Comparative study of different approaches for multivariate image analysis in HPTLC fingerprinting of natural products such as plant resin

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ABSTRACT

Considering the introduction of phytochemical fingerprint analysis, as a method of screening the complex natural products for the presence of most bioactive compounds, use of chemometric classification methods, application of powerful scanning and image capturing and processing devices and algorithms, advancement in development of novel stationary phases as well as various separation modalities, high-performance thin-layer chromatography (HPTLC) fingerprinting is becoming attractive and fruitful field of separation science. Multivariate image analysis is crucial in the light of proper data acquisition. In a current study, different image processing procedures were studied and compared in detail on the example of HPTLC chromatograms of plant resins. In that sense, obtained variables such as gray intensities of pixels along the solvent front, peak area and mean values of peak were used as input data and compared to obtained best classification models. Important steps in image analysis, baseline removal, denoising, target peak alignment and normalization were pointed out. Numerical data set based on mean value of selected bands and intensities of pixels along the solvent front proved to be the most convenient for planar-chromatographic profiling, although required at least the basic knowledge on image processing methodology, and could be proposed for further investigation in HPLTC fingerprinting.

1. Introduction

Thin-layer chromatography (TLC) is commonly used technique for screening of low-molecular mass compounds from complex food, pharmaceutical and environmental samples [1,2]. It took priority over other chromatographic methods, like high-performance liquid chromatography (HPLC) and gas chromatography (GC), due to its simplicity, flexibility, accessibility and cheapness. With development of high-performance adsorbent layers and sophisticated instrumentation for sample application, chromatogram development, derivatization and chromatogram evaluation, high-performance thin-layer chromatography (HPTLC) became very popular.

Among numerous application of HPTLC as rapid, and effective chromatographic method for analysis of complex mixtures, its employment in fingerprint analysis is of special interest [3]. Chemical fingerprint is chemo-profiling that establishes a characteristic chemical pattern for the material or extract, which help in its identification. The entire chromatogram is treated as unique multivariate fingerprint, i.e. multidimensional vector, without special identification of single peaks. Concerning a small amount of sample needed for analysis, high number of samples that could be separated in parallel on the same plate resulting in a high throughput, better precision and accuracy caused by simultaneous analysis of both samples and standards under the same conditions, and wide choices of adsorbents and developing solvents, TLC is often a method of choice in chemo-profiling of natural products [3]. Chromatographic data can be obtained from the chromatograms by the means of classical densitometry, by densitometric scanning with a small slit (the signal response represents the signal from one pixel – simulation of scanning with camera [4]), or by collecting a photo of selected chromatogram and extracting a video-densitogram of the target track by the means of appropriate software. Photos of TLC plate can be taken by several devices such as commercially available DigiStore 2 image analyzing system, equipped with 12-bit CCD Camera with excellent color fidelity, or in-house made apparatus equipped with digital camera or by flat-bed scanner [5]. Although the digital camera is much cheaper, easily available and could be used to perform quantitative analysis with accuracy that is surprising with such equipment [6–9], the main disadvantage remains manual positioning of plate and illumination system (not uniform illumination [6–9], the main disadvantage remains manual positioning of plate and illumination system (not uniform illumination [5]) that could significantly affect further chemometric evaluation [10].
For ensuring the quality of the image photo the optical parameters for shot work should be adjusted and recorded in order to ensure the photo looks like the original image as much as possible.

Stored images could further be processed with several software packages such as ImageJ [11], Just TLC [12], Sorbil TLC Videodensitometer [13], MATLAB [14], etc [8,15–19]. The role of the software in TLC fingerprinting are: (a) post-correction of the photo, since the chromatographic process in TLC goes under an open system and produce some unexpected contamination that could be occurred during the development and visualization; (b) quantification by calculating peak area of characteristic bands on chromatograms, separately [20], or by measuring the intensities of pixels along the solvent front [21,22], or by determining mean value of each zone [17]. Correction of the photo should be made appropriately encompassing processes like baseline removal, denoising, target peak alignment and normalization [10]. Regardless the procedure of image processing, the result must closely resemble the original image [16].

Great amount of information for a large number of samples (up to 20 samples in a single run), obtained from thin-layer chromatogram, require the use of statistical procedures in order to efficiently extract the maximum useful information from the data. Once the data have been properly recorded, extracted, and pre-treated, several classification and regression methods such as principal component analysis (PCA), hierarchical cluster analysis (HCA), linear discriminant analysis (LDA), partial least square discriminant analysis (PLS-DA), k-nearest neighbours (KNN), classification and regression trees (CART), artificial neural networks (ANN), and partial least square regression (PLS), have been applied in TLC fingerprint studies [9,16–22].

Due to an increasing scientific interest for the use of HPTLC in combination with multivariate analysis, as a tool for fingerprint of different natural products, it is important to indicate the procedure for image processing. In that sense three commonly used image analysis procedure for transformation of HPTLC chromatograms to numerical data set were studied in detail on the example of plant resins chromatograms. Resins from wide range of different plants were chosen as test samples, as a good input data for image processing and multivariate analysis due to the vast number of different colored bands corresponding to their rich phenolic profiles. The aim of this study is to present TLC in the light of fingerprint and chemometric methodology, to highlight the most important steps in image processing prior to application of multivariate data analysis, and to compare different approaches for multivariate image analysis.

2. Materials and methods

2.1. Chemicals and materials

2-Aminoethyl diphenylborinate (NTS) was purchased from Fluka (Steinheim, Germany), toluene and ethyl acetate from Merck (KGaA, Darmstadt, Germany), polyethylene glycol (PEG) and methanol from Sigma-Aldrich (Steinheim, Germany), and formic acid from Kemika (Zagreb, Croatia). All solvents used for extraction, for mobile phase preparation and plate derivatization were of analytical purity grade.

2.2. Plant resin samples

A total of 50 plant resins samples were collected from different regions of Serbia (Table S1, Supplementary material) during spring 2014. Approximately 2 g of plant resin sample (deciduous trees – Populus nigra, Betula pendula, Populus tremula, Quercus Robur, Salix herbaceae, Syringa and fruit trees – Fraxinus avium, Pyrus, Malus domestica, Cidonia oblongata, Prunus persica, Prunus armeniaca, Prunus mahaleb) was cut, mix with 20 mL of a mixture of ethanol - water (16:4, v/v), and the solution was ultrasonicated for 45 min. Subsequently, extracts were filtered and evaporated to dryness. Dried residuals were redisolved in 5 mL of methanol and stored in fridge (4 °C) prior to analysis.

2.3. High-performance thin-layer chromatography

The 2 μL of plant resins extracts were applied to the 20 cm×10 cm silica gel HPTLC plates (Art. 105641, Merck, Darmstadt, Germany) as 8 mm band by using Automatic TLC sampler 4 (ATS4, CAMAG, Muttenz, Switzerland). Plates were developed with a mixture of toluene - ethyl acetate - formic acid (6:5:1, v/v/v) in the saturated (20 min) vertical twin chamber up to the distance of 70 mm. Developed plates were dried for 5 min with hairdryer. The plates were then heated for 3 min at 100 °C on TLC Plate Heater III (CAMAG) and immediately dipped in 0.5% solution of NTS in ethyl acetate for 1 s, by using Chromatogram Immersion Device III (CAMAG). After 5 min of drying in the air, the plates were immersed in 5% solution of PEG 400 in dichloromethane for 1 s, for enhancement and stabilization of fluorescent zones. Images were captured at 366 nm with DigiStore 2 device image analyzing system in conjunction with Reprostar 3. Four apertures with exposure time of 30 ms and frame of 2 mm were applied. The photos were stored as TIF files for further image processing.

2.4. Image processing and multivariate data analysis

Images of the plates were processed with the MATLAB processing program, ImageJ, and Sorbil TLC Videodensitometer [11,13,14].

ImageJ is a freely available Java-based program for digital picture manipulation which could be used for simple picture transformations such as resizing, cropping, and rotating or advanced ones such as filtering, smoothing, background subtraction, auto balance, or grayscale conversion and other signal transformations. ImageJ software contains many built-in algorithms, which are proved to be sufficient for correct processing of images of TLC plates [2,11,22]. It provides an option for plotting intensity associated with each pixel for particular chromatogram, as well as raw data export which is particularly useful for further chemometric data handling.

Sorbil TLC Videodensitometer is of limited power in signal profiling and data exporting but can be very useful in quantitation and peak identification.

Principal component analysis was carried out by PLS ToolBox [14]. The data were additionally pre-processed by using mean centering, which is the preferred option when the classification of the samples is based on variables that are all measured in the same unit. PCA was carried out as an exploratory data analysis by using a singular value decomposition algorithm and a 0.95 confidence level for Q and T2 Hotelling limits for outliers. It was performed in order to reduce dimensionality of data hyperspace, visualize the structure of data (i.e., confirm any possible samples clustering based on HPTLC fingerprint analysis), identify important variables, and underline the presence of outliers. A PCA was applied on the results obtained for each channel, separately.

3. Results and discussion

Natural products' extracts are complex mixtures that contain vast number of compounds whose thin-layer chromatographic profile is usually taken into consideration for an assay of authenticity and quality. However, HPTLC analysis is generally affected by noise, related to grainy nature of plates, non-uniformity of the plate caused by derivatization, progressive degradation of spot color, etc. All these drawbacks should be properly corrected before chemometric analysis in order to extract the maximum valuable information from chromatographic profile. In the current study, important steps in image processing, baseline removal, denoising, target peak alignment and normalization were pointed out in detail as it is still a main short-coming of majority fingerprint studies. Results of HPTLC fingerprinting of fifty plant resins were analyzed by three the most commonly used
Plant resins are complex mixtures of phenolic and isoprenoid compounds secreted by plants to provide protection against predators and pathogenic microorganisms [23,24]. The chemical composition of resins is complex, varies within and among plant families and is mostly unknown. Botanical sources of bee-collected resins could be identified applying classical chemical analysis by sampling at the colony level. However, these methods are, however, hard to apply on unknown sample due to the amount of a priori information required and a fact that bees collect resins from more than one plant and mix them in the hive [24]. The exact identity of characteristic set of compounds must be determined for a large number of samples in order to uncover differences among complex mixtures such as resins. Generally, little is known about the botanical origin of resins in many regions or the benefits of specific resins to bees. A group of authors used metabolomic method as a type of environmental forensics to track individual resin forager behavior through comparisons of global resin metabolite patterns [24]. We used HPTLC fingerprint analysis to seek for a characteristic pattern of high number of resins samples with wide range of botanical origin, by using advantages of multivariate image processing and chemometrics. Resins from deciduous trees from Salicaceae family (Populus sp. and Salix sp.), some fruit trees from Rosaceae family (Prunus sp.) and few other species were analyzed.

Evaluation of the chromatographic profile of plant resin samples was based on application of HPTLC conditions optimized regarding the resolution of the phenolic acids and flavonoids. Although a large variety of solvent systems, adsorbents, and chromatographic techniques were employed in TLC analysis of phenolics, the most frequent are combinations of silica gel stationary phase and developing solvents consisting of mixtures of three, four, or even five solvents of various polarities. Typically, normal-phase system was employed to separate highly and medium polar phenolics. Moreover, formic acid is added to the mobile phase to suppress the ionization of acidic groups and improve the shape of chromatographic bands [25].

Visual examination of the obtained HPTLC chromatograms (Fig. 1.) revealed a difference in chemical composition of plant resins’ extracts. A group of samples belonging to Populus sp. is rich in phenolic compounds and has a pattern dominated by blue and orange color bands, while the other chromatograms also contain red and green bands all with lower intensity compared to Populus sp. group. Colorful picture-like HPTLC chromatograms, such as those obtained for plant resins’ samples, are excellent input data for image processing and further chemometric analysis. Splitting images through the red (R), green (G), and blue (B) channels we could increase selectivity and differentiate compounds according to their fluorescent colors [26] enabling the exact identity of characteristic signature compound of each plant resin.

3.2. Data acquisition and chemometric analysis

Chromatographic data set for further analysis was generated by collecting photos of HPTLC chromatograms obtained by image analyzing system. Compared with conventional slit-scanning densitometry, where a small part of the TLC plate is irradiated through a slit of defined dimensions, in video recording the whole plate is irradiated and the image is captured by an image-acquisition device (charge coupled device, CCD, camera) [6,21]. Owing to the uniform lighting of surfaces, short time of scanning, high optical resolution, and multi-channel scanning capabilities, video recording is suitable for fast and efficient collecting of TLC chromatograms. Final results of chromatographic analysis depend on the quality of the captured image. In that sense, camera settings such as aperture, frame accumulation, and exposure time were optimized to improved image quality.

3.2.1. Data based on mean value of selected zone

Multivariate analysis performed on data based on mean value of selected HPTLC bands was successfully used for classification of propolis samples from Germany and other locations based on their phenolic compound profile [17]. The results obtained by the chemometric evaluation of HPTLC and DART-MS data provided complementary information, while the complexity, expense, and image analysis time were significantly reduced due to the use of statistical tools for evaluation of fingerprints. This approach significantly reduced the relevant effort for pattern recognition and categorization of samples which turned out to be time-consuming especially for unclear sample assignments.

Image analysis of HPTLC profiles of plant resins were performed according to the method described in [17]. The HPTLC plate images of...
analyzed plant resins were exported from the winCATS software (CAMAG) to MATLAB. The RGB-scale images were converted into double precision (im2double) and then transformed into 8-bit monochromatic grayscale by eliminating the hue and saturation information while retaining the luminance. Each HPTLC image was, on this way, converted into the data matrix. Obtained data matrices were transferred into Microsoft Office Excel document in order to generate HPTLC profiles of all investigated plant resins by calculating $R_F$ values of the target zones and accompanying intensities for each sample’s image. Characteristic peaks were chosen as variables for further multivariate models. Data matrix was composed of averaged intensities on targeted $R_F$ values as independent variables for each object considered [17]. Spatial differences of the location of the selected bands were not confirmed and image warping procedure was not applied before chemometric analysis.

PCA was applied on data set consisted of fifty samples and fifteen carefully selected variables of gray scale intensities at $R_F$ values: 0.03, 0.04, 0.07, 0.08, 0.19, 0.30, 0.44, 0.47, 0.48, 0.52, 0.54, 0.57, 0.65, 0.79, and 0.82. Variables at marked $R_F$ values were chosen as input data after visual examination of line profile plots of all investigated plant species. The line profile plot displays a two-dimensional graph of the intensities of pixels along a chromatogram. Each sample was characterized by the presence of the selected peaks. Variables at low $R_F$ values were selected due to the presence of highly polar phenolic compounds on chromatograms of fruit trees, which was characterized by different colored bands. The data were additionally pre-processed by using mean centering. Statistical parameters of models (number of principal components (PCs), percent of total variance explained by model, and a share of each PC in the overall data variance) are presented in Table 1. Mutual projections of factor scores for the first two PC s and loading plot for first and second principal components (PC1 and PC2, respectively) are presented in Fig. 2. PCA resulted in a two-component model which explains 99.98% of total variance. The first principal component, PC1, accounted for 98.94% of the overall variance. It is quite unusual that for such a high number of natural samples PC1 explain so high percent of data variability. Score plot (Fig. 2A) reveals several groups of samples separated according to plant resin variety alongside the PC1 direction. The *Populus* sp. samples formed distinct cluster on the left side of PC score diagram, while other plant resins are mainly positioned on right side of score plot. Although of very similar chemical composition, *Prunus* sp. samples were clearly separated into six groups. The number of samples of other plant species was too small for classification but still positioned on score plot distant from *Populus* and *Prunus* sp. samples. The corresponding loadings plot displays relationships between variables and can be used to identify variables that contribute to the positioning of the objects on the scores plot and, hence, influence any observed groups in the data set. The zones at $R_F$ values 0.57, 0.54, 0.48, 0.19 and 0.08 were variables that exhibit the most positive impact on PC1 direction and differentiate plant resins according to their botanical origin (Fig. 2B).

3.2.2. Data based on intensities of pixels along the solvent front

Data based on intensities of pixels along the solvent front was previously used by authors for HPTLC phenolic profiling of Serbian propolis samples [22]. All studied samples were classified in two major types of European propolis confirming successful application of this approach.

Image analysis of HPTLC profiles of plant resins were performed according to the method described in [22] by ImageJ software for image preprocessing. Prior to chemometric analysis, each chromatogram was subjected to following steps: baseline removal, denoising, target peak alignment, and normalization.

Baseline removal step was skipped since differences of the background intensity between images were not confirmed. There are two main sources of noises in the HPTLC research: the first is related to the non-uniformity and damages of the stationary phase, while the second source is the imperfectness of the detection [28].

Denoising of the images was carried out using three pixels median filter; using higher width to much information was lost. The applied procedure enables to get plots with distinct peak borders, which lead to fewer mistakes in assessing areas under overlapping peaks. The line profile plots of chromatograms obtained with application of ImageJ software, for one selected plant resin sample, and adjusted to its three RGB channels, are presented in Fig. S1 (Supplementary material). Only certain channels are responsible for the color value of a given point from the chromatographic plate.

Peak alignment was employed to correct the inter- and intra-plate peak shift due to different vapor fluctuations variations in mobile phase composition, inefficient drying of the application zones, humidity, temperature, local change of the stationary phase, instrumental instability as well as human error. In general terms, warping aims to enhance the similarity of profiles by shifting and/or stretching and/or compressing them along their $x$-axis [29]. Many warping techniques such as Correlation Optimized Warping (COW), Dynamic Time Warping (DTW), and Fuzzy Warping, have been applied in order to remove the negative impact of band shifts. The HPTLC chromatograms were warped to the reference by deleting or adding baseline segments near the selected signals using COW, so that the peak $R_F$ values were equalized [18,29]. In the case of COW, there are two important parameters such as section length (N) and slack parameter (t) that must be optimized to achieve a satisfactory chromatogram alignment. Further, the selection of a target sample is crucial when chromatograms are used for the construction of discrimination or classification models. There are several proposed ways for choosing of target sample: 1) the chromatogram with the highest mean correlation coefficient among all tracks in it, 2) chromatogram which contains the highest number of common chemical constituents, 3) alignment of all chromatograms with a randomly selected chromatogram, 4) chromatogram that is most similar to the loading plot of PC1. The best results for HPTLC chromatograms of plant resins were obtained using following optimal COW parameters, $N =50$ and $t=5$. Phenolic profiles of plant

**Table 1**

| A          | B          | C          |
|------------|------------|------------|
| Gray       | Red        | Blue       |
| Number of PCs | 2 | 3 | 2 |
| RMSEC      | 0.0041     | 0.2448     | 0.4055     |
| RMSECV     | 0.0246     | 10.2649    | 10.7675    |
| % Variance captured – Total | 99.98 | 79.30 | 61.16 |
| % Variance captured | 98.94% | 57.31% | 45.53% |
| PC1/PC2    | 1.04       | 13.48      | 15.62      |

A – Data based on mean value of selected zone.
B – Data based on intensities of pixels along the solvent front.
C – Data based on peak area of characteristic bands.
resins were mutually quite different making difficult the selection of reference chromatogram. In that sense, target sample for warping of extracts was set to be randomly selected.

Normalization was applied in order to remove the undesired effects due to the unequal amounts of samples applied on the stationary phase. Normalization is not obligatory preprocessing procedure and it is recommended to compare the results with and without normalization. The standard normal variate (SNV), as commonly applied normalization technique, normalizes the variables from each row with the standard deviation of all variables.

The scaling of samples was applied to each spectrum individually by subtracting the spectrum mean and scaling with the spectrum standard deviation. Scaling converts all the concentrations to fluctuations around zero instead of around the mean of the phenolic amount. It adjusts for differences in the offset between high and low abundant metabolites [29,30].

Data set used for chemometric analysis was consisted of 50 samples \( \times 516 \) variables matrix. Variables represent the intensities of pixels along the 516 length lines. Before multivariate data analysis the data were additionally pre-processed by using mean centering. This step is independent of scaling of each spectrum and is used to centre all data together.

Statistical parameters of models obtained for three channels are listed in Table 1. Mutual projections of factor scores for the first two PCs and loading plot for PC1 and PC2 for each channel are presented in

3.2.3. Data based on peak area of characteristic bands

The literature search revealed that multivariate analysis performed on data based on peak area of characteristic bands of HPTLC chromatograms was used exclusively for quantitative evaluations
Peak area of characteristic bands on HPTLC chromatograms were calculated by Sorbil TLC Videodensitometer software and further used as input data for PCA. Pretreatment of the images preceding the data analysis, baseline correction and denoising, seems to be simpler compared to ImageJ software. In this study, baseline removal step was skipped due to the reason explained in previous section, but the main steps in processing will be emphasized in order to indicate main differences with ImageJ software. In Sorbil TLC, inhomogeneous plate illumination could be removed with the application of 'Background approximation' function. Operator should select at least 10–30 markers on plate’s brightness places free from spots, chose 'Calculate background function' and 'Correct background' and software corrects the whole image brightness [33]. Contrary, in ImageJ software, operator should apply bandpass filter for baseline-drift correction and optimize the number of pixels for removing high spatial frequencies (blurring the image) and low spatial frequencies (similar to subtracting a blurred image), which require at least the basic knowledge on image processing methodology. Furthermore, track arrangement in ImageJ is performed by rectangular selection tool, baseline could only be set manually and the track width is automatically compensated. In order to obtain quantitative data the track width must be the same, contrary to Sorbil where its width could differ. In Sorbil, tracks could be arranged manually or automatically [33].

Statistical parameters of models obtained for three channels are presented in Table 1. Mutual projections of factor scores for the first and third principal component (PC3) and loading plot for PC1 and PC3, for blue channel, which gave the best classification results, are presented in Fig. 4. Two other models could be found in Supplementary material (Fig. S3). Score plot revealed the existence of one cluster consisting of Populus sp. samples, which was dissipated in a broader range and positioned close to other samples. Among resins of other...
botanical origin Prunus avium and Prunus persica samples formed two clusters but overlapped with other species among which could not be observed any classification. The highest influence on such grouping had bands on $R_f$ values 0.11, 0.14, 0.16, 0.30, 0.39, 0.65 and 0.74 (Fig. 4B).

### 3.3. General remarks

The latest trend in TLC analysis of natural products, pharmaceuticals and environmental samples is the usage of ‘image analysis’. It should be emphasized that image analysis of HPTLC profiles, beside the three previously mentioned approaches, could also be performed by measuring the zone area and color intensity using Just TLC software. However, this software has no options for image pre-processing such as denoising, baseline removal, target peak alignment or normalization which could be a major shortcoming to this method. Previously stated facts connected to the drawbacks of program are supported by the results of PCA applied on the HPTLC chromatograms of plant resins. Namely, statistical parameters of models and mutual projection of PCs results of PCA applied on the data obtained after ImageJ processing. However, good clustering of plant resin samples after application of first approach on 8-bit monochromatic grayscale images is probably due to the fact that we selected bands that have the similar color on the certain $R_f$ value.

### 4. Conclusion

The combination of HPTLC and image processing is still in progress and demands further improvements. However, the relatively low price of instrumentation, small amount of sample needed for analysis, high number of samples that could be separated in parallel on the same plate, better precision and accuracy caused by simultaneous analysis, wide choices of adsorbents and developing solvents and short time required for analysis make this method an optimal choice for profiling of natural products.

Development of efficient and reliable HPTLC fingerprint method requires application of experimental design and optimization techniques for the separation step, data acquisition, signal manipulation, and classification and modelling. Serious lack in application of aforementioned techniques could be observed, particularly in the part of image processing.

The conclusion based on the analysis of data obtained after application of three commonly used approaches for image processing on plant resins samples, is that first two are able to characterized samples according to their HPTLC fingerprint, while third approach should be rather use just for quantitative determinations. It should be stressed, however, that ImageJ software is the most difficult to perform due to the high number of parameters that should be properly adjusted which demand enviable knowledge of the operator. However, if image processing with this program is performed adequately, the results of HPTLC fingerprint analysis could be remarkable. Sorbfil TLC Videodensitometer is simple to perform but, according to our results, not suitable for thin-layer profiling. Splitting images through the R, G, and blue B channels is recommended due to the presence of different colored bands at the same $R_f$ value, which is confirmed with the results of PCA applied on the data obtained after ImageJ processing. However, good clustering of plant resin samples after application of first approach on 8-bit monochromatic grayscale images is probably due to the fact that we selected bands that have the similar color on the certain $R_f$ value.

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Appendix A. Supplementary material

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