Journal of the Serbian Chemical Society
JSCS-info@shd.org.rs • www.shd.org.rs/JSCS
J. Serb. Chem. Soc. *82* (1) 63–72 (2017)
JSCS–4947
UDC 633.15:635.074:547.562+543.544.5.068.7+621.383.5
Original scientific paper

Determination of free phenolic acids from leaves within different colored maize

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(Received 12 May, revised 22 November, accepted 29 November 2016)

Abstract: Along with other plant parts, maize leaves are widely used for making fermented food for cattle, known as silage. Since there have only been a few reports on studies concerning the extraction and determination of phenolic acids from maize leaves, the main goal of this investigation was to evaluate the content of free phenolic acids in the leaves of fifteen different maize inbred lines. Reverse-phase, high performance liquid chromatography (RP--HPLC), with a photodiode array detector (DAD), was performed. Under the optimized chromatographic conditions, referring to short time of sample preparation, small quantities of solvent and direct injection of the extract into HPLC, phenolic acids (*i.e.*, gallic, protocatechuic, caffeic, *p*-coumaric and ferulic acid) were successfully separated in less than 25 min, indicating that the method could be applied for routine analysis. The efficiency and validation of the method was evaluated by measuring the rate parameters: linearity, limit of detection and quantification, accuracy and precision. The obtained results showed that the most abundant free phenolic acid was *p*-coumaric acid (23.57 μg g^{-1} dry weight), followed by ferulic and caffeic acids (21.27 and 20.78 μg g^{-1} dry weight, respectively). Principal Component Analysis (PCA) revealed the existence of a link.

Keywords: HPLC-DAD; method validation; phenolic acids; corn.

INTRODUCTION

Maize (*Zea mays* L.) is one of the oldest cultivated grain cereals and one of the most productive species worldwide, with an average yield of about 5.5 t ha^{-1}.[1] Maize has food, feed, and industrial uses. Namely, a high proportion of the produced maize is used in livestock feed as green chop, dry forage, silage or grain.[2,3] In addition, differently processed and unprocessed maize grain is used in human diet.[4] The rest of the plant is mostly used for the preparation of fermented

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doi: 10.2298/JSC160512104M

high-moisture preserved food used in livestock feed (silage) or is discarded wherein a potentially valuable source of phytochemicals is lost.

Besides providing functional dietary micronutrients and fibers, plants are rich sources of phytochemicals, such as phenolic compounds. These are aromatic secondary plant metabolites that include a large number of naturally occurring compounds divided into several groups. Due to their antioxidant and other biological properties that can promote human health, phenolic compounds in food have been constantly investigated over the past few years.[5] In many studies the inhibitory effect of phenolics to oxidative damage, which could lead to atherosclerosis and cancer, were reported.[6–8] The antioxidant activity of phenolic compounds can be explained by several possible mechanisms, such as their ability to chelate metals, inhibit lipoxygenase, modulate peroxide concentration, scavenge free radicals and stimulate the enzyme systems of antioxidative defense.[9] Due to harmful properties of synthetic antioxidants, such as butylated hydroxyanisole (BHA) and hydroxytoluene (BHT),[10] demands of the food industry for antioxidants of natural origin, especially from industrial residues, have never been greater.[11–13]

Phenolic acids are one of the main classes of phenolics and, according to their chemical structure, represent derivatives of benzoic and cinnamic acids. Generally, they could be described as phenols with carboxylic acid and hydroxyl groups, the positions of which affects their antioxidative properties.[14] Phenolic acids in different concentrations are distributed in seeds, leaves, roots and stems.[15] Their functions in the plant are linked with photosynthesis, synthesis of proteins, enzyme activity and allelopathy.[16] Various factors affect the quantity and quality of the phenolic acids present in plant foods, including plant genetics, soil composition and growing conditions, maturity state and post-harvest conditions.[17,18] Many studies have reported the extraction of phenolic acids from the leaves of different plant species,[19–21] but only a few from maize leaves.[22]

According to all the findings mentioned above, the aim of this study was to evaluate the content of free phenolic acids extracted from maize leaves. For this purpose, a new reverse phase HPLC method with diode array detection was developed for identification and quantification of five phenolic acids. The method was performed on fifteen maize inbred lines. In addition, principal component analysis (PCA) was performed to determine the relationship between compositions of the free phenolic acids in the leaves and the color of the maize kernel.

EXPERIMENTAL

Plant material and chemicals

In this study, fifteen maize inbred lines (from IL1 to IL15) from the gene bank of the Maize Research Institute “Zemun Polje”, Belgrade, Serbia, were used for the analysis. For each genotype (nine white, four orange and two red maize inbred lines), plant samples (*i.e.*, leaf of the uppermost ear) were taken at flowering. Leaves were dried to constant weight at 60

°C, milled (Perten 120, Sweden) into powder (particle size <500 μm) in order to obtain greater surface contact,[23] and stored at –20 °C prior to analysis.

Methanol and formic acid, purchased from J.T. Baker (Netherlands), were of HPLC grade. Ethanol and standards of tested phenolic acids (*i.e.*, gallic, protocatechuic, caffeic, *p*-coumaric and ferulic) were HPLC grade and purchased from Sigma–Aldrich. For the analysis, ultrapure water (Thermo Fisher TKA Micro Pure water purification system, 0.055 μS cm^{-1}) was used. Syringe filters (17 mm, PTFE membrane 0.45 μm) were purchased from Thermo Scientific (Germany).

Samples preparation and extraction of free phenolic acids

The method used for phenolic acids extraction was a slightly modified method proposed by Sultana *et al*.[24] Approximately 0.3 g of the leaves powder was extracted (IKA HS 501, Germany) twice with 3 mL of 80 % methanol, for 30 min at 300 rpm, at room temperature. The collected extracts were centrifuged at 3000 rpm for 5 min, filtered through a 0.45-μm PTFE membrane filter and directly injected into the HPLC.

Calibration curve and linearity

Working solutions were made by diluting the initial mixture containing all analyzed phenolic acids (100 μg mL^{-1}) to the final concentration: 0.1, 0.3, 0.5, 1, 3, 5, 10 and 20 μg mL^{-1}. Calibration curves were obtained in MS Excel, by plotting the peak areas (detector response) *versus* the concentration of the standard solutions. Obtained correlation coefficients were used for determination of the linearity of the method.

Limit of detection and quantification

Limit of detection (*LOD*) and limit of quantification (*LOQ*) were calculated from the following equations:

$$LOD = 3\frac{SD}{b} \tag{1}$$

$$LOQ = 10\frac{SD}{b} \tag{2}$$

where *SD* is the standard deviation of the response (standard error value for coefficient *b*) and *b* is the slope of the calibration curve obtained from the linear regression.

Precision and accuracy

The repeatability of the method was determined by triplicate measurement of the relative standard deviation (*RSD*) of both the peak area for each phenolic compound (at a concentration of 1.0 μg mL^{-1}), and two randomly chosen samples, on an intra- and inter-daily basis. For the calculation, the following equation was used:

$$RSD\,/\,\% = 100\frac{SD}{X} \tag{3}$$

where *SD* is standard error value for the peak area and *X* is the average value of the peak area.

The accuracy of the method was express as percentage of recovery. Two samples were spiked with the working solution of the phenolic acids mixture (at a concentration of 1 μg mL^{-1}), in three replicates. Recovery (*R* / %) was determined from the equation:

$$R\,/\,\% = 100\frac{F}{F_0 + A} \tag{4}$$

where F represents the concentration of phenolic acid in the spiked sample, F_0 is the concentration of phenolic acid in the unspiked sample and A is the added amount of phenolic acid (*i.e.*, 1.0 μg mL^{-1}).

HPLC method

Chromatographic separation of five phenolic acids was performed using a Dionex UltiMate 3000 liquid chromatography system (Thermo Scientific, Germany), consisting of a quaternary pump (LPG-3400), autosampler (WPS-300SL), column compartment (TCC--300SD) and a photodiode array detector (DAD–3000). The analytical column used was Acclaim Polar Advantage II, C18 (150 mm× 4.6 mm, 3 μm) from Thermo Fisher Scientific, operated at 25 °C. The mobile phase (flow rate 0.8 mL min^{-1}) contained 0.1 vol. % aqueous formic acid solution (A) and pure methanol (B). The linear gradient program was as follows: 0.0–10.0 min, 15–45 % B; 10.0–20.0 min, 45–65 % B; 20.0–25.0 min, 65–15 % B. The injection volume was 5 μL. The UV detection wavelengths were set at 278, 280, 290 and 300 nm. Phenolic acids were identified according to characteristic retention time and absorption spectra, whilst calibration curves of the corresponding standards were used for quantitative calculations. Chromeleon software package (version 7.2) was used for instrument control, as well as for data acquisition and analysis. The contents of the phenolic acids are expressed as μg per g of dry weight (DW). Data are reported as the mean value of three independent injections.

Statistical analysis

All analyses were performed on triplicate measurements (n = 3) and the results are presented as mean values. The data were subjected to one-way analysis of variance (ANOVA). The F-test was used for comparison of the means at the 0.05 probability level.

Principal component analysis (PCA) was performed using the PLS Toolbox software package v.6.2.1, for MATLAB 7.12.0 (R2011a). To prevent the predominance of components existing in higher concentrations, compared to those present in lower concentrations, data were mean-centered and auto-scaled to unit variance before statistical processing. The singular value decomposition (SVD) algorithm was used at the 0.95 confidence level for Hotelling T2 limits.

RESULTS AND DISCUSSION

Extraction of free phenolic acids

Determination of extraction efficiency was performed using pure methanol, pure ethanol and mixtures of methanol–water and ethanol–water (both in volume ratio of 8:2, data not shown). The mixture of methanol–water exhibited the highest extraction efficiency, which is in agreement with the results of Shabir *et al.*,[25] and was used for further analysis.

Validation of the method

The applied chromatographic method encompassed separation of all tested phenolic acids with good resolution, with a total separation time of 25 min. Chromatograms of phenolic acids standards are shown in Figs. 1a and b, recorded at 278 and 300 nm, respectively. The chromatograms obtained from maize leaves, also recorded at 278 and 300 nm, are presented in Fig. 2a and b, respectively.

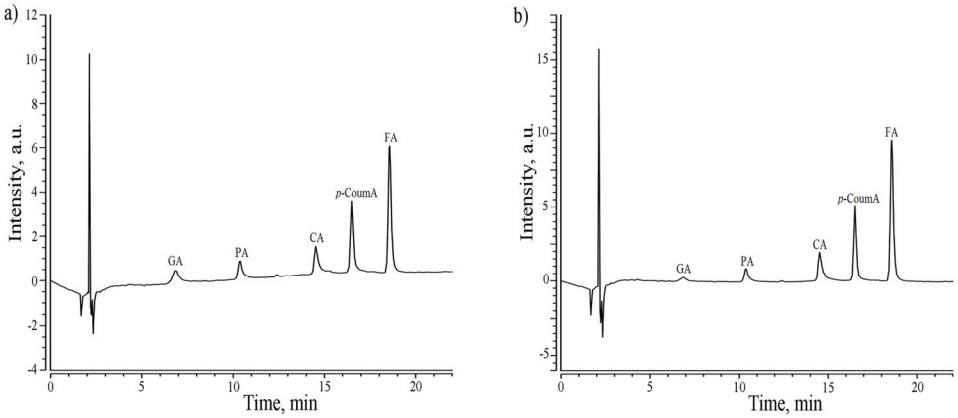

Fig. 1. Chromatograms of the phenolic acid standards, recorded at 278 nm (a) and 300 nm (b).

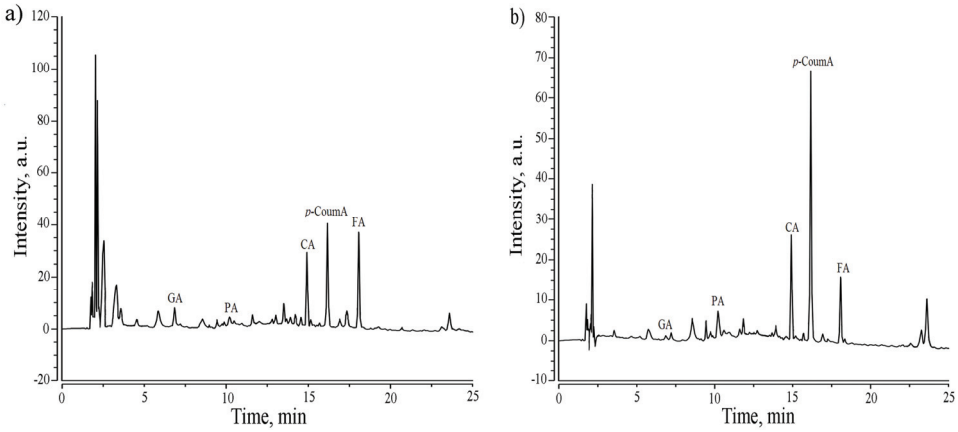

Fig. 2. Chromatograms for phenolic acids from maize leaves, recorded at 278 nm (a) and 300 nm (b).

According to obtained maximum absorbance, the wavelength for protocatechuic acid (PA) and *p*-coumaric acid (*p*-CoumA) was set at 300 nm, for ferulic acid (FA) and caffeic acid (CA) at 290 nm and for gallic acid (GA) at 278 nm. These values of wavelengths are in line with those used in the study of Nour *et al*.,[26] but different to those used by Lee *et al*.[27] and Kovacova *et al*.,[28] when all phenolic acids were monitored at 280 nm.

Although recorded at 278 nm (Fig. 2a), the peak of gallic acid had diminished absorbance at 300 nm, as is shown in Fig. 2b. This was also confirmed in Fig. 1a and b, obtained from phenolic standards chromatograms. Retention time for GA, PA, CA, *p*-CoumA and FA were 6.78, 10.35, 14.51, 16.46 and 18.19 min, respectively.

The parameters of linear regression (*i.e.*, slope, intercept, coefficient of determination (r^2), *LOD* and *LOQ*), were obtained for the phenolic acids standards, based on their peak area, as presented in Table I. The obtained linear correlation coefficients for all standards were not lower than 0.997, indicating good linearity of the method. The obtained *LOD* values ranged from 16 to 52 ng mL^{-1} and those of *LOQ* from 54 to 173 ng mL^{-1}. The obtained values were lower to those reported in method proposed by Nour *et al.*,[26] indicating higher sensitivity of the method used in this study.

TABLE I. Parameters of linear regression, *LOD* and *LOQ* for the phenolic acid standards

Phenolic acid	Intercept	Slope	r^2	*LOD* / ng mL^{-1}	*LOQ* / ng mL^{-1}
GA	–0.1180	0.3344	0.999	27	90
PA	–0.0495	0.3426	0.999	32	107
CA	–0.1424	0.7078	0.997	52	173
p-CoumA	–0.0133	1.0225	0.999	29	97
FA	–0.1181	2.0032	1.000	16	54

The repeatability of the method was investigated using intra-day and inter-day data obtained from the standards and two samples (Table II). For standards, the *RSD* of the intra-day (n = 5) and inter-day (n = 3) analysis were 0.17–1.19 and 0.81–2.42 %, respectively. For sample I, the *RSD* of the intra-day (n = 5) analysis was 0.21–1.41 %, being 1.13–3.05 % for the inter-day (n = 3) analysis. For sample II, the *RSD* of the intra-day (n = 5) analysis was 0.32–1.18 %, being 1.69–2.93 % for the inter-day (n = 3) analysis.

TABLE II. The obtained *RSD* (%) for the phenolic acids and the tested samples

Phenolic acid	Standard		Sample I		Sample II	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
GA	0.2	0.8	0.2	1.1	0.3	1.7
PA	1.2	2.4	0.7	1.9	0.6	2.1
CA	0.4	1.7	0.9	3.0	1.2	1.9
p-CoumA	0.9	2.0	1.0	2.5	0.8	2.9
FA	0.7	1.5	1.4	2.4	1.0	1.8

The standard additional method was used for recovery (R) determination, which represents the accuracy of the method. R for GA, PA, CA, *p*-CoumA and FA were 100.5, 97.8, 102.4, 99.7 and 98.9 %, respectively, indicating the good accuracy of the preformed method.

Free phenolic acids contents in relation to the maize kernel color

The content of free phenolic acids in the leaves for each of the fifteen evaluated maize inbreds, as a well as total average values per parameter, are pre-

sented in Table III. The obtained results indicate that the most abundant evaluated free phenolic acid was *p*-coumaric acid, followed by ferulic and caffeic acids.

TABLE III. Obtained content of phenolic acids in the tested maize leaves

Sample	Maize kernel color	GA μg g $^{-1}$	PA μg g $^{-1}$	CA μg g $^{-1}$	*p*-CoumA μg g $^{-1}$	FA μg g $^{-1}$
IL1	White	10.3014	7.1970	34.8993	21.8526	20.4395
IL2	White	10.2993	5.5556	24.7301	21.0614	18.1406
IL3	White	9.1026	7.0338	15.1407	18.0995	21.7998
IL4	White	9.5499	7.0916	22.5808	16.2327	23.0608
IL5	White	10.3803	11.4546	17.4689	23.5272	24.9282
IL6	White	10.4429	9.8031	25.9565	21.3246	17.0967
IL7	White	12.298	11.228	31.8369	17.2297	9.9062
IL8	White	10.8847	6.3156	22.7485	19.0469	13.1095
IL9	White	8.6345	8.5403	19.5884	15.6033	9.7336
Average[a]		10.1412	8.2466	23.8833	19.3309	17.5794
IL10	Orange	15.3776	22.0641	4.7345	18.2032	12.7739
IL11	Orange	14.1058	18.2494	27.705	28.665	28.9746
IL12	Orange	13.6257	19.7144	19.1517	35.3965	28.5013
IL13	Orange	13.2097	15.0719	17.8619	15.977	16.611
Average[a]		14.0797	18.775	17.3633	24.5604	21.7152
IL14	Red	9.0262	7.7717	20.9281	43.1607	29.5398
IL15	Red	9.1026	12.9652	6.4191	38.1813	44.4828
Average[a]		9.0644	10.3685	13.6736	40.671	37.0113
Total average		11.0479	11.3371	20.7834	23.5708	21.2732

[a]Average phenolic acids content of leaves

Total average values for GA, CA and FA contents obtained from all tested fifteen genotypes (*i.e.* 11.05, 20.78 and 21.27 μg g^{-1} DW, respectively), were shown to be higher than those in study on cob leaves (10.99, 6.0 and 1.87 μg g^{-1} DW, respectively), reported by Pandey *et al.*[22] Average amounts of *p*-CoumA, FA and CA (*i.e.*, 23.57, 21.27 and 20.78 μg g^{-1} DW, respectively), obtained from all analyzed genotypes, was higher than those in commercial red wine.[29] Mean values for PA and GA contents (*i.e.*, 11.34 and 11.05 μg g^{-1} DW, respectively) obtained from leaves of all tested maize, was lower compared to leaf extracts of Gold Mohar (*Delonix regia* (Bojer ex Hook.) Raf.), having antimicrobial and antifungal properties and being widely used in folk medicine.[25] On the contrary, the average contents of the other phenolic acids (i.e., p-CoumA, GA and FA) were higher.[25]

Different kernel color in maize generally originates from the carotenoids and anthocyanins concentration, with positive correlations found between the antioxidant activity and the color of maize.[30–32] Similar studies showed that the color of the samples is also related to the content of phenolic acids.[33,34] In this context, PCA was performed in order to examine the possible relationship between the

content of free phenolic acids in the leaves and the color of the maize kernel. PC analysis resulted in a four-component model that explains 98.93 % of the total variance. The first two principal components explain 42.80 (for PC1) and 36.23 % (for PC2) of the overall data variance. Mutual projections of the factor scores and their loadings for these PCs are shown in Fig. 3a and b, respectively.

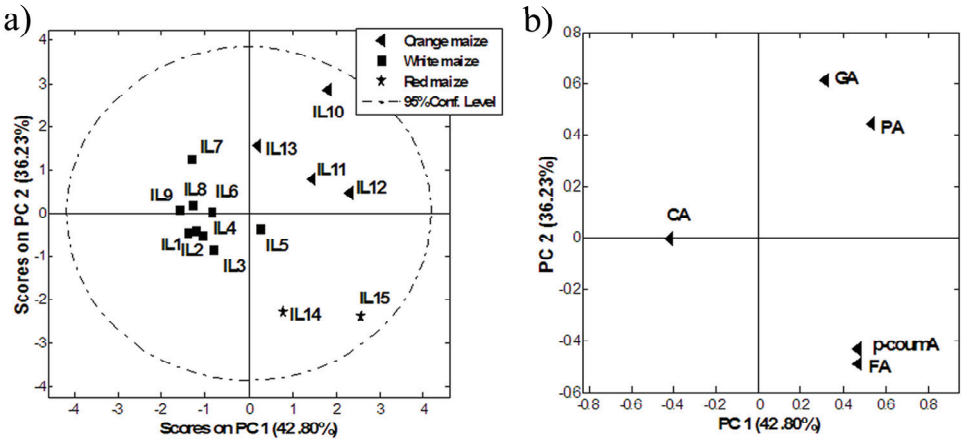

Fig. 3. PCA score (a) and loading plots (b).

Considering PC1 and PC2 score values (Fig. 3a), three well-separated groups of samples (according to kernel color) were formed. This indicates that the leaves from different colored maize possibly have unique contents of phenolic acids. White kernel maize (IL1–IL9) formed a group in the plot center, while the group of orange kernel maize (IL10–IL13) was allocated in the upper right part of the plot. Red kernel maize (IL14 and IL15) were separated in the lower right part of plot. The loading plot (Fig. 3b) revealed that the most efficient parameters for distinguishing white kernel maize was CA, for orange maize GA and PA, while for red kernel maize FA and *p*-CoumA. These results are in agreement with the mean values for phenolic acids obtained in the leaves from different colored maize (Table III). Leaves from white kernel maize had the highest average content of CA (*i.e.*, 23.88 μg g^{-1} DW) compared to the leaves from orange and red maize (*i.e.*, 17.36 and 13.67 μg g^{-1} DW, respectively). Similarly, the leaves from the orange kernel maize showed the largest average content of GA and PA (*i.e.*, 14.08 and 18.77 μg g^{-1} DW, respectively), compared to the leaves of white and red maize. Moreover, leaves of the red kernel maize had the highest average concentration of *p*-CoumA and FA (40.67 and 37.01 μg g^{-1} DW, respectively), compared to the leaves from other colored maize. Among the phenolic acids evaluated, the highest value for FA was found in red wheat, as was reported by Ma *et al.*[34]

CONCLUSIONS

A new RP-HPLC method with DAD was developed for the quantification of five phenolic acids in maize leaves, due to the lack of information on this subject. The observed validation parameters confirmed that the performed method is of good accuracy and precision, with relatively low values for the *LOD* and *LOQ*. This indicates that the method developed in this study could be usefully applied in further, more detailed analyses on phenolic acids content in maize leaves.

The performed PCA distinguished a relationship between the concentrations of phenolic acids in the leaves and the color of the maize kernel. In leaves, the most abundant phenolic acid was CA for white maize, and GA and PA for orange maize. Moreover, the leaves from red maize showed the highest concentrations of FA and *p*-CoumA.

Acknowledgement. This research was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (Projects TR31068 and TR31028).

И З В О Д

ОДРЕЂИВАЊЕ СЛОБОДНИХ ФЕНОЛНИХ КИСЕЛИНА У ЛИСТУ КУКУРУЗА РАЗЛИЧИТО ОБОЈЕНОГ ЗРНА

ЈЕЛЕНА З. МЕСАРОВИЋ, ВЕСНА Д. ДРАГИЧЕВИЋ, СНЕЖАНА Д. МЛАДЕНОВИЋ ДРИНИЋ, ДАНИЈЕЛА С. РИСТИЋ и НАТАЛИЈА Б. КРАВИЋ

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Лист кукуруза, заједно са другим деловима биљака, доста се користи у производњи ферментисане хране за стоку, познате као силажа. С обзиром на то да је само неколико студија објављено на тему изоловања и квантификације фенолних киселина из листова кукуруза, главни циљ овог рада је одређивање садржаја слободних фенолних киселина у листовима петнаест различитих самооплодних линија кукуруза. Коришћена је реверсно-фазна високо ефикасна течна хроматографија са DAD детектором. Под оптимизованим хроматографским условима, као што су кратко време припреме узорака, мале количине растварача и директно инјектовање екстракта узорка, фенолне киселине (тј. гална, протокатехинска, кафеинска, *p*-кумаринска и ферулинска киселина) успешно су раздвојене за мање од 25 min, што указује на могућу примену методе у рутинским анализама. Ефикасност и валидација методе су процењене мерењем параметара као што су: линеарност, граница детекције и квантификације, тачност и прецизност. Добијени резултати указују да је најзаступљенија слободна фенолна киселина *p*-кумаринска киселина (23,57 μg g^{-1} суве масе), праћена ферулинском и кафеинском киселином (21,27 и 20,78 μg g^{-1} суве масе, редом). Анализом главних компонената (PCA) процењен је однос садржаја слободних фенолних киселина у листу и боје зрна кукуруза.

(Примљено 12. маја, ревидирано 22. новембра, прихваћено 29. новембра 2016)

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