant extracts are complex mixtures that contain hundreds of compounds, although some of them only exhibit biological activity. For the quality control of herbal medicines, it is important to derive as much information from a sample as possible (Fan *et al*., 2006). The chromatographic profile (fingerprint) and the most active constituents are usually taken into consideration for an assay of authenticity and quality of herbal medicines (Liang *et al*., 2004). Recently, the concept of developing the activity and biological fingerprints has been proposed to adequately reveal the quality characteristics of medicinal plants and herbal preparations (Zhang *et al*., 2005; Wang *et al*., 2007; Chang *et al*., 2008). A potency fingerprint that can quantitatively analyse the antioxidant activity of individual constituent and provide the total antioxidant activities of the samples has recently been proposed (Chang *et al*., 2009). The method involved the use of high-performance liquid chromatography coupled with ultraviolet and pyrogallol–luminol chemiluminescence detection.

Because of the wide spectrum of its biological activity, the *Salvia* genus has been widely investigated. Several studies were performed to assess the total antioxidant activity of selected *Salvia* species (Tepe *et al*., 2004; Orhan *et al*., 2007; Kelen and Tepe, 2008; Kivrak *et al*., 2009). An on-line HPLC chemiluminescence technique was proposed for detection of the free radical scavengers in the preparation containing *Salvia milthiorizza* (Chang *et al*., 2008). Moreover, several chromatographic techniques were proposed to construct fingerprints of different *Salvia* species (Gu *et al*., 2004; Liu *et al*., 2007; Ma *et al*., 2007; Jin *et al*., 2008; Rzepa *et al*., 2009).

In this study, the chemical and free radical scavenging TLC fingerprints were used for the purposes of the quality control of 19 *Salvia* species. Fractions of differentiated polarity were investigated for the presence of substances exhibiting free radical scavenging potential. The main advantages of the presented technique are its simplicity, flexibility, speed and the possibility to present the results in form of a picture that can easily be processed with an image-processing program. To the best of our knowledge, chemical thin-layer chromatographic and the free radical scavenging fingerprints have not yet been reported for the quality control of herbal medicines.

Experimental

Apparatus and reagents

The following standards: gallic acid (Ga), hiperoside (H) and rutin (Ru) were obtained from Aldrich (St. Louis, MO, USA), and the remaining ones, i.e. caffeic acid (C), chlorogenic acid (Cl) and rosmarinic acid (R), were purchased from ChromaDex (Santa Ana, USA). *n*-Hexane, methanol, toluene, ethyl acetate and formic acid used as the extracting solvents or the mobile phase components were manufactured by Merck (KGaA, Darmstadt, Germany). Sulfuric acid was obtained from Polish Reagents (Gliwice, Poland). All solvents were of the analytical purity grade. Vanillin and DPPH were purchased from Aldrich.

TLC was performed on 10 cm × 20 cm glass-backed silica gel TLC $60F_{254}$ plates (lot no. OB535150), purchased from Merck. Solutions of all test substances and plant extracts were applied to the chromatographic plates band-wise by means of a Camag automatic TLC sampler (Camag, Muttenz, Switzerland) and developed in the horizontal DS chambers (Chromdes, Lublin, Poland). Location of the bands was carried out after derivatisation in visible light. Chromatograms were documented using a 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. The obtained videoscans were processed using of the Image J image-processing program (available on-line, and elaborated by the National Institutes of Health, USA).

Standard substance solutions and plant extracts

All reference substances were dissolved in methanol to prepare the 1.0 mg/mL solutions. Extracts from 19 dried *Salvia* species were obtained from the plants collected in the Pharmacognosy Garden (Lublin, Poland) in the course of the vegetation periods of 2007 and 2008. The list of the investigated species is given in Table 1. Botanical material was authenticated and voucher specimens were deposited at the Department of Pharmacognosy, Medical University of Lublin. The fresh material was oven-dried at a temperature not exceeding 40°C for 40 h. The dried plant material remained frozen until the time of extraction and chromatographic analysis. Accelerated solvent extraction (ASE) was performed with the ASE extractor (Dionex, Sunnyvale, CA, USA). First, the samples were extracted with *n*-hexane to get rid of chlorophylls, then extraction was performed with use of methanol. The optimised 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 (with both solvents); static extraction time, 5 min (with both solvents); solvent volume, 40 mL (with both solvents); number of cycles, 2 (with both solvents). A detailed description of the extraction procedure of the plant material can be found in our earlier work (Cieśla *et al*., 2010).

Thin-layer chromatography

Standard and sample solutions were applied to the 10 × 20 cm silica gel TLC 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). The 5 µL aliquots of standard solutions and the 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 development

Table 1. The investigated *Salvia* species

Sample no.	Species
1	*Salvia officinalis*
2	*Salvia triloba*
3	*Salvia canariensis*
4	*Salvia lavandulifolia*
5	*Salvia farskohlei*
6	*Salvia sclarea*
7	*Salvia verticilatta*
8	*Salvia pratensis*
9	*Salvia tesquicola*
10	*Salvia deserta*
11	*Salvia hians*
12	*Salvia cadmica*
13	*Salvia glutinosa*
14	*Salvia atropatana*
15	*Salvia azurea*
16	*Salvia staminea*
17	*Salvia jurisicii*
18	*Salvia amplexicaulis*
19	*Salvia nemorosa*

was carried out in the horizontal DS chambers (Chromdes, Lublin, Poland) at ambient temperature of 20 ± 1°C. For the less polar constituents, as mobile phase we used solvent system I, composed of toluene–ethyl acetate–formic acid, 60:40:1 (v/v/v). For the medium and highly polar substances, solvent system II was applied composed of ethyl acetate–water–formic acid–acetic acid, 100:26:11:11 (v/v/v/v). Pre-saturation of the chromatographic chamber was performed only in the case of solvent system II and it lasted for 15 min. The plates were developed to the distance of 90 mm. Then the plates were dried at room temperature for 15 min, prior to derivatisation.

Derivatisation and DPPH assay

The vanillin–sulfuric acid reagent was prepared by mixing 1 g vanillin with 20% sulfuric acid in methanol. The plates were sprayed with this reagent using the automatic spraying device (Merck) and then heated for 5 min at 105°C. Plate images were collected in visible light. They were also scanned with densitometer at the wavelengths $\lambda = 254$ and 366 nm, with the slit parameters 1×0.1 mm. Separated bands in the analysed samples were identified by matching their R_F values and UV–vis spectra, respectively, with those obtained for the standards.

In order to check free radical scavenging properties of the investigated samples, the chromatographic plates were sprayed with an 0.2% DPPH reagent in methanol and left at ambient temperature for 30 min. Yellow spots obtained as a result of bleaching the purple color of the DPPH reagent were interpreted as a positive free radical scavenging activity (Kivrak *et al*., 2009). Images of the plates were processed with the Image J processing program, and the substances with free radical scavenging activity were identified as negative peaks.

Method validation

The method was validated for its specificity, precision (repeatability and intermediate precision), stability and robustness according to the recognised AOAC guidelines for the qualitative TLC procedures (Reich *et al*., 2008).

Precision was expressed as precision of the positions (the R_F 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 each time a fresh portion of mobile phase. 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 the R_F values for several 'marker zones' across each plate, and variability of the average R_F 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 1, 2, and 3 h prior to the development with the fingerprint obtained for the sample applied just before the development. The stability of the sample in solution was determined by multiple measurements of one 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 I and II were checked.

The influence of deliberate, small changes in the chromatographic parameters, e.g. mobile phase composition, heating temperature and drying time, on the final results was also checked. A more detailed description of the validation procedures was given in our earlier work (Cieśla *et al*., 2010).

Results and Discussion

The first part of the experiment was focused on the development of chromatographic fingerprints, based partially on the results obtained in our earlier work (Cieśla *et al*., 2010). As the methanol extracts of the investigated *Salvia* species are very complex, two different chromatographic systems were applied, focusing on resolution of the low, medium and high polarity compounds. For the less polar fraction, solvent system I was applied, and for the medium and highly polar fractions, solvent system II was used. Different visualising agents were tested and for both the less polar and the medium and highly polar fractions, and the best results were obtained with the vanillin–sulfuric acid reagent. Its usage proved particularly advantageous with the medium and highly polar fraction, allowing a vast number of chromatographic bands to appear in the respective fingerprints. In that way, the presence of rosmarinic, gallic and caffeic acid was confirmed in the analysed extracts (Fig. 1). For almost all sage species (with the exception of *S. deserta*), the bands present between the application point ($R_F = 0$) and the band at $R_F = 0.33$ are fully analogous and they can be considered as characteristic of the *Salvia* spp. Two other bands at the R_F values of 0.63 and 0.67 also appear in all chemical profiles. Chromatographic patterns characteristic of the individual sage species appear in the lower R_F range of the less polar chemical fingerprints and they can be applied for differentiation of these species.

Application of solvent system II (with relatively high elution strength) resulted in the separation of medium and highly polar constituents, mainly the more polar phenolic acids, and the different flavonoid aglycones and their glycosides (Fig. 2). Chromatographic fingerprints obtained with aid of the visualising reagent were quite similar for the majority of the investigated species. As a characteristic feature, we considered the presence of several dark zones in the lower R_F range, which can be attributed to very polar glycosides. At $R_F = 0.06$, an intensive dark band appeared in the respective chromatograms of *S. officinalis*, *S. farskohlei*, *S. glutinosa* and *S. jurisicii*. As can be seen from Fig. 2, chromatograms of these species are characterised by a lower number of bands just above the aforementioned zone than the remaining analysed species. It could happen that these dark bands originate from the products of partial deglycosilation of polyphenolics in the course of the plant drying process. Also the four bands at the R_F values equal to 0.10, 0.31, 0.76, and 0.80,

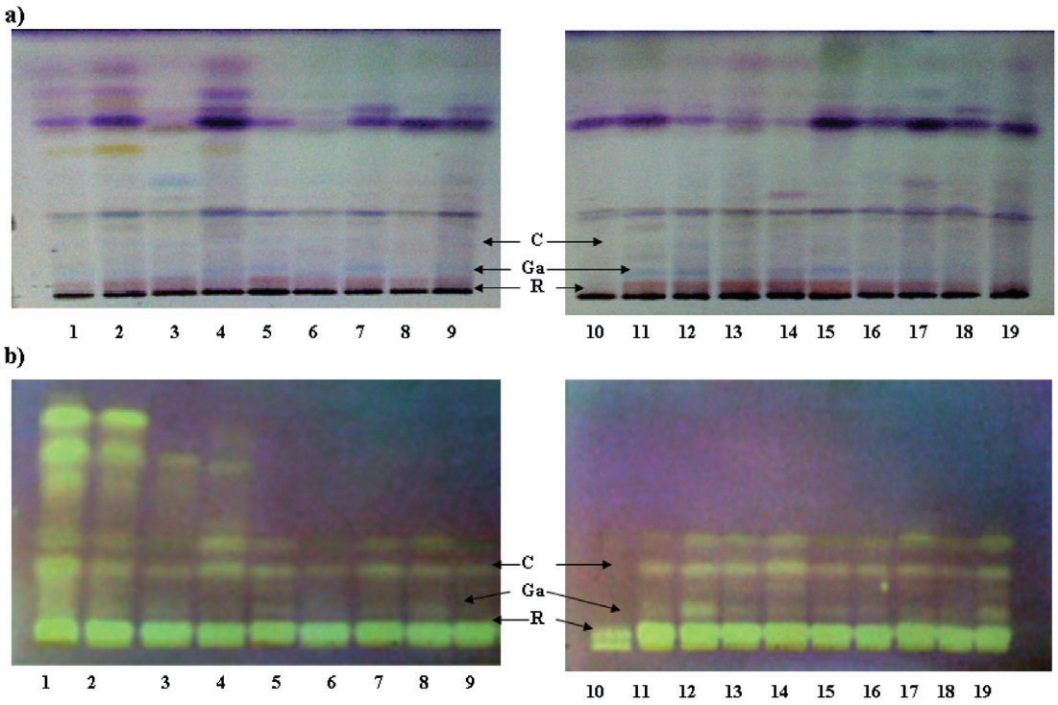

Figure 1. TLC images of compounds resolved with use of solvent system I, after spraying with (a) vanillin–sulfuric acid reagent, and (b) DPPH methanolic solution. For symbols, see Experimental.

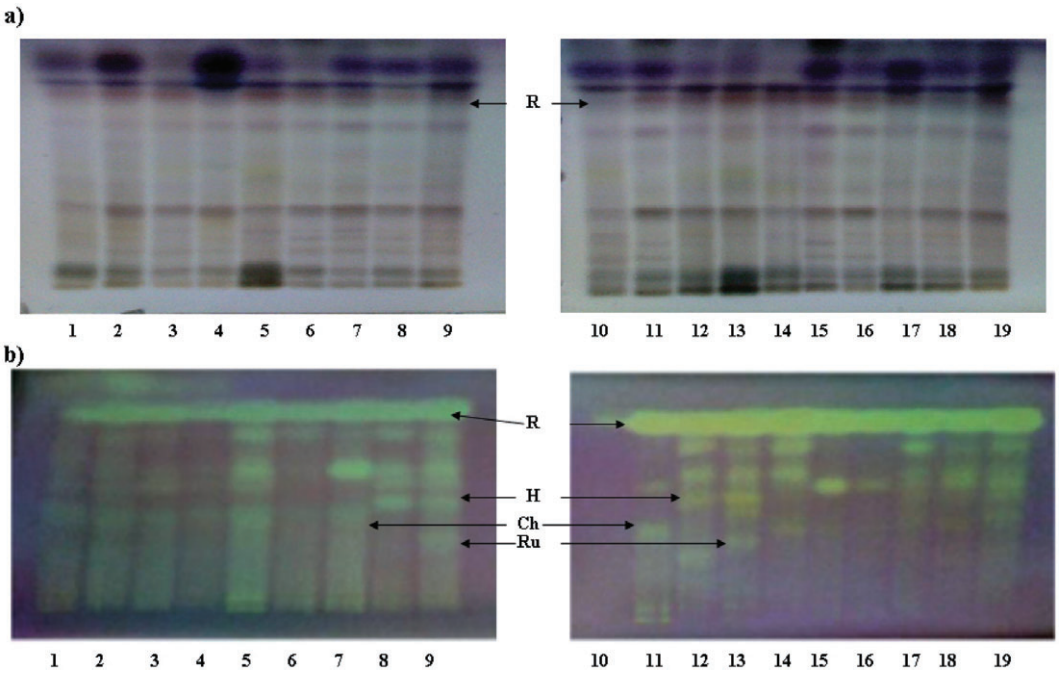

Figure 2. TLC images of compounds resolved with use of solvent system II, after spraying with (a) vanillin–sulfuric acid reagent, and (b) DPPH methanolic solution. For symbols, see Experimental.

respectively, which appear in all fingerprints, can be considered as characteristic of the chemical profiles of the *Salvia* species.

The second part of our study focused on a scrutiny of free radical scavenging properties with the separated sage constituents. To this effect, the developed plates were sprayed with the DPPH solution in methanol. Substances that exhibit antiradical potential appeared on the chromatograms as yellow zones on the purple background (Figs 1 and 2). With the less polar fraction, the majority of the analysed species characterise with the fully analogous free radical scavenging profiles, i.e. showing four bands at R_F values equal to 0.06, 0.12, 0.22 and 0.41. In this particular fraction, three free phenolic acids (i.e. rosmarinic, gallic

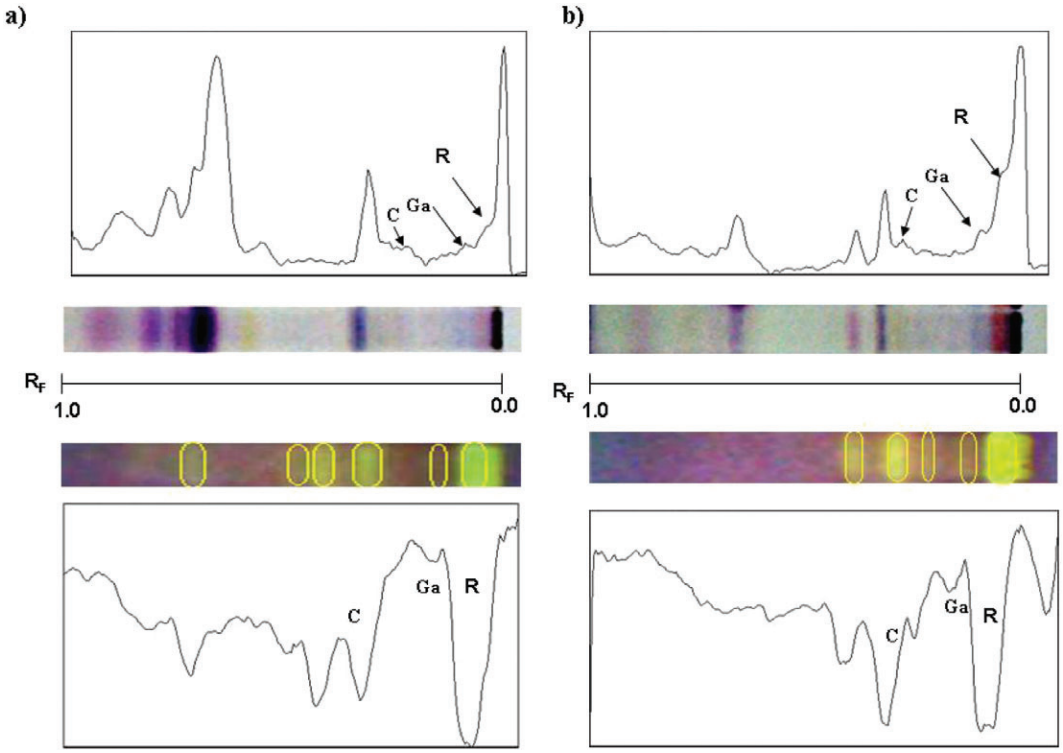

Figure 3. Comparison of chemical and free radical scavenging TLC fingerprints obtained with use of solvent system I, for the extracts prepared from two *Salvia* species: (a) *S. lavandulifolia* and (b) *S. atropatana*. For symbols, see Experimental.

and caffeic acid) were identified as exhibiting free radical scavenging properties. With all the investigated species, the strongest free radical scavenging activity was observed for rosmarinic acid. This can be concluded from the size of yellow zone corresponding to this compound, after spraying the chromatogram with the DPPH solution (Figs 3 and 4). Also for the band of caffeic acid derived from *S. officinalis* and *S. atropatana*, relatively strong free radical scavenging properties were observed (Figs 1 and 3). These results remain in a good agreement with the data referring to these two polyphenolics as to the most potent hydrogen donating free radical scavengers in the *Lamiaceae* family (Lu and Foo, 2002). The least pronounced free radical scavenging properties were found with the *Salvia deserta* extract, where the band of rosmarinic acid alone is clearly visible in the respective fingerprint. The less polar fractions derived from the four *Salvia* species (i.e. *S. officinalis*, *S. triloba*, *S. lavandulifolia* and *S. canariensis*) were characterised by the presence of a greater number of free radical scavengers having their respective R_F values equal to 0.57, 0.70 and 0.74. *S. officinalis* and *S. triloba* were the species containing the highest amounts of the constituents with the free radical scavenging properties. Almost identical chemical and free radical scavenging fingerprints suggest that these two species can possess similar chemical and antioxidant properties (as concluded in our earlier study as well, Cieśla *et al*., 2010). In Poland, *S. officinalis* is the only pharmacopoeial species and, hence, it seems quite sensible to introduce *S. triloba* as its equivalent for medical purposes. Further justification of this statement can be the fact that *S. triloba* is reported to contain less α- and β-thujone than *S. officinalis* (Länger *et al*., 1996; Arikat *et al*., 2004). Control of the content of thujone in *Salvia* is very important, as thujone exhibits neurotoxic properties. The antioxidant potential of the sage extracts has been ascribed mainly to the presence of phenolic acids (e.g. rosmarinic, gallic or caffeic acid) and flavonoids. However, the results obtained in this study show that, with several species, the free radical scavenging activity is strongly pronounced with the constituents of the less polar fraction too.

Apparently, medium and highly polar substances that remained at the start when solvent system I was used also gave intensive yellow zones, indicating their potential as free radical scavengers (Fig. 1). Thus the ability to scavenge free radicals was studied for the constituents of the more polar fraction derived from the investigated *Salvia* species and developed with solvent system II. In this fraction, the following constituents were identified: chlorogenic acid ($R_F = 0.36$), rosmarinic acid ($R_F = 0.70$), hiperoside ($R_F = 0.43$) and rutin ($R_F = 0.28$; see Fig. 2). Substances exhibiting free radical scavenging properties are located in the upper R_F range of the chromatograms obtained with solvent system II. Although the sage extracts are rich in medium and highly polar constituents, in the chromatograms the visualised yellow spots did not appear in the upper R_F range. This result can be attributed to the fact that these polar constituents are highly glycosylated, and the glycosides are reported as the less potent free radical scavengers than aglycones (Rice-Evans *et al*., 1996). Medium and highly polar fractions of *S. farshohlei*, *S. tesquicola*, *S. cadmica*, *S. glutinosa*, *S. atropatana* and *S. nemorosa* were characterised by the greatest amounts of free radical scavengers. The least pronounced free radical scavenging properties of the polar fraction were observed with *S. lavandulifolia*, *S. sclarea*, *S. azurea* and *S. staminea*. In the case of *S. deserta*, the band of rosmarinic acid alone was detected. The R_F values of the common compounds from the investigated *Salvia* species are summarised in Table 2.

For the development of fingerprints, the identity of all the resolved bands does not need to be known. Application of the

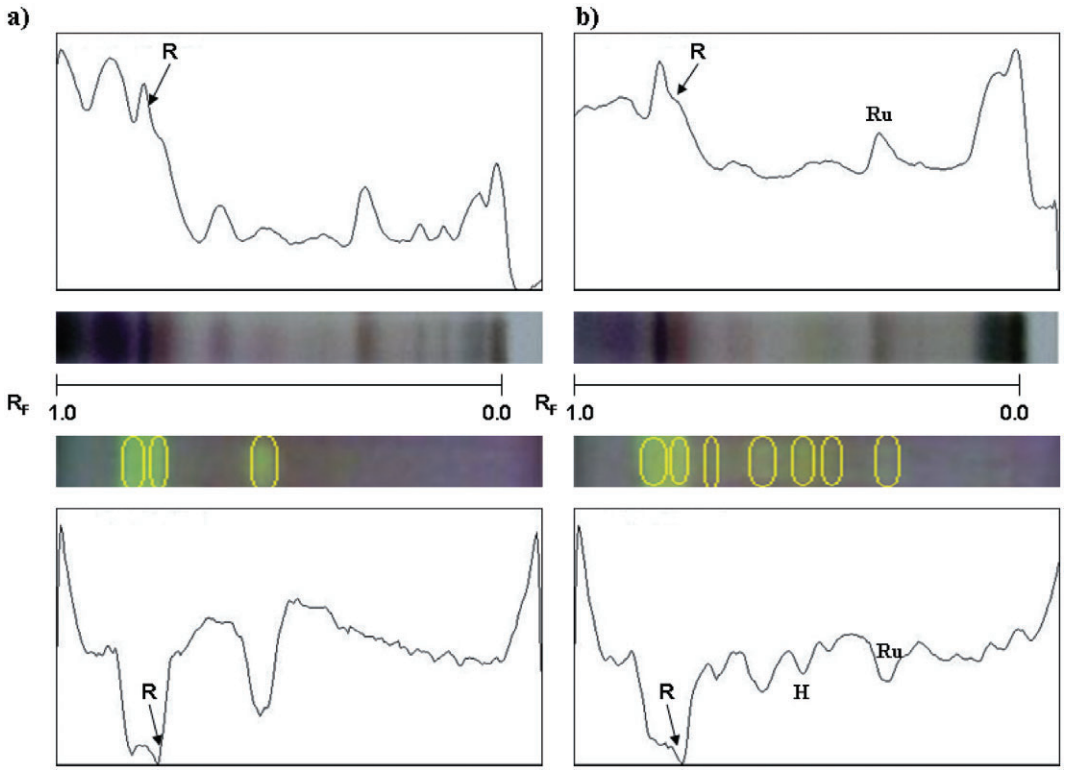

Figure 4. Comparison of chemical and free radical scavenging TLC fingerprints obtained with use of solvent system II, for the extracts prepared from two *Salvia* species: (a) *S. azurea* and (b) *S. glutinosa*. For symbols, see Experimental.

Table 2. R_F values of the common compounds characteristic for the investigated *Salvia* species' fingerprints

Sample no.	Solvent system I R_F										Solvent system II R_F								
	0.06	0.12	0.22	0.33	0.41	0.57	0.63	0.67	0.70	0.74	0.06	0.10	0.28	0.31	0.36	0.43	0.70	0.76	0.80
1	X	X	X	X	X	X	X	X	X	X	X	X		X	X		X	X	X
2	X	X	X	X	X	X	X	X	X	X	X	X		X	X		X	X	X
3	X	X	X	X	X	X	X	X	X	X	X	X		X	X		X	X	X
4	X	X	X	X	X	X	X	X	X	X	X	X		X	X		X	X	X
5	X	X	X	X	X		X	X			X	X	X	X	X		X	X	X
6	X	X	X	X	X		X	X			X	X		X			X	X	X
7	X	X	X	X	X		X	X			X	X		X	X		X	X	X
8	X	X	X	X	X		X	X			X	X		X		X	X	X	X
9	X	X	X	X	X		X	X			X	X	X	X		X	X	X	X
10	X	X	X	X	X		X	X			X	X		X			X	X	X
11	X	X	X	X	X		X	X			X	X		X	X		X	X	X
12	X	X	X	X	X		X	X			X	X		X		X	X	X	X
13	X	X	X	X	X		X	X			X	X	X	X		X	X	X	X
14	X	X	X	X	X		X	X			X	X		X	X		X	X	X
15	X	X	X	X	X		X	X			X	X		X	X		X	X	X
16	X	X	X	X	X		X	X			X	X		X			X	X	X
17	X	X	X	X	X		X	X			X	X		X	X	X	X	X	X
18	X	X	X	X	X		X	X			X	X		X	X	X	X	X	X
19	X	X	X	X	X		X	X			X	X	X	X	X	X	X	X	X

image-processing program makes it possibile to comprehensively investigate both the chromatographic and the free radical scavenging profiles. Figures 3 and 4 show the peak profiles obtained from the videoscans of the selected *Salvia* species. As can easily be noticed, several bands that are barely seen in the TLC images (e.g. caffeic and gallic acid) produce intensive negative peaks in the DPPH test, showing a considerable free radical scavenging performance.

Validation of the method was performed according to the recognised international standards (Reich *et al.*, 2008). The proposed

method characterises with good specificity, as the analysed *Salvia* species produce distinctive fingerprints. Fingerprints obtained in the repeatability and intermediate precision test were identical in terms of the amount of peaks appearing in the individual chromatograms. Variability of the R_F values was, as follows: for the repeatability, it did not exceed 0.01, and for an intermediate precision, it was not greater than 0.03. Samples were stable when undergoing the chromatographic procedure, and showed signs neither of decomposition nor of chemisorption. The use of the vanillin–sulfuric acid visualising reagent produced more stable results (i.e. the band colors did not considerably change with time), than with the use of sulfuric acid alone. The proposed technique is also robust, i.e. significant changes in bands' colours or their R_F values were not observed when slightly changing the experimental conditions. The way of performing the validation experiments was described in detail in our earlier work (Cieśla *et al*., 2010).

Construction of the chromatographic and the free radical scavenging fingerprints gives the possibility to comprehensively investigate the plant-derived products. The use of this procedure allows a search for the species that contain the highest amounts of free radical scavengers. This procedure can be applied for the search for new plant species that can also be used as potential herbal remedies. The proposed chromatographic procedure can be adapted to the quality control of the other plant extracts and plant-derived products.

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