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HPTLC-based metabolomics for the investigation of metabolic changes during plant development: The case study of *Artemisia annua*

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Abstract: The application of high performance thin layer chromatography (HPTLC)-based non-targeted metabolomics as a holistic approach to compare fingerprints of metabolite changes during *Artemisia annua* development is described. Images of HPTLC chromatograms obtained after derivatization with anisaldehyde-sulphuric acid reagent were used as a dataset for multivariate analysis. Principal component analysis and orthogonal partial least squares discriminant analysis confirmed the differentiation of samples belonging to vegetative phase, flowering stage, and seed formation stage of the plant development. The obtained results showed that the HPTLC-based metabolomics approach can be a very reliable technique for the investigation of metabolic changes during plant development, complementary to gas chromatography–mass spectrometry and nuclear magnetic resonance-based metabolomics.

Keywords: plant metabolomics; PCA; OPLS-DA.

INTRODUCTION

In this study, the cultivated *Artemisia annua* was used as a model system for the assessment the application of high performance thin layer chromatography (HPTLC) based untargeted metabolomics to probe unique metabolites during the life cycle of the plant. Since the discovery of its constituent antimalarial artemisinin, *A. annua*, which is the only viable resource of this drug, has been the

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subjects of extensive research on its chemical composition. More than 600 secondary metabolites have been identified throughout the plant including several sesquiterpenoids, triterpenoids, monoterpenoids, steroids, flavonoids, coumarins, alkaloids and benzenoids.[1,2] The method of untargeted metabolomics considers simultaneous measurement of as many metabolites as possible from each sample, thus providing a holistic, general overview of a sample composition.[3] HPTLC is an emerging analytical tool in metabolomics research in the last decade.[4,5] Even conventional thin layer chromatography (TLC) offers the advantages over other analytical methods, such as short measuring time and capability of parallel analysis, and the availability of chemical reagents. Many improvements in HPTLC over conventional TLC such as high resolution and data robustness, also improved some metabolomics and profiling studies. Furthermore, metabolites which are difficult to be identified by nuclear magnetic resonance (NMR) or mass spectrometry (MS)-based methods could be visualized on HPTLC plates and isolated from the plate for further chemical elucidation.[6] The main idea of this study is the application of an HPTLC-based non-targeted metabolomics as a holistic approach to compare fingerprints of metabolites changing during the plant development.

EXPERIMENTAL

General methods

All used solvents were of analytical grade. Glass HPTLC silica gel 60 CN F254s plates 10 cm×20 cm were purchased from Merck (Darmstadt, Germany); anisaldehyde-sulphuric acid used as a spray reagent was freshly made according to Wagner *et al.*[7] Artemisinin standard was isolated previously in the laboratory of Bulgarian Academy of Sciences. All NMR spectra (^{1}H, ^{13}C, correlated spectroscopy (COSY), heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond coherence (HMBC)) were recorded in $CDCl_3$ (with tetramethylsilane (TMS) for referencing) on a Bruker Avance III 500 NMR spectrometer operating at a proton NMR frequency of 500.26 MHz, equipped with a 5 mm broadband probe (BBI) probehead. Gas chromatography (GC)/MS analyses were performed according to Stankovic *et al*. (2019)[8] with some modifications of the temperature program and split ratio. The oven temperature was programmed from 60 to 240 °C at 3 °C/min, then from 240 to 310 °C at 35 °C/min, and then held isothermally for 8 min.

Injection volume was 1 μL, split ratio, 20:1. For lipid fraction the oven temperature was kept constant at 40 °C for 1 min, then was linearly programmed from 40 to 315 °C at 10 °C/min and then held isothermally for 6.5 min. Injection volume was 1 μL, split ratio 50:1.

Plant material

Artemisia annua L. is grown at Institute of Field and Vegetable Crops, Novi Sad. The plants samples were collected 14 times at intervals of 7 or 14 days, successively in different phenophases – from May 27 to November 3, 2018. Thus, vegetative phase (collections 1–6), flowering stage (collections 7–11), and seed formation stage (collections 12–14) were collected. The Voucher specimens (determined by Milica Rat, M.Sc., as No. 2-1514) were confirmed and deposited at the Herbarium of the Department of Biology and Ecology (BUNS Herbarium), Faculty of Natural Sciences, University of Novi Sad.

Extraction of plant material

Dry and ground plant materials of each collection (150 mg) were extracted with a mixture of hexane:ethyl acetate, 95:5 volume ratio (1 mL) for 15 min in an ultrasound water bath. The extracts were centrifuged for 15 min at 13,600 rpm. The extraction procedure was independently repeated with four biological replicates of each collection. The obtained extracts were used for HPTLC analysis.

HPTLC analysis

The 3 μL of the extracts were sprayed with a 25-μL syringe as an 7-mm band on the HPTLC plate using the Linomat 5 (CAMAG, Switzerland). The application position *Y* was 8.0 mm, the first application position *X* was 10.0 mm, and the distance between tracks was 10.0 mm. The plates were developed in a previously saturated CAMAG automatic developing chamber 2 with a mobile phase consisting of hexane:ethyl acetate:formic acid (19:11:0.5 volume ratio). After a drying time of 4 min developed HPTLC plates were derivatized by dipping in the anisaldehyde reagent (CAMAG chromatogram immersion device 3), followed by heating at 100 °C for 5 min.

Fractionation by dry-column flash chromatography

Dry and ground average samples of all collections (167.0 g) were extracted with hexane:ethyl acetate, 95:5 volume ratio(2×600 mL) for 15 min in an ultrasound water bath. The extracts were combined, filtered, and concentrated under reduced pressure to give 1.1 g of residue. Dry-column flash chromatography fractionation was performed in a glass column (300 mm×22 mm) packed with silica gel (100 g Merck, <0.08 mm) using a water vacuum pump. The column was equilibrated with the mobile phase *n*-hexane:ethyl acetate (95:5 volume ratio) mixture. The extract (m = 1.1 g) was diluted with the mobile phase (1:1) before applying to the column. The elution was performed with hexane:ethyl acetate mixture with increasing polarity (Table S-I, Supplementary material to this paper) and 34 fractions were collected.

Methylation of non-polar lipid fraction with MeOH/H_2SO_4

A mixture of the lipid fraction (30 mg) and MeOH (6 mL+ two drops of conc. H_2SO_4) was refluxed in a test tube fitted with condenser for 2 h. After neutralization with saturated sodium bicarbonate solution, the methyl esters were extracted with *n*-hexane. The organic layer was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure in a rotavapor.

Image and multivariate data analysis

The HPTLC chromatograms were processed with the Image J processing program (version 1.47q). The chromatograms were denoised using the median filter function with two pixels width filter, and the tracks were outlined with rectangular selection tool. The line profile plots (gray scale) were generated using Plot Profile option for each sample. On this way, two-dimensional graphs containing the pixel intensities toward distance along the line were obtained. The HPTLC image data were then normalized to the total area, mean centred and pareto scaled. Principal component analysis (PCA) and orthogonal partial least squares to latent structures – discriminant analysis (OPLS-DA) method was performed with SIMCA software (version 15, Sartorius, Göttingen, Germany).

RESULTS AND DISCUSSION

The HPTLC conditions regarding amount of sample applied and mobile phase composition were optimised to detect the maximum number of resolved

bands per sample. Four biological replicates for each of the 14 collections of *A. annua* were then fingerprinted using the HPTLC method. Thus, vegetative phase (collections 1-6), flowering stage (collections 7–11), and seed formation stage (collections 12–14), were covered. The fingerprint patterns visualized after derivatization with anisaldehyde-sulphuric acid reagent are depicted in Fig. 1.

Fig. 1. HPTLC metabolic fingerprint of 14 samples (red) *A. annua* collected in different developmental stages.

For the identification of the components found in the studied extracts, dry-column flash chromatography of the plant extract was used, followed by NMR and/or GC/MS analyses. As a result of the purification, the isolated fractions appeared as a single band on HPTLC plate. Each band of the purified fraction was then connected to those of *A. annua* extracts by co-chromatography comprising their R_F values and colours after derivatization (Table I). The identity of the purified metabolites was then performed by NMR and/or GC/MS analyses (Supplementary material). Only artemisinin was not isolated as a pure compound, and its HPTLC band was compared to that of the standard compound.

TABLE I. Identified metabolites in *A. annua* extracts

No.	R_F value	Identified metabolites	Colour after derivatization	Identification
1	0.94	Triacylglycerol	Dark green	NMR; GC/MS after transesterification
2	0.86	α-Pinene, α-copaene, caryophyllene E and β-selinene	Purple	GC/MS
3	0.78	Artemisia ketone	Orange	NMR; GC/MS
4	0.68	1,8-Cineole	Purple	GC/MS
5	0.66	Caryophyllene oxide	Purple	GC/MS
6	0.58	Artemisia alcohol	Dark green	GC/MS
7	0.46	Artemisinin	Pink	Comparison to standard
8	0.20	Arteannuin B	Pink	GC/MS

The line profile plots generated from the images obtained using visible light were used as a dataset for multivariate analysis. Firstly, the principal component analysis (PCA), as a variable reduction technique to develop a smaller number of novel variables that will account for most of the variation in the observed variables, was performed. It has resulted in five principal components (PCs) model explaining 84.3 % of the total data of variances.

Based on PCA score plot (Fig. 2A), three groups of samples were separated to some extent, according to the plant development stages.

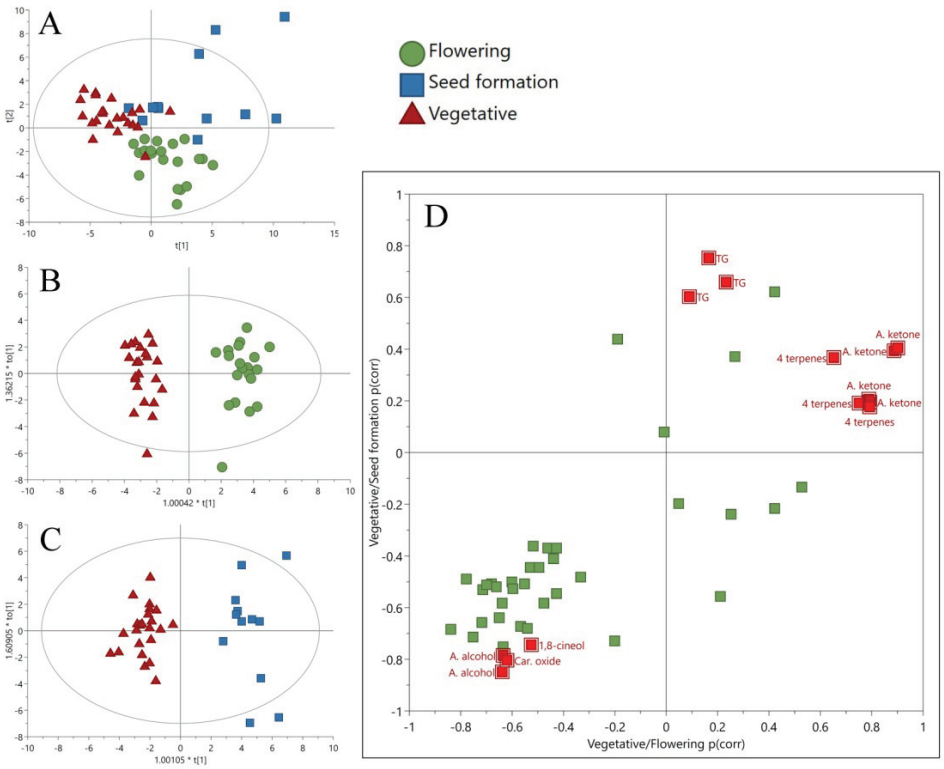

Fig. 2. A) PCA score plot; B) OPLS-DA score plot containing vegetative and flowering phase of *A. annua*; C) OPLS-DA score plot containing vegetative and seed formation phase of *A. annua*; D) OPLS-DA SUS-plot, all obtained from HPTLC fingerprints; four (4) terpenes: α-pinene, α-copaene, caryophyllene E and β-selinene.

Next, the orthogonal partial least squares to latent structures–discriminant analysis (OPLS-DA) was applied, where novel variables will account for the maximum separation between predefined classes. An additional advantage of the orthogonal model is the facilitated interpretation due to separation of the systematic variation of the variables into two parts: one linearly related to class information and one orthogonal to the class information.[9] Two OPLS-DA models were

created containing HPTLC data of: a) vegetative phase versus flowering stage and b) vegetative phase versus seed formation stage of *A. annua* development. The quality of the obtained models was assessed by goodness of fit (R^2) indicating how well the variation of variables is explained using the predictive components and predictive ability of the model (Q^2), indicating how well the model predicts new data, estimated by cross validation. In both created OPLS-DA models, large R^2 and Q^2 values over 0.5 and close to 1 indicated high goodness of fit and good predictivity (Table II).

TABLE II. Parameters of the OPLS-DA models

Model	Number of components (predictive + orthogonal)	R^2	Q^2	p (CV-NOVA)	F (CV-ANOVA)
Vegetative/ Flowering	1 + 4	0.951	0.897	4.88×10^{-13}	28
Vegetative/ Seed formation	1 + 3	0.918	0.784	6.45×10^{-7}	12

The models were validated by permutation tests and CV-ANOVA, the significance of the models was clearly shown with p values far less than 0.05 (Table II). In the score plots (Fig. 2B and C) a clear separation between the samples was obtained along to the predictive components. The most influential variables were selected based on the variable influence on projection (*VIP*) scores of the predictive components, and the loadings scaled as a correlation coefficient (p(corr)). $VIP > 1$ and $|p(\text{corr})| > 0.5$ were considered as important for the separation. Shared and unique structure plot (SUS-plot) containing p(corr) from both OPLS-DA models was used to reveal the changes in the metabolomes in each of the three predefined group of samples (Fig. 2D). The unique metabolites were found close to either the X or Y axis, and the shared were located on the diagonals. Also, the metabolites which are on the extreme ends of the axes contribute more significantly than those close to the centre. Thus, the accumulation of triacylglycerol (**1**) is characteristic for the seed formation phase since it is an unique metabolite in Vegetative/Seed formation model. Similarly, four terpenes (α-pinene (**2**), α-copaene (**2**), caryophyllene E (**2**) and β-selinene (**2**)) and artemisia ketone (**3**) were accumulated in the flowering stage since they are unique metabolites in the vegetative/flowering model. Artemisia alcohol (**6**), caryophyllene oxide (**5**), and 1,8-cineole (**4**) were the main metabolites in the vegetative stage, since they are shared in both models. For artemisinin (**7**) and arteannuin B (**8**) there was no significant change in their amount during plant development.

Metabolomics fingerprinting of *A. annua* in different stages of development has also been performed previously by GC/MS.[10] Our objective was to highlight the advantages of HPTLC: no need for derivatization prior to chromatography,

and thus detection of thermolabile and non-volatile metabolites, which is not possible using GC/MS. HPTLC is also complementary technique to NMR-based metabolomics, offering solutions where crowded regions in the NMR spectra are difficult to resolve.[4]

CONCLUSION

This work presents an efficient way for analysing metabolic fingerprinting data of *A. annua* L. generated by HPTLC. As a result, we demonstrated that HPTLC may be applied as a simple and reliable untargeted approach to rapidly differentiate the extracts originating from various developmental stages of the plant. HPTLC also demonstrated to be the method of choice for the routine study of complex matrices and could be used as an alternative and/or complementary method to techniques, such as GC/MS or NMR.

SUPPLEMENTARY MATERIAL

Additional data and information are available electronically at the pages of journal website: https://www.shd-pub.org.rs/index.php/JSCS/article/view/10744, or from the corresponding author on request.

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ИЗВОД

МЕТАБОЛОМИКА БАЗИРАНА НА HPTLC ЗА ИСПИТИВАЊЕ МЕТАБОЛИЧКИХ ПРОМЕНА ТОКОМ РАЗВОЈА БИЉКЕ: СТУДИЈА СЛУЧАЈА *Artemisia annua*

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Описана је холистичка примена нетаргетне метаболомике базиране на високоефикасној танкослојној хроматографији (HPTLC) која омогућава поређење метаболичких профила *Artemisia annua* и праћење њихових промена током развоја биљке. HPTLC хроматограми након развијања анизалдехидом и сумпорном киселином су коришћени за генерисање података за мултиваријантну анализу. Анализа главних компоненти и ортогонална дискриминанта анализа најмањих квадрата су потврдиле разлике између узорака који припадају различитим фенофазама – вегетативној фази, фази цветања и фази формирања семена. Добијени резултати указују да метаболомички приступ заснован на HPTLC методи која је комплементарна са GC/MS и NMR анализом, може бити веома поуздана техника за анализу промене током развоја биљке.

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