In the previous paper from this series, we proposed mass spectrometric fingerprinting of complex botanical samples upon the examples of the pharmacologically important phenolic acids and flavonoids selectively extracted from *Salvia lavandulifolia*. In this study, we explore fingerprinting efficiency with a novel two-dimensional analytical system composed of the reversed-phase thin-layer chromatography and the reversed-phase high performance liquid chromatography with mass spectrometric detection (2D RP-TLC–RP-LC–MS). We also compare its efficiency with that of the one-dimensional analytical system (the reversed-phase thin-layer chromatography with mass spectrometric detection; 1D RP-TLC–MS). As our present study is basically focused on the method development, we considered it as justified to carry out our comparison with the phenolic acid extracts selectively derived from the *Salvia lavandulifolia* species, similar as it was done in Part II from this series. Upon the results obtained, it was established that the 1D RP-TLC–MS mode and the 2D RP-TLC–RP-LC–MS mode can be applied to fingerprinting of herbal extracts, and that the 2D RP-TLC–RP-LC mode can provide a more abundant information than that originating from the 1D RP-TLC mode.

**Abstract**

In the previous paper from this series, we proposed mass spectrometric fingerprinting of complex botanical samples upon the examples of the pharmacologically important phenolic acids and flavonoids selectively extracted from *Salvia lavandulifolia*. In this study, we explore fingerprinting efficiency with a novel two-dimensional analytical system composed of the reversed-phase thin-layer chromatography and the reversed-phase high performance liquid chromatography with mass spectrometric detection (2D RP-TLC–RP-LC–MS). We also compare its efficiency with that of the one-dimensional analytical system (the reversed-phase thin-layer chromatography with mass spectrometric detection; 1D RP-TLC–MS). As our present study is basically focused on the method development, we considered it as justified to carry out our comparison with the phenolic acid extracts selectively derived from the *Salvia lavandulifolia* species, similar as it was done in Part II from this series. Upon the results obtained, it was established that the 1D RP-TLC–MS mode and the 2D RP-TLC–RP-LC–MS mode can be applied to fingerprinting of herbal extracts, and that the 2D RP-TLC–RP-LC mode can provide a more abundant information than that originating from the 1D RP-TLC mode.

**Introduction**

A mass spectrometric detector can be off-line coupled with the thin-layer chromatographic plate by means of the TLC–MS interface (Camaq, Muttenz, Switzerland). In that way, TLC gains an additional analytical dimension which enhances its performance, thus becoming an even more flexible and better performing separation and identification tool than before. With use of the TLC–MS interface, certain amount of analytical tasks has already been accomplished, basically focused on the separation of the less complicated mixtures of compounds and identification thereof upon their molecular ions. A considerable contribution to this area was done by Morlock et al. [e.g., (1–6)].

In a previous paper (7), the one-dimensional low-temperature thin-layer chromatography with mass spectrometric detection (1D LT TLC – MS) was reported, for the first time applied to the fingerprinting of the complex essential oil samples derived from several sage (*Salvia*) species. In papers (8,9), a novel possibility was introduced of the two-dimensional chromatographic mode making use of the TLC–MS interface and applied to the low-temperature fingerprinting of the essential oil fraction (8) and of the room temperature fingerprinting of the phenolic acid and flavonoid fractions (9) selectively extracted from *Salvia lavandulifolia*. In papers (8,9) the experimental results derived from the one-dimensional analytical system composed of the adsorption thin-layer chromatography coupled with mass spectrometry (1D NP TLC–MS) were presented, and also those for the first time derived from the two-dimensional system composed of the adsorption thin-layer chromatography and the reversed phase high-performance liquid chromatography coupled with mass spectrometric detection (2D NP TLC–RP-LC–MS).

The aim of this study is to investigate the fingerprinting efficiency of the two novel analytical systems, that is, the one-dimensional analytical system composed of the reversed-phase thin-layer chromatography with mass spectrometric detection (1D RP-TLC–MS) and the two-dimensional analytical system composed of the reversed-phase thin-layer chromatography and the reversed-phase high performance liquid chromatography with mass spectrometric detection (2D RP-TLC–RP-LC–MS). These two systems were tested upon the practical example of the phenolic acids fraction selectively extracted from *Salvia lavandulifolia*. Phenolic compounds of the sage origin are widely recognized for their antioxidant, antimicrobial, antiviral, and even...
anticancer properties, hence it seems purposeful to test our novel analytical systems upon these pharmacologically important secondary metabolites of the plant origin (10). Successful fingerprinting of complex samples of botanical origin can prove particularly helpful for identification and quality assurance operations, especially when handling commercial batches of medicinal plants traded in a powdered form.

**Experimental**

**Materials and reagents**

Sample of the *Salvia lavandulifolia* species was harvested in the Pharmacognosy Garden of the Medical University, Lublin, Poland, in July, 2009. The plant material comprised all parts of the plant (i.e., roots and the aerial parts) and it was dried for 40 h in an oven with a forced air flow at 35°C to 40°C. Then the obtained dry material was stored in the deep-freeze compartment of refrigerator until the commencement of the analysis. Finally, the necessary amounts of the plant material were weighed and powdered in a porcelain mortar. Three replicates of the sage sample were processed in an identical way.

Metamnine was purchased from Pharma Cosmetics (Krakov, Poland), and methanol, toluene, acetone, ethyl acetate, acetic acid, formic acid, and hydrochloric acid used for the experiments were of analytical purity grade and purchased from POCh (Gliwice, Poland). Water was double distilled and de-ionized in the laboratory conditions by means of Elix Advantage model Millipore system (Molsheim, France).

**Selective extraction of phenolic acids from the *Salvia* species**

Ten grams of the finely powdered plant material was placed in the 250-mL round-bottomed flask on the water bath under the reflux and the sample was three times extracted with the consecutive 50-mL portions of methanol. The methanol extracts were combined and then evaporated under the reduced pressure to dryness. Dry residue was dissolved in 20 mL hot water and then left in the refrigerator for 24 h. Finally, the precipitate was filtered off, and the filtrate was placed in volumetric flask and made up to 100 mL with water.

**1D RP-TLC–densitometry**

The reversed-phase thin-layer chromatographic separations were performed on the commercial glass plates (20 cm × 20 cm) precoated with 0.25 mm layers of RP-18 F 254s (Merck; cat. no. 1.15423). The phenolic acid extracts and the respective standards were spotted onto the thin layer in the 10-µL aliquots, using an AS 30 model autosampler (Desaga, Heidelberg, Germany). For the sake of comparison of the retardation factor (Rf) values and the mass spectrometric fingerprints, we also chromatographed the following standard samples (5 mg/mL solutions in methanol): p-coumaric acid, caffeic acid, chlorogenic acid, ferulic acid, rosmarinic acid, protocatechuic acid, *trans*-cinnamic acid, ellagic acid, vanillic acid, syringic acid, and *p*-hydroxybenzoic acid. All standards were purchased from Chromadex (Irvine, CA).

Development of the planar chromatograms was carried out with the ternary mixture methanol–water–acetic acid, 5:5:0.1 (v/v) at 21 ± 0.5°C for a distance of 15 cm. All planar chromatograms were developed in the sandwich DS chambers (Chromdes, Lublin, Poland), previously saturated with mobile phase vapor for 15 min.

The developed chromatograms were dried for 3 h at ambient air and eventually evaluated by means of densitometry. Acquisition of the densitograms was carried out with a Desaga CD 60 model densitometer equipped with Windows compatible ProQuant software (Desaga). Concentration profiles of the development lanes for the sage samples were recorded in reflected ultraviolet (UV) light from a deuterium lamp. The dimensions of the rectangular light beam were 2.0 mm × 0.1 mm. Each TLC analysis was performed in triplicate. Then the developed and scanned chromatographic plates were utilized at the next experimental step, which was either direct analysis of the separated chromatographic bands with use of mass spectrometer (1D RP-TLC–MS), or an indirect analysis of these bands by further separating them with use of high-performance liquid chromatography followed by mass spectrometric detection (2D RP-TLC–RP-LC–MS).

Like in the study presented in papers (8,9), application of TLC – densitometry was an indispensable initial step in each of the procedures introduced in this study. Firstly, it enabled group separation of complex mixtures contained in the investigated samples into several less complex mixtures. Secondly, it allowed localization of the separated chromatographic bands on the plate surface. Consequently, positions of individual chromatographic bands could be marked on the adsorbent surface, indicating the areas for the consecutive elution with use of the TLC–MS interface.

**1D RP-TLC–MS**

The first chromatographic mode assumed in this study consisted of a direct elution of a given band from the chromatographic plate with use of the TLC–MS interface (Camag), which enabled an introduction of a given band to mass spectrometer. This mode is referred to as the reversed-phase thin-layer chromatography with mass spectrometric detection (1D RP-TLC–MS) and it has been devised to fingerprint the preliminarily fractionated mixture of phenolic acids. Here it needs to be emphasized that all thin-layer chromatograms were performed on the fresh and clean chromatographic plates. However, each plate can absorb humidity and the other volatile compounds (e.g., carbon dioxide) from the environment. Moreover, the residual amounts of the applied mobile phase can also be found on its surface, when eluting a given band even from the dried chromatogram to the mass spectrometer. Thus in order to avoid misinterpretation of the obtained mass spectra, we purposely avoided in our further “Results and Discussion” sections any comments on the signals in the m/z range below 150.

In this study, elution was carried out at ambient temperature either with use of pure methanol, or methanol + acetic acid, 99.5:0.5, v/v (the flow rate equal to 0.2 mL/min). The eluate was directly introduced to the Varian 500-MS model mass spectrometer (Varian, Harbor City, CA) and the samples were analyzed in the ESI mode (full ESI-MS scan, negative ionization, spray chamber temperature 45°C, drying gas temperature 200°C, drying gas pressure 25 psi, capillary voltage 80 V, needle voltage 5 kV). Varian MS Workstation v. 6.9.1 software was used for data acquisition and processing. Prior to the registration of the spectra, the mass spectrometer was carefully calibrated.
according to the procedures prescribed for such calibration by the manufacturer (Varian). Besides, the baseline of the mass spectra was strictly controlled and corrected prior to acquisition of the spectra.

2D RP-TLC–RP-LC–MS

The second chromatographic mode was aimed at mass spectrometric fingerprinting of the phenolic acid extracts derived from the sage species. Owing to the TLC–MS interface, we developed the two-dimensional liquid chromatography system equipped with mass spectrometric detection, off-line coupling the thin-layer chromatographic system with the high-performance liquid chromatograph (TLC–LC) (8,9). In this two-dimensional system, the RP-TLC part provides preliminary group separation of the investigated extract, with individual separated fractions one by one transported with aid of the TLC-MS interface not directly to mass spectrometer, but to the high-performance liquid chromatograph first. Eventually, we used mass spectrometer as a detector, to obtain the 2D RP-TLC–RP-LC–MS tandem system.

The working conditions of TLC-densitometry and MS employed in this second analytical mode were exactly the same as in the first mode. Liquid chromatographic analysis with mass spectrometric detection (LC-MS) was carried out using an LC–MS System Varian (Varian, Palo Alto, CA) equipped with a Varian ProStar model pump, Varian 100-MS mass spectrometer, and Varian MS Workstation v. 6.9.1 software for data acquisition and processing.

The LC analyses were carried out in the isocratic mode, using a Pursuit XRs 3-C18 column (50 mm × 2.0 mm i.d.; Varian; cat. no. A6001050C020) and either pure methanol, or methanol–acetic acid, 99.5:0.5 (v/v) as mobile phase at the flow rate of 0.20 mL/min. Mass spectrometric detection and processing of the obtained results were carried out using the same working parameters as those described in the preceding subsection.

Figure 1. The densitogram and videoscan obtained from the analytical thin layer chromatogram developed at 21 ± 0.5°C for the fraction of phenolic acids selectively extracted from Salvia lavandulifolia, with the bands of interest indicated by numerals 1, 4, 5, and 8 (A), and the mass spectra of band 1 (B), band 4 (C), band 5 (D), and band 8 (E) eluted from the chromatographic plate with use of the TLC-MS interface.
Results

1D RP-TLC–MS

Separation of the phenolic acids extract derived from *Salvia lavandulifolia* was carried out with use of the reversed-phase thin-layer chromatography (RP-TLC) on the C18 stationary phase and with the aqueous eluent. Videoscan of the chromatogram showing the developed phenolic acids was recorded at 366 nm and it is shown in Figure 1A. In the same Figure 1A, the densitogram recorded from the chromatographic plate is presented, with the eight bands (1–8) indicated on it.

In an attempt to ascribe the separated chromatographic bands to individual phenolic acids, it was necessary to use the retardation parameter (RF) values of the employed standard compounds (see Table I). From our experiment it can be assumed that the majority of the applied standards were not detected in the investigated *Salvia lavandulifolia* extract on the thin-layer chromatographic C18 plate. Further, it can be assumed that band 4 contains caffeic acid (as convincingly proved by its intensely blue fluorescence both with the standard and the extract sample, when the chromatogram was irradiated with the 366 nm light), band 6 contains rosmarinic acid, and band 7 contains chlorogenic acid (see Table I). None of the employed standards could be ascribed to bands 1–3, 5, and 8. The sequence of the identified phenolic acids on the considered chromatogram remains in rational agreement with the chemical structure of the detected compounds. Thus, caffeic acid identified in band 4 (with its two hydroxyl groups in a vicinal position and therefore apt to form intramolecular H-bonds), certainly has greater affinity to the non-polar stationary phase than rosmarinic acid identified in band 6 (which contains five hydroxyl groups in its molecule and hence, shows less affinity to this stationary phase than caffeic acid). The lowest affinity to C18 is observed with chlorogenic acid (band 7), due to the six hydroxyl groups per one acid molecule.

The mass spectra shown in this study refer to the selected thin-layer chromatographic bands only (and not to all bands separated and denoted in Figure 1A as bands 1–8), as our methodical goal was to demonstrate the selected examples of a direct mass spectrometric evaluation (with a possibility of the future fingerprinting kept in mind) of the thin-layer chromatograms of the complex natural mixtures of botanical origin, and also to compare these preliminary fingerprinting result of the 1D RP-TLC–MS mode with the 2D RP-TLC–RP-LC–MS one. Repeated acquisition of the mass spectra from the different thin-layer chromatograms performed for one and the same phenolic acids extract was carried out, in order to verify the repeatability of the assumed approach. Based on visual inspection and comparison of the obtained mass spectra, very good repeatability was confirmed in this sense that the analogous signals were present in the compared mass spectra, and also the general profiles (or envelopes) were very similar.

Thus, in Figure 1B–1E, mass spectra are presented valid for bands 1, 4, 5 and 8, respectively (as denoted on the densitogram.

<table>
<thead>
<tr>
<th>Standard</th>
<th>Structural formula</th>
<th>Molecular weight</th>
<th>RF no.</th>
<th>Peak no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ellagic acid</td>
<td><img src="image" alt="Ellagic acid" /></td>
<td>302.20</td>
<td>0.15</td>
<td>–</td>
</tr>
<tr>
<td>trans-Cinnamic acid</td>
<td><img src="image" alt="trans-Cinnamic acid" /></td>
<td>148.17</td>
<td>0.19</td>
<td>–</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td><img src="image" alt="Ferulic acid" /></td>
<td>194.18</td>
<td>0.42</td>
<td>–</td>
</tr>
<tr>
<td>p-Coumaric acid</td>
<td><img src="image" alt="p-Coumaric acid" /></td>
<td>164.16</td>
<td>0.44</td>
<td>–</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td><img src="image" alt="Vanillic acid" /></td>
<td>168.15</td>
<td>0.52</td>
<td>–</td>
</tr>
<tr>
<td>Syringic acid</td>
<td><img src="image" alt="Syringic acid" /></td>
<td>198.17</td>
<td>0.53</td>
<td>–</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td><img src="image" alt="Caffeic acid" /></td>
<td>180.16</td>
<td>0.54</td>
<td>4</td>
</tr>
<tr>
<td>p-Hydroxybenzoic acid</td>
<td><img src="image" alt="p-Hydroxybenzoic acid" /></td>
<td>138.12</td>
<td>0.55</td>
<td>–</td>
</tr>
<tr>
<td>Rosmarinic acid</td>
<td><img src="image" alt="Rosmarinic acid" /></td>
<td>360.31</td>
<td>0.58</td>
<td>6</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td><img src="image" alt="Protocatechuic acid" /></td>
<td>154.12</td>
<td>0.68</td>
<td>–</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td><img src="image" alt="Chlorogenic acid" /></td>
<td>354.31</td>
<td>0.71</td>
<td>7</td>
</tr>
</tbody>
</table>

* Their respective structural formulas and molecular weights, the retardation factor (RF) values for the selected standard compounds, and a possible occurrence thereof in chromatographic peaks indicated in Figure 2.
and videoscan), eluted with use of the TLC–MS interface and the methanol-acetic acid eluent. Prior to a more detailed discussion of these results, let us make the following general statement. All three phenolic acids identified by means of TLC in the *Salvia lavandulifolia* extract (i.e., caffeic acid, rosmarinic acid, and chlorogenic acid) are made of the caffeic acid building block, as it can easily be seen from a comparison of molecular structures given in Table I. In all mass spectra given in Figure 1B–1E, characteristic signal at m/z 198 can be observed and it seems largely probable that this intense signal is closely related to the aduct of caffeic acid with water. Another characteristic feature of the recorded mass spectra (see Figure 1D–1E) is the signals which appear at the values higher than m/z 400.

### 2D RP–TLC–RP–LC–MS

Similarity between the mass spectra originating from the TLC–MS and the TLC–LC–MS mode is evident and in a sense expected. For all RP–TLC–RP–LC–MS spectra, the presence of a

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**Figure 2.** Liquid chromatogram recorded by means of the LC–MS system for band 1 (Figure 1) eluted with use of the TLC–MS interface from the chromatographic plate, with the positions of recording the mass spectra indicated by numerals 1 and 2 (A), and the mass spectra of band 1 (A) and band 2 (B) from the liquid chromatogram.

**Figure 3.** Liquid chromatogram recorded by means of the LC–MS system for band 4 (Figure 1) eluted with use of the TLC–MS interface from the chromatographic plate, with the positions of recording the mass spectra indicated by numerals 1 and 2 (A), and the mass spectra of band 1 (A) and band 2 (B) from the liquid chromatogram.
large number of signals with the relatively high m/z values seems characteristic (see Figures 2–5). These signals can originate from the caffeic acid-derived trimers. For example, the signal at m/z 535.52, present in the spectrum given in Figure 2B, can originate from the (sagecoumarin – H)⁻ anion and the signal at m/z 539.77, present in Figure 2C, can be attributed to the (yunnaneic acid D – H)⁻ anion. In Figure 2B, a very intense signal at m/z 560 can be seen, which can originate from the caffeic acid dimers, or from rosmarinic acid (and more exactly, from the rosmarinic acid derivatives, e.g., its glucosides).

Discussion

The hydrophobic surface of the C₁₈ thin layer employed in this study has proved better suited for the analysis of phenolic acids than the highly active silica gel adsorbent, employed for the same purpose in our previous study (9) and evidently able to induce structural transformation of these acids. As a result, even after a short exposure of the silica-gel chromatograms to ambient air, the separated bands have been turning yellowish-brown, which made a comparison between these bands and those of the standards hardly possible. In our present study, the C₁₈ layer enabled an easy classical usage of the external standards to identification of the bands separated by means of TLC.

At the same time, we are aware of the fact that an agreement between the retardation factor (Rᶠ) values of the standard compounds and the respective bands on the chromatogram of the Salvia lavandulifolia extract cannot be regarded as an undeniable identification proof, although it can serve as a strong suggestion, especially when combined with the observations summarized below.

Right from the beginning of our experiment, we realized that most of the phenolic acid standards (i.e., ellagic, ferulic, p-coumaric, vanillic, syringic, caffeic, p-hydroxybenzoic, protocatechuic, and chlorogenic acid) applied in this study have never before been identified in Salvia lavandulifolia, except for rosmarinic acid (11). Our present results largely confirmed the previous findings, except for caffeic acid and chlorogenic acid. What is perhaps most important, from the review on polyphenolics identified in the Salvia genus (12) it comes out that with all Lamiaceae, the presence of chlorogenic acid is surprisingly uncommon and prior to our own research, it has not been reported in Salvia lavandulifolia (which seems a rather valuable contribution of this study to identification of its phenolic acids composition).

Chlorogenic acid on the C₁₈ plate was tailing (maybe due to the hydrolysis of this glycoside under the influence of acetic acid contained in mobile phase, and partial release of the caffeic acid aglycone and the sugar moiety). Another (or maybe complementary) reason of this tailing could be the fact that the molecule of chlorogenic acid contains five hydroxyl (-OH) groups. Three of them belong to the sugar moiety in the chair conformation and appear, respectively, in the axial and equatorial position, thus unable to build intramolecular H-bonds. They can, however, interact through the intermolecular H-bonds with residual non-bonded silanols (Si-OH) of C₁₈, in that way contributing to the observed “smearing” effect.

Now let us refer to the mass spectrometric results. As it comes out from the literature (12), trimers derived from caffeic acid constitute the largest group of the Salvia genus metabolites. They include such compounds, as lithospermic acid (m.w. 538), sagecoumarin (m.w. 536), salvianolic acids H, I, J, and K, with their respective molecular weights equal to 531, 531, 526, and 555, and yunnaneic acids C, D, E, and F, with their respective molecular weights equal to 538, 540, 572, and 597. Thus, the
mass spectrometric signals which appear in Figure 1C–1E at the values higher than m/z 400 could probably be ascribed to certain phenolic acid trimer(s).

Finally, it can be concluded that the results obtained with use of the two analytical techniques discussed in this study (i.e., TLC–MS and TLC–LC–MS) are important for the future fingerprinting of any given herbal material. Evident complexity of the mass spectrometric fingerprints makes their direct visual inspection and comparison rather difficult and hence, an immediate necessity arises to develop an adequate chemometric approach that might enable a more accurate utilization of the rich information contained therein.

**Conclusion**

The hydrophobic C$_{18}$ thin layer has proved a right adsorbent for the group separation of the phenolic acids fraction selectively extracted from the *Salvia lavandulifolia* sample and for the classical usage of the external standards.

Upon the obtained TLC results, it can be deduced that the *Salvia lavandulifolia* extract might probably contain chlorogenic acid never before reported to occur in this particular sage species.

Mass spectrometric fingerprinting of the phenolic acids extract preliminarily separated with use of both, the 1D RP-TLC and the 2D RP-TLC–RP-LC mode is possible and it furnishes an abundant data set, which can prove very helpful in chemotaxonomy and for the plant identification purposes.

Due to considerable complexity of the mass spectrometric fingerprints, a chemometric approach has to be developed, able to facilitate their rapid and non-biased usage.

Attribution of certain mass spectrometric signals to the selected constituents of the investigated plant extract can be considered as tentative and provisional only, as no rigid identification protocol has been followed in this study.

**Acknowledgement**

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