

Thin Layer Chromatography and Chemometric Studies of Selected *Potentilla* Species

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Abstract

Herbal medicinal products have a long-standing therapeutic record. To ensure the quality of herbal products specific identification tests which allow experts to discriminate related species and/or potential adulterants/substitutes are required. The purpose of the research was to recommend an original and simple method for the separation of closely related five triterpenic acids (ursolic, oleanolic, tormentic, euscaphic, pomolic acid) and its application to chemotaxonomy studies. 17 standard samples of *Potentilla* species and 3 test samples were chromatographed (with or without prechromatographic derivatization) on silica gel plates using the mobile phase: chloroform-diethyl ether-methanol-formic acid (30:10:1:0.2 v/v/v/v); they were subsequently derivatized, and visualized in UV 366 nm light. After images received pre-treatment (montaging stack, removal of noise, background subtract, horizontal equalization, two types of warping) exploratory analysis of the investigated *Potentilla* species fingerprints was processed. The method without prechromatographic derivatization was used to create differential fingerprints. Good results were obtained by the application of r-project iterative algorithm for subtract background and our simple algorithm for warping to image processing of TLC chromatograms for the first time.

Keywords

Pomolic Acid, Ursolic Acid, Euscaphic Acid, Tormentic Acid, Oleanolic Acid, Triterpene Acids, TLC Fingerprint, *Potentilla*

1. Introduction

Herbal medicinal products have a long-standing therapeutic record and are still used as cure for various diseases

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all over the world. It is the quality of herbal medicinal products that is of major importance since chemical complexity of herbs varies greatly due to a wide range of factors such as species variation, hybrids, growth location and condition, climate, harvesting season and condition as well as drying and storage condition. To ensure the quality of herbal medicinal products, European Medicines Agency (EMA) suggest establishing good agricultural and collection practice for herbal starting materials (GACP) [1] and emphasize specific identification testing which allows experts to discriminate related species and/or potential adulterants/substitutes [2]. Herbal fingerprinting was adopted as a comprehensive qualitative methodology in which the entire chromatogram is assessed during data analysis and enables to distinguish different species occurring within the same herbal family [3]. By definition, the chromatographic fingerprint of herbal medicinal products is, in practice, the chromatographic pattern of the extract of some common chemical components of pharmacologically active and or chemically characteristics [4]. Thin layer chromatography (TLC) is frequently used as a chromatographic technique in fingerprint analysis of herbal medicines. The advantages of TLC method in the construction of such fingerprints are its simplicity, versatility, high velocity, specific sensitivity and simple sample preparation. Therefore, TLC is considered to be a very convenient method in quality determination and in tracing possible adulteration of herbal products [4].

The aim of this paper is to elaborate TLC method for the separation five triterpene acids (ursolic acid (UA), oleanolic acid (OA), pomolic acid (PA), tormentic acid (TA), euscaphic acid (EA)) present in the *Potentilla* species. The method was applied to construct the fingerprints of dichloromethane extracts of roots and rhizomes in the *Potentilla* species. This type of secondary metabolites was chosen on purpose because of its wide spectrum of pharmacological activities. Triterpene acids are reported to have an interesting bioactivity, including anti-HIV [5], hepatoprotective [6] [7], antimalarial [8], antimicrobial and anti-inflammatory effect [9], anti-tumoral activity [10] [11], anti-atherogenic [12] and hypoglycemic effect [13].

The separation of triterpene acids by thin layer chromatography was carried out using selected methods. The research found in recent papers points to simultaneous separation of various triterpene acids such as: oleanolic acid, β -amyrin and β -amyrin acetate on silica gel HPTLC plates using hexane-dichloromethane-methanol-water (4:5:0.9:0.1) [14], oleanolic and ursolic acid on silica gel HPTLC plates using petroleum ether-ethyl acetate-acetone (8.2:1.8:0.1) after pre-chromatographic iodine derivatization [15], ursolic acid and oleanolic acid and 8 other triterpenoids and sterols (α -amyrin, β -amyrin, δ -amyrin, lupeol, lupenon, lupeol acetate, cycloartenol, cycloartenol acetate, stigmaterol and β -sitosterol) on C18 RP-HPTLC plates using acetone-acetonitrile (5:1) or ethyl acetate-acetonitrile (3:2) and on silica gel HPTLC plates using n-hexane-ethyl acetate (5:1) [16], oleanolic, ursolic and betulinic acids on C18 RP-HPTLC plates using n-hexane-ethyl acetate (5:1) after pre-chromatographic bromine derivatization [17].

The aim of this paper is to elaborate TLC separation method of pomolic, ursolic, oleanolic, euscaphic and tormentic acid and apply it to chemotaxonomy studies of various *Potentilla* species. The method as such hasn't been reported yet. The studies of this plant genus were carried out to find the specific method for identification of both species and subspecies in the *Potentilla* genus. TLC fingerprints (videoscans) of dichloromethane extracts obtained from roots and rhizomes of various *Potentilla* species were chemometrically processed with free open source software. Our current research shows new strategies for image processing and thorough analysis of fingerprints.

2. Materials and Methods

2.1. Chemicals

All chemicals including solvents were at least of analytical grade. Dichloromethane, chloroform, methanol, ethanol, acetone, diethyl ether, formic acid, iodine crystals, sulphuric acid ($\geq 95\%$) were purchased from Polish Chemical Reagents (POCH, Gliwice, Poland).

The standard of ursolic acid (UA) ($\geq 98.5\%$), oleanolic acid (OA) (98.7%), stigmaterol (96.5%) were from Sigma-Aldrich (St. Louis, MO, USA), tormentic acid (TA) (98%), euscaphic acid (EA) (95%) and pomolic acid (PA) (98%) were from LGC Standards (BioBioPha Co, Ltd., China). β -sitosterol (97.8%) was from LGC Standards (Chromadex, USA). Chemical structures of triterpene acids are presented in **Figure 1**.

The plates were developed in horizontal Teflon DS chambers (Chromdes, Lublin, Poland). A rotary evaporator (Laborata 4003, Heidolph) was used for extracts evaporation. The ultrasonic bath Elmasonic S30H (Elma GmbH&Co. KG, Singen, Germany) was used for the extraction of compounds from *Potentilla* species.

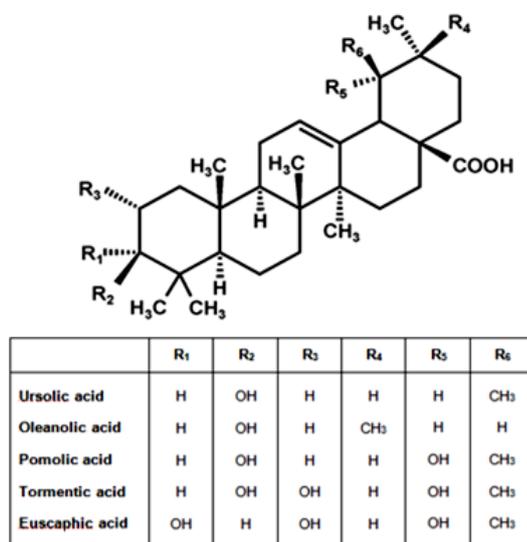


Figure 1. The structure of triterpene acids.

2.2. Preparation of Standard Solutions

Standard solutions of EA, PA, OA and UA were prepared by dissolving 1 mg of each compound in 1 mL of acetone. Standard solution of TA were prepared by dissolving 0.5 mg in 1 mL of acetone. All standard solutions were stored at 4°C.

2.3. Sample Preparation

Plant material was collected in Maria Curie-Skłodowska University Botanical Garden, Lublin, Poland. Roots and rhizomes of the *Potentilla* species were examined and treated as standard samples (see [Table 1](#)). Three test samples collected by authors in different parts of Europe and in different time (the Białystok Region-Poland, harvest year: 2008, the Zamosc Region-Poland, harvest year: 2010, central France, harvest year: 2011) were also examined.

Plant material was dried at room temperature for 10 days during which it was turned over every day and subsequently reduced to powder (mesh 0.315 mm), using a microfine grinder drive IKA Werke MF 10 basic (Staufen, Germany), and stored at room temperature.

3.00 g of dry powdered roots and rhizomes of the *Potentilla* species were extracted three times with 50 mL portions of dichloromethane within 30 min in an ultrasonic bath at the temperature of 30°C. The extracts were combined and evaporated to dryness using rotary evaporator under reduced pressure. Dry residues were further dissolved in acetone to fixed volume of 10 mL.

2.4. Preparation of Dipping Detection Reagent

10% (v/v) H₂SO₄ in ethanol detection reagent was prepared by mixing concentrated sulphuric acid (≥95%) (10 mL) and ethanol (90 mL). The mixture was cooled in an ice bath.

2.5. Prechromatographic Derivatization

One plate (with *P. megalantha* and all standards), see [Figure 2\(b\)](#), was developed with 1% iodine solution in chloroform at the distance of 1.2 cm. Next, the starting zone was covered by a glass strip and the plates were placed in darkness for 10 min. When the reaction was completed, the plates were dried in the stream of warm air to remove the excess of iodine.

Due to the changes in the appearance of chromatograms after iodine derivatization (the appearance of new, non-typical spots or disappearance/weakening of the already existing ones) we decided to apply the method without prechromatographic derivatization for the construction of *Potentilla* species fingerprints.

Table 1. *Potentilla* species.

<i>Potentilla</i> species	Collecting conditions
<i>Potentilla anserina</i> L.	Botanical Garden, Lublin, Poland, 2012
<i>Potentilla argyrophylla</i> Wall. ex Lehm.	Botanical Garden, Lublin, Poland, 2012
<i>Potentilla atrosanguinea</i> G. Lodd. ex D. Don	Botanical Garden, Lublin, Poland, 2012
<i>Potentilla bifurca</i> L.	Botanical Garden, Lublin, Poland, 2012
<i>Potentilla collina</i> Wibel	Botanical Garden, Lublin, Poland, 2012
<i>Potentilla crantzii</i> (Crantz) Beck ex Fritsch	Botanical Garden, Lublin, Poland, 2012
<i>Potentilla desertorum</i> Bunge	Botanical Garden, Lublin, Poland, 2012
<i>Potentilla erecta</i> (L.) Raesch	Botanical Garden, Lublin, Poland, 2012
<i>Potentilla fulgens</i> Wall. ex Lehm.	Botanical Garden, Lublin, Poland, 2012
<i>Potentilla grandiflora</i> L.	Botanical Garden, Lublin, Poland, 2012
<i>Potentilla hirta</i> L.	Botanical Garden, Lublin, Poland, 2012
<i>Potentilla megalantha</i> Takeda	Botanical Garden, Lublin, Poland, 2012
<i>Potentilla montenegrina</i> Pant.	Botanical Garden, Lublin, Poland, 2012
<i>Potentilla neumanniana</i> Rchb	Botanical Garden, Lublin, Poland, 2012
<i>Potentilla recta</i> L. subsp. <i>obscura</i> (Willd.) Arcang.	Botanical Garden, Lublin, Poland, 2012
<i>Potentilla recta</i> L.	Botanical Garden, Lublin, Poland, 2012
<i>Potentilla reptans</i> L.	Botanical Garden, Lublin, Poland, 2012
Test sample 1	Bialystok Region, Poland, 2008
Test sample 2	Central France, 2011
Test sample 3	Zamosc Region, Poland, 2010

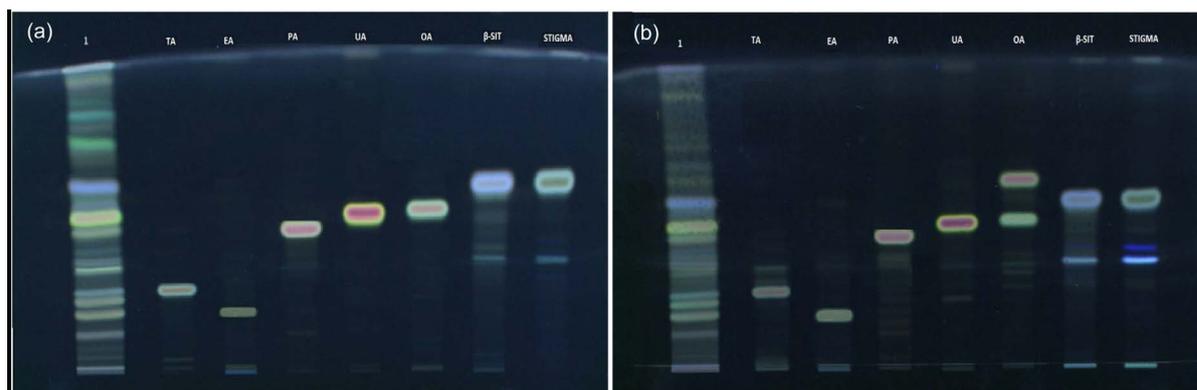


Figure 2. TLC silica gel 60F₂₅₄ developed in Teflon DS chamber using chloroform-diethyl ether-methanol-formic acid (30:10:1:0.2 v/v) after derivatization with 10% H₂SO₄ in ethanol detection reagent in UV 366 nm light. (a) Without prechromatographic derivatization; (b) With prechromatographic derivatization with 1% iodine solution in chloroform; Tracks: 1-*P. megalantha*, tormentic acid (TA), euscaphic acid (EA), pomolic acid (PA), ursolic acid (UA), oleanolic acid (OA), β -sitosterol (β -SIT), stigmasterol (STIGMA).

2.6. Thin-Layer Chromatography

TLC was performed on 10 cm \times 20 cm plates coated with silica gel 60F₂₅₄ Art. No. 1.05554 (Merck, Darmstadt, Germany). 10 μ L of standard solutions of ursolic acid, oleanolic acid, tormentic acid, euscaphic acid, pomolic acid, β -sitosterol, stigmasterol and 10 μ L of herbal extracts were spotted on the plates. To construct fingerprint, twenty samples of *Potentilla* extracts were applied on four plates. The same species or subspecies were on the same plate (tracks of the first plate: 1, *P. anserina*; 2, *P. hirta*; 3, *P. fulgens*; 4, *P. crantzii*; 5, *P. grandiflora*; tracks of the second plate: 6, *P. montenegrina*; 7, *P. recta* subsp. *obscura*; 8, *P. recta*; 9, *P. atrosanguinea*; 10, *P. atrosanguinea*; tracks of the third plate: 11, *P. erecta*; 12, *P. desertorum*; 13, *P. collina*; 14, *P. megalantha*; 15, *P. bifurca*; tracks of the fourth plate: 16, *P. neumanniana*; 17, *P. reptans*; 18, *Bialystok Region*, 2008; 19, *France*, 2011; 20, *Zamosc Region*, 2010).

The plates were developed in horizontal Teflon DS chambers (Chromdes, Lublin, Poland) with a mixture of chloroform, diethyl ether, methanol, formic acid (30:10:1:0.2 v/v) as a mobile phase at the distance of 8 cm.

Standards and sample solutions were applied as 15 mm bands, 20 mm from the left and right edge, 10 mm from the bottom edge and 35 mm apart by means of Linomat 5 (CAMAG, Muttenz, Switzerland), operated with winCATS (Planar Chromatography Manager) version 1.4.0. (CAMAG, Muttenz, Switzerland), and syringe 100 μ l (No. 695.0014) (Hamilton, Bonaduz, Switzerland).

After being dried in the stream of warm air the plates were sprayed with 10% (v/v) H_2SO_4 in ethanol detection reagent by means of TLC sprayer GW1 108540 (Merck, Darmstadt, Germany), dried for 5 min and then heated at 105°C for 3 min by means of TLC plate heater III (CAMAG, Muttenz, Switzerland).

The documentation of chromatographic plates was obtained with using a TLC Visualizer CAMAG in white light and UV 366 nm light by means of DigiStore 2 Documentation System (CAMAG, Muttenz, Switzerland), operated with winCATS Version 1.4.1.8154 software. The plates were documented within 10 min, afterwards progressive degradation was observed.

To ensure the repeatability of results we carried out the analysis on the same TLC plates (the same producer and batch lot). The samples were applied by means of Linomat 5. The chromatograms were developed in horizontal Teflon DS chambers with a mobile phase prepared in the amount sufficient to develop all plates. The plates were sprayed by means of TLC sprayer with 10% H_2SO_4 in ethanol detection reagent, prepared in the amount sufficient to spray all plates. The time of drying, heating as well as the time before taking a photo were strictly obeyed.

Such developed analytical method of the identification of five triterpenic acids was validated by the determination of specificity, repeatability and robustness.

The most common type of fingerprint analysis relied upon the data of methanol extracts analysis. Such extracts contain a number of compounds with different chemical structure, which facilitates the differentiation of the species of the same genus. In our study we used dichloromethane extracts which contained a relatively small group of compounds, which caused that the development of methods indicating the interspecies differences was much more difficult. The chemometric attempt shows that, for such difficult cases to develop, the method using image processing can be very effective.

3. Results and Discussion

3.1. Data Pre-Treatment

As mentioned above, for fingerprint construction four original TLC images with 5 samples of *Potentilla* extracts on each plate in UV 366 nm light were prepared. The original TLC images were exported from winCATs TLC workstation (CAMAG) to ImageJ (1.48c version), public domain, Java-based image processing program developed at the National Institute of Health. ImageJ was designed with open architecture that provides extensibility via Java plugins and recordable macros. From every track on the plate the subtracks was cut out (715 pixel length and 60 pixel width for each subtrack). Next the stack of all subtracks (as RGB color images) was montage. Three RGB channels were always processed separately, parallel. The stack of all subtracks was decomposed to three independent channels for red, green and blue primary color components before every step of image processing. The montaging stack should be a basic step in processing large number of images. For each stage of image processing special macro instruction was written. These executing algorithms were designed to automate all actions and make this process less tedious and less error-prone.

Subsequently, to increase the signal-to-noise ratio without greatly distorting the signal we used the Savitzky-Golay filter [18] which performs a simple local least squares regression and provides its fitted value to the output. The removal of noise was done using the Savitzky Golay Filter Plugin. This type of filter was previously performed for the removal of noise in Raman spectra [19], in densitometry [20] or TLC videoscans [21] TLC analysis is affected by noise (e.g. related to grainy nature of TLC plates, non-uniformity of the plate spraying, progressive degradation of spot color). Therefore, the increase the signal-to-noise ratio is required. As it proves previously [21] the charged coupled device (CCD) camera noise is almost uncorrelated (white one) and it can be treated as homoscedastic (almost no correlation of signal amplitude with noise amplitude is observed). For this reason, the optimal Savitzky-Golay parameters were selected without checking its autocorrelation. The half width of the filter (the length of the window) and the degree of the fitted polynomial (order of filter) were optimized. The difference between the surface plot of original TLC image and the surface plot of a smoothed ver-

sion of TLC image depending on the parameters used is presented in **Figure 3**. Optimal parameters which still retain features of TLC image (without over smoothing) were 8:3 (half width of filter: order of filter).

After smoothing the digital data in stack we subtracted the background. The built-in roller ball algorithm to remove the background baseline is implemented in ImageJ. The background estimated in this algorithm depends on the ball diameter, which has to be larger than the peak or the spot width. We decided to estimate of baseline (to remove it) by using another algorithm, based on “fill peak algorithm” described in baseline r-project package (free software environment for statistical computing and graphics). In this method, local windows of buckets the minimum of the mean and the previous iteration is chosen as the new baseline. In these windows of buckets the minimum of the mean and the previous iteration is chosen as the new baseline. The width of local window and the number of iterations in suppression loop were optimized. The line, which is created by this algorithm, is treated as the background estimate (see **Figure 4(b)**). The subtract background procedure is illustrated in **Figure 4**.

Subsequently, horizontal equalization for image defects removal was adopted. Each color of RGB subtracks was compressed (using compressed) to the size of 715 pixel length and 1 pixel width. Afterwards, the size of subtracks was adjusted to primary size (715×60) and in the next step red, green and blue stacks merged. The horizontal equalization allowed us to eliminate the image defects that occurred during TLC analysis (e.g. alignment of color spots, accidental dots, spots not aligned horizontally) or during the warping (e.g. narrowing of the image).

Therefore, the aim of the study was also the chemometric processing of 20 chromatograms (precisely 20 subtracks in stack). For this reason it is required to perform the alignment of chromatograms by warping. The warping was carried out using bUnwarpJ developed as an ImageJ plugin [22], previously used for TLC videoscans [23]. This is an algorithm for elastic and consistent image registration and it performs a simultaneous registration of two images, source image and target image (source image is elastically deformed in order to look as similar as it is possible to target image). The images like ours (RGB Color images) were converted to grayscale during the registration process. The resulting transformations were applied to the original color images.

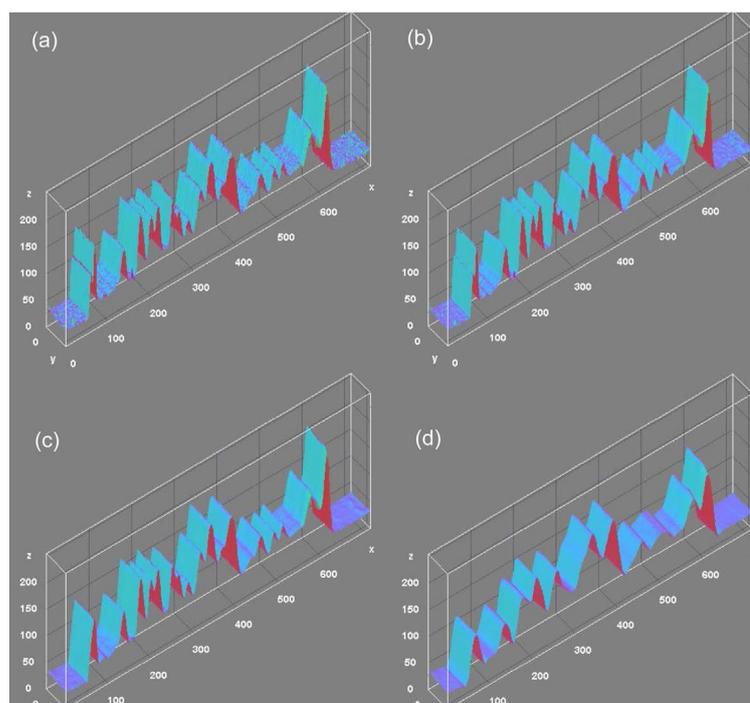


Figure 3. The Savitzky-Golay filter (half width of filter: order of filter). (a) Surface plot of original *P. anserina* TLC image; (b) Surface plot of a smoothed version *P. anserina* TLC image (6:4); (c) Surface plot of a smoothed version *P. anserina* TLC image (8:3); (d) (b) Surface plot of a smoothed version *P. anserina* TLC image (20:3).

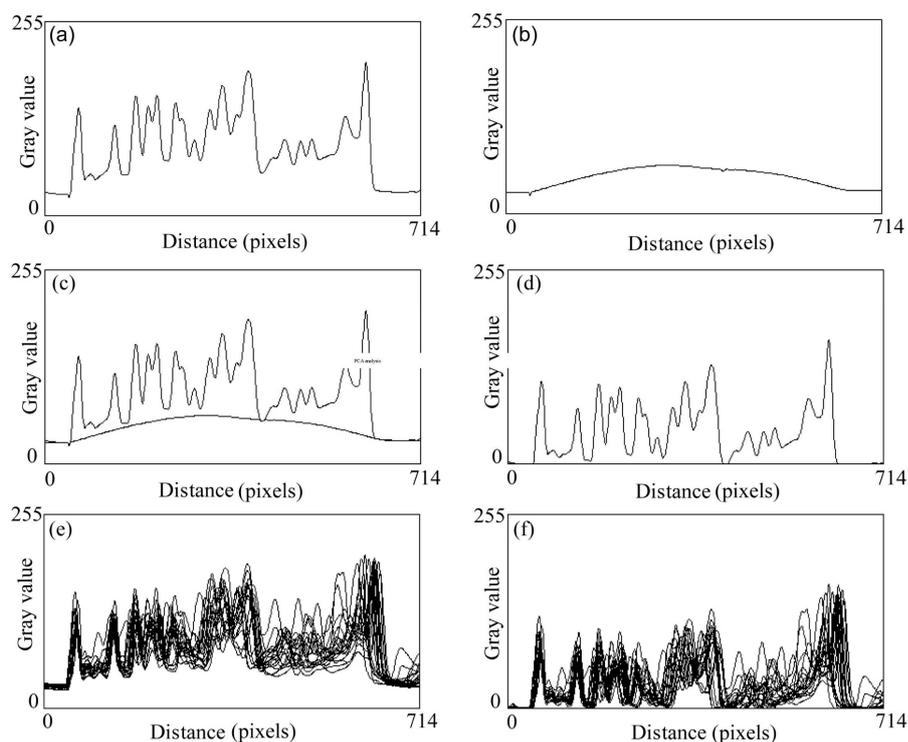


Figure 4. Subtract background: (a) *P. anserina* plot with background; (b) *P. anserina* background estimate plot; (c) *P. anserina* plot with background and background estimate; (d) *P. anserina* plot after subtract background; (e) Plot of all subtrack in stack; (f) Plot of all subtracks in stack after subtract background.

The first step of warping is the choice of target image. Following previously research [24], we selected the image with the highest mean correlation coefficient with remaining ones (in our research that was *P. hirta* chromatogram (subtrack 2) as the target image. The optimization of image registering parameters was the second step. We selected the registration mode “Mono” which makes the program perform only unidirectional registration, *i.e.* from source image to target image. As it was observed, the great impact on the final result of warping seems to have three parameters: initial deformation option, sample subsample factor and curl weight. The initial deformation lists permit selecting the coarsest and finest scale of the spline deformation field (where “very coarse” option corresponds to 4 splines (one in each corner of the image)). Sample subsample factor enables subsampling input images. Next registration was calculated using the subsampled versions of images. The results were applied to the original ones (e.g. image dimensions can be reduced by the factor between 0 ($2^0 = 1$) and 7 ($2^7 = 128$)). We also observed that the warping result was different for the same stack, target image and that same warping parameters, but with different position of stack (vertical or horizontal) or with or without invert color before warping. The warping effect was then incorrect (R_f value of the spots identified as the same substance was changed) or with image defects (*i.e.* large image narrowing, arched spots). Only the use of markers significantly improved the quality of the process. The points being exactly in the middle of each image corresponding to the start line, finish line, and part of the spots of previously identified compounds (ursolic acid, β -sitosol + stigmasterol) were selected as markers (landmarks).

To examine such characteristics, this observation prompted us to test TLC images to design our own simple algorithm for alignment. The algorithm was not based on B-spline but on segmental elastic compression or decompression of images between fixed markers. In addition to the landmarks previously used in warping, two points corresponding to the beginning and end of each subtrack were added. Because its simplicity the algorithm, didn't cause distortion of the image, and could be used practically without data smoothing, background subtract and horizontal equalization. This algorithm can be successfully used for similar TLC chromatograms, especially in cases where we have spots with confirmed identity preferably distributed in different parts of the chromatogram.

Warping procedures are illustrated in **Figure 5** and **Figure 6**. The comparison of two warping algorithms applied can be seen in the *P. desertorum* chromatogram example in **Figure 6(b)** (black plot—before warping, pink plot—after bUnwarpJ warping, blue plot—after our algorithm warping). Mean Pearson’s correlation coefficient of all chromatograms (subtracks) increased from 0.582 before warping to 0.844 after bUnwarpJ warping, and to 0.859 after our algorithm warping.

3.2. Exploratory Analysis of the Investigated *Potentilla* Species Fingerprints

After data pre-treatment of fingerprints (removal of noise, subtract background, horizontal equalization,

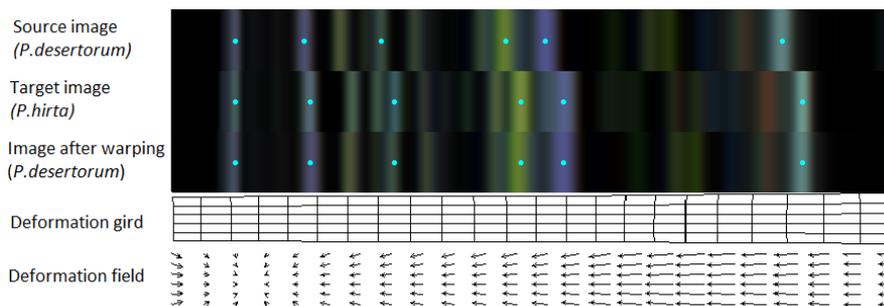


Figure 5. bUnwarpJ warping with landmarks (blue dots in the pictures).

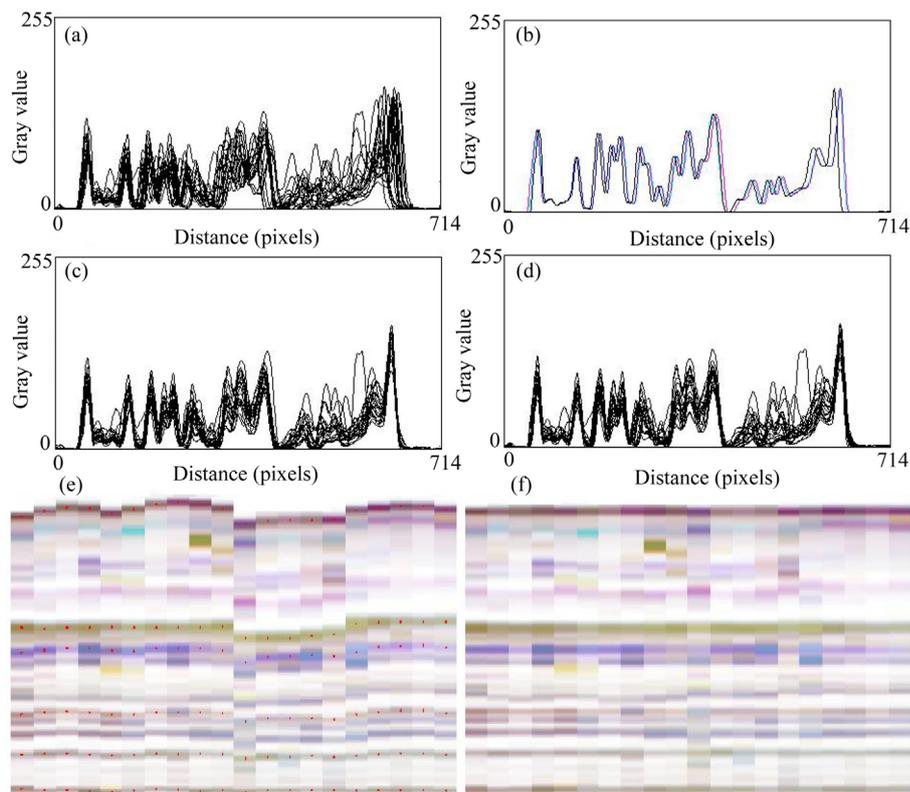


Figure 6. Warping with landmarks (red dots in the pictures). (a) Plot of all subtracks in stack after subtract background; (b) Plot of *P. desertorum* before and after warping (description in text); (c) Plot of all subtracks in stack after subtract background and bUnwarpJ warping; (d) Plot of all subtracks in stack after subtract background and simple, elastic algorithm warping; (e) Invert color subtracks images montage before warping; (f) Invert color subtracks images montage after simple, elastic algorithm warping.

warping), differences among the *Potentilla* samples were explored. All the data processing was used to remove the undesired part of the data variation and ensure that all variability of data is related to chemical variability among samples.

The main purpose of our study was to create a fully automatic image processing model that enables quick identification of selected species of the *Potentilla* genus. Fully processed images of standard samples of the *Potentilla* species were presented in **Figure 7(a)**. The botanical identity of standard samples was confirmed. Next, three samples which were collected by authors in different time and part of Europe (see **Figure 7(b)**) were also processed. The final observation of the comparison of each tested sample to the standard samples resulted in highest Pearson correlation coefficient value and the number of standard sample to which the test sample is adjusted (see **Table 2**).

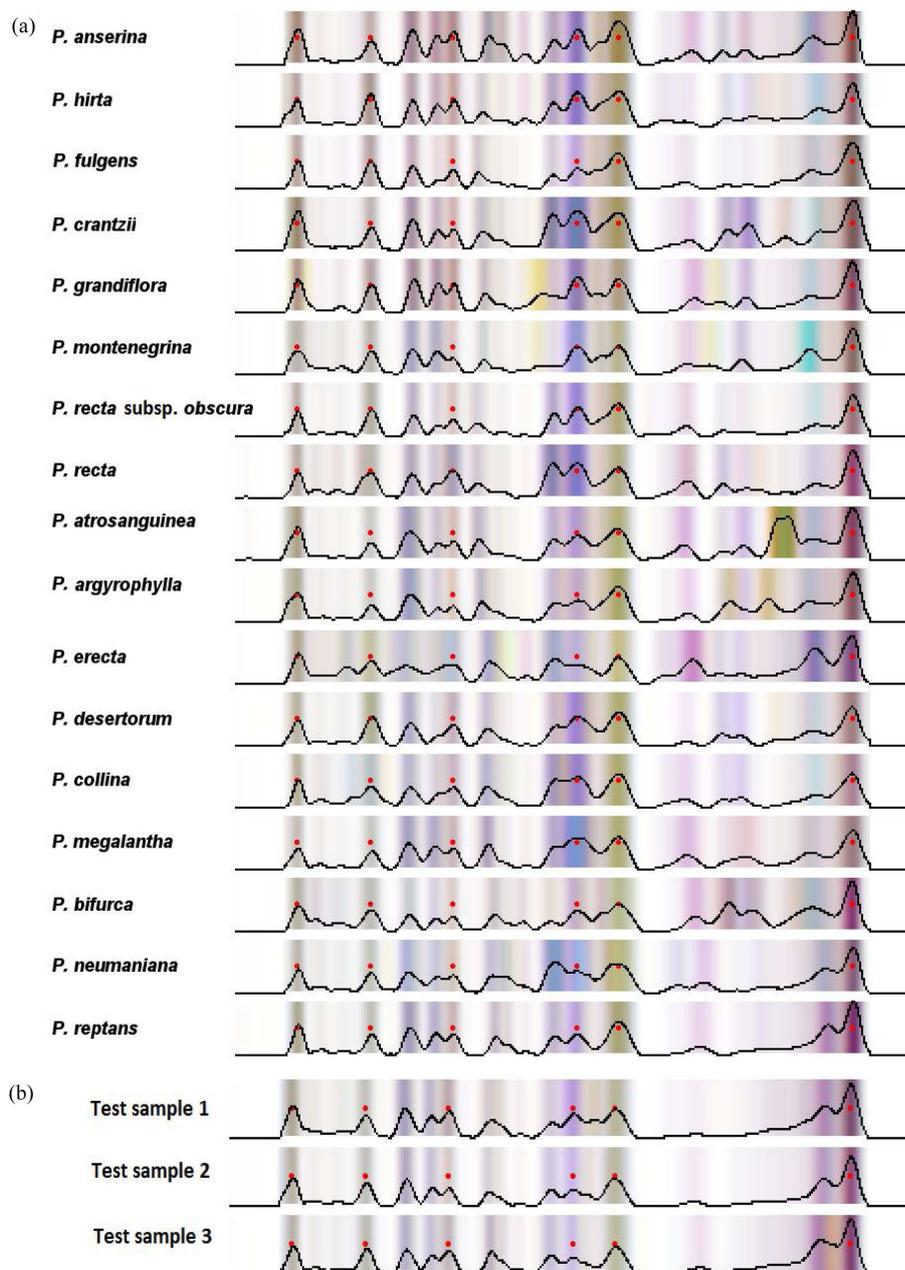


Figure 7. Fully processed invert color images of all *Potentilla* chromatograms; landmarks (red dots in the pictures). (a) Standard samples; (b) Test samples.

Table 2. The final result of the comparison of tested sample.

Test sample	First match	
	Number of best-fit standard sample	r
Białystok Region, 2008	17	0.943
France, 2011	17	0.971
Zamosc Region, 2010	17	0.933

As it is observed, all test samples are best correlated with the standard sample number 17 (corresponding to *P. reptans*). The most important issue is that test samples were classified as one species, with a correlation coefficient of no less than 0.90. The correlation coefficient changed in the range from 0.933 to 0.971 for the same species collecting in different places and time. The second best fit standard sample correlation was below 0.9. Through the use of macros, all image processing (including the removal of noise, subtract background, horizontal equalization, warping) and exploratory analysis (including indicating the number of best-fit standard sample and value of correlation coefficient) from beginning to the end lasted no longer than 30 seconds. To confirm the validity of results, additional botanical analysis of test samples was carried out, thus confirming the accuracy of the obtained results.

Previous observations were also confirmed by the principal component analysis (with auto scaling and mean centering), done on fully processed images. The PCA of processed images showed the grouping of samples of the same species (samples 17, 18, 19 and 20 corresponding to *P. reptans* collected at different times and places). The first three principal components explain 92% of total variance. PC2-PC3 loadings plot for all TLC chromatograms is presented in [Figure 8](#).

In the present study, the original and simple method for the separation of closely related five triterpenic acids was presented. The analyzed phytosterols (β -sitosterol, stigmasterol) did not interfere. Four triterpenic acids (ursolic/oleanolic acid, pomolic acid and two optical isomers—euscaphic and tormentic acid) were separated without prechromatographic derivatization (see [Figure 2\(a\)](#)). The described method of separating optical isomers is simple, rapid and inexpensive on a silica gel TLC plate, without using expensive impregnated plates, chiral mobile phase or chiral stationary phase. The method is based on different physical properties (unlike enantiomers) and different chemical reactivity of diastereomers. For the separation of oleanolic and ursolic acid, the previously described prechromatographic derivatization method we used [15]. All five triterpene acids can be separated only with prechromatographic derivatization with 1% iodine solution in chloroform (see [Figure 2\(b\)](#)). It is very important iodine derivatization didn't change retention factors of euscaphic, tormentic and pomolic acid or analyzed sterols.

4. Conclusions

The method without prechromatographic derivatization was used to create differential fingerprints. The methodology was validated and may be successfully used to assess the identity of test samples in other laboratories. It seems to be particularly important in this kind of research since fingerprint evaluation method may be used to create a digital database of validated methods with image processing. Such assessment allows identifying different plant species. In our study we proved that the analysis of extracts containing small group of chemical compounds with displaying similar chemical character was sufficient for error-free identification of test samples.

Good results were obtained by the application of r-project iterative algorithm for subtract background and our simple algorithm for warping to image processing of TLC chromatograms for the first time. Fully automated digital data pre-treatment and exploratory analysis of the investigated *Potentilla* species fingerprints has proved to be fast, simple and effective mechanism for differentiating the investigated plant species. The combination of image processing techniques and statistical evaluation of the collected data proved to be an additional source of interesting conclusions. For this purpose, Pearson's correlation coefficient and the PCA analysis were used.

Fully automated digital data pre-treatment and chemometric analysis has proved to be fast, simple and effective mechanism for differentiating the investigated plant species. This fingerprint evaluation method with image processing may be used to create a digital database for plant identification.

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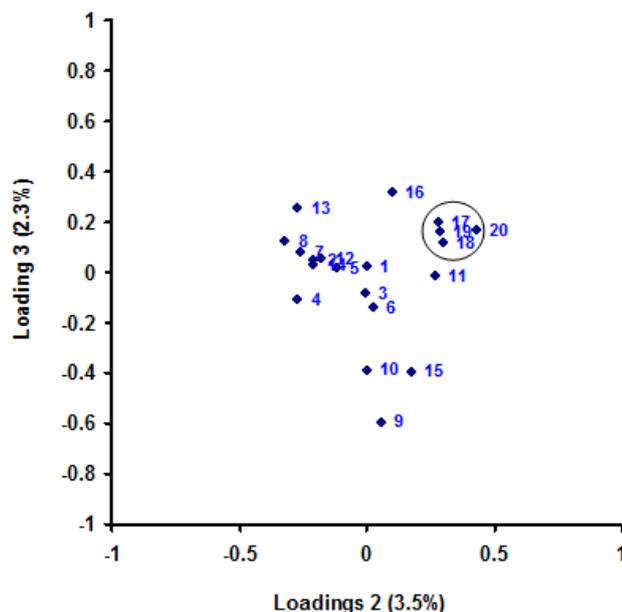


Figure 8. PCA analysis.

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