SHORT REPORT

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Phenolic acid contents of *Salvia poculata* Nab by LC-MS/MS

Seda Damla Hatipoğlu*

Department of Chemistry, Faculty of Science and Letters,Istanbul Technical University, 34469, Istanbul, Türkiye,
TÜBİTAK UME, Chemistry Group Laboratories, 41400, Kocaeli, Türkiye

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Abstract: Phenolic acids, biological aromatic secondary plant metabolites, are widely distributed throughout the plant kingdom. Due to the biological importance of phenolic acids as secondary metabolites, development of analytical methods for the determination of phenolic acids crucial. In the present study, a simple, rapid LC-MS/MS method was developed and validated for phenolic acid determination in methanol extract of *Salvia poculate* Nab from Turkey. The relative standard deviations (RSD) were found to be 3.01, 5.99, 3.21, 2.07 and 5.59 % for phenolic acids caffeic acid, fumaric acid, *p*-coumaric acid, syringic acid and *t*-ferulic acid respectively. The correlation coefficient was greater than 0.99 for each analytes in the calibration range. The uncertainty of the measurements for phenolic compounds were 7.31%, 9.27%, 7.34, 6.78% and 9.11% respectively.

Keywords: *Salvia*; phenolic acids; LC-MS/MS; method development and validation; uncertainty.

1. Plant Source

The plant of *Salvia poculata* Nab were collected from Liçan Village Van in Turkey, during 2001, and identified by a senior botanist Dr. Tuncay Dirmenci, in Department of Biology, Balıkesir University, Balikesir, Turkey. The voucher specimen (No: 103273) were deposited in the Herbarium, Faculty of Pharmaceutical Science (ISTE), Istanbul University, Turkey.

2. Previous Studies

Salvia L., one of the largest genera of the family, is represented by over 900 species and is widely distributed in different regions of the world [1]. S*alvia* genus is represented by 89 species and 97 taxa in Turkey, 45 of which are endemic [2]. S*alvia* species, having antibacterial, antioxidant, antidiabetic and antitumor properties are being used as folk medicine across the world[3]. Phytochemical investigations have shown that *Salvia* species are mostly rich in diterpenoids[4-6], triterpenoids [7] flavonoids [8] and other phenolic compounds [9]. Phenolic acids also function as reducing agents, quenchers of singlet oxygen formation and free radical scavengers. The phenolic acids as well as their components play important roles in the control of in plants and human health [10].

* Corresponding author: E-Mail: E-Mail: sedadamla.hatipoglu@tubitak.gov.tr, Tel.:(+90) 262 – 679 50 00 Fax: (+90) 262 – 679 50 01

3. Present Study

In this study, the contents of phenolic acids of the methanol extract of aerial parts of *Salvia poculata* Nab collected from, and a novel method was developed for the chromatographic identification and LC-MS/MS quantification of phenolic acid in extracts. The method developed was validated and the uncertainties caluculated via the bottom-up method.

LC and MS Conditions: Experiments were conducted using the Zivak® HPLC and Zivak® Tandem Gold Triple quadrupole (Istanbul, Turkey) mass spectrometer. Synergy Max C18 Gravity column (250×2 mm i.d., 5 μm particle size) was used for the separation. The mobile phase A was composed of water (A, 0.1% formic acid) whereas the mobile phase B was composed of methanol (B, 0.1% formic acid). The gradient program of separation is given below; 45% Solvent B at 0 min, 45% B at 1.00 min, 100% B at 1.01 min, 100% B at 20.00 min, 45% B at 20.01 min and 45% B at 23.00 min . The flow rate of the mobile phase was 0.25 mL/min, and the column temperature was set to 30 °C. The injection volume was 10 μL.

Quantification was performed on a triple quadrupole mass spectrometer via selected ion monitoring (SRM). The optimized ESI parameters are as follows; CID gas pressure 2.40 mTorr, ESI needle voltage of 5000 V, ESI shield voltage of 600 V, drying gas temperature of 300 °C, atmospheric pressure ionization (API) housing temperature 50°C, Nebulizer gas pressure of 55 psi, and the drying gas pressure of 40 psi. Additional information for the LC-MS/MS parameters are given in Table 1.

Table 1. LC-MS/MS parameters of phenolic acids

Compounds	Retention time (min)	Parent ion	Daughter ion	Collision energy (V)	ESI mode
Caffeic acid	6.29	179	135	14	Negative
p-Coumaric acid	8.39	163.2	118.7	14	Negative
Fumaric acid	3.91	115	71	8	Negative
Syringic acid	6.43	196.7	181.4	12	Negative
t-Ferulic acid	8.59	193	133	15	Negative
Curcumin*	17.99	369.3	176.9	20	Negative

*****Used as internal standart

Sample and calibration solution preparation: The aerial parts of *Salvia poculata* of about approximately 1 g were chopped into small pieces and incubated in 100 mL methanol at room temperature for extraction, for two weeks. The yield of methanol extract was 34.3 mg.

Preparation of calibration and test solutions; caffeic acid (%98), *p*-coumaric acid (98%), fumaric acid (99%), syringic acid (95%), *t*-ferulic acid (95%) were purchased from Sigma-Aldrich. Formic acid purchased from Merck. All solvents and reagents were of the highest purity needed for each application. Stock solutions of approximately 10 mg/kg were prepared in methanol. Calibration solutions were prepared by serial dilutions in methanol for the linear range of 0.1, 0.25, 0.5, 2.5 and 5 mg/kg. The solutions were stored at -20 °C in glass containers. 100 mg/kg of curcumin solution was used as an internal standard and was freshly prepared each time, from which 50 μL was used as an internal standard (IS) in all experiments.

Method validation: In validation experiments of all of the compounds, curcumin was used as an internal standard. The validation parameters to be determined are linearity, repeatability, LOD (limit of detection) and LOQ (limit of quantification).

LOD and LOQ of the LC-MS/MS methods for the above compounds were determined as0.002mg/kg and 0.25 mg/kg respectively. The LOD and LOQ were calculated as 3 and 10 times of the standard deviation respectively. The LOD and LOQ values of phenolic acids for LC-MS/MS method are given in Table 2.

The linearity for each compound for the reported method was determined by the analysis of the corresponding standard solutions. Peak areas versus the analyte concentrations in mg/kg were plotted

to obtain the calibration curves for phenolic acids. Linearity was evaluated using linear regression analysis of a six-point linear plot. The plot was consisted of three replicates per point and squared correlation coefficients, r^2 was estimated for each analyte. The correlation coefficients (r^2) for all analytes were found to be ≥0.99. Table 2 shows the linear regression equations of each compound. , The developed LC-MS/MS method was found to be linear from 0.1 to 5.0 mg/kg. The repeatability (RSD%) values for phenolic acids were calculated using the corresponding peak area of 3 replicate analyses performed for sample. The RSD% ranged between 2.07-5.99.

Estimation of uncertainty sources: In analytical methods the results needs to be assigned with an uncertainty budget for the method in order to express the accuracy of the method. For the calculation of the uncertainty correlated to the final measurement results the sources of the standard uncertainties need to be described before the measurements [11]. In case of phenolic acids it was considered that the concentrations of phenolic acids were mainly affected by the following sources of uncertainties for LC-MS/MS method: standard uncertainty associated with the sample weighing, preparation of the calibration standard solutions, interpolation of the sample reading in the calibration graph and the repeatability. The equation below was used to convert the calculated concentrations to µg/g of the crude sample;

$$Amount = \left(\frac{C_a \, x \, V_{final}}{m \, x \, V_{final}}\right) x \, 1000 \qquad \textbf{(1)}$$

In the equation C_a stands for the analyte concentration obtained by calibration curve (in mg/kg), m for the amount of extract in gram and where as V_{final} and $V_{initial}$ for the final diluted volume before the analysis and the initial sample volume respectively.

For the evaluation of the sources of the standard uncertainities and the uncertainty calculations the EURACHEM/CITAC guide was used [12]. It was found that the maximum contribution of uncertainty comes from the calibration curve. Detailed procedures of uncertainty evaluation were reported previously in the literature [13].

Table 2. Validation and uncertainty parameters for the LC/MSMS method developed for the phenolic acids.

Compounds	Linear regression equation	R^2	LOD (mg/kg)	LOQ (mg/kg)	RSD (%)
Caffeic acid	y= 1.818x+0.0067	0.9952	0.083	0.250	3.01
Fumaricacid	y= 0.338x+0.016	0.9942	0.069	0.208	5.99
p-Coumaric acid	y= 1.558x+0.157	0.9917	0.059	0.178	3.21
Syringic acid	y= 0.157x+0.0155	0.9906	0.002	0.006	2.07
t-Ferulic acid	y= 0.388x+0.031	0.9929	0.030	0.090	5.59

It is well known that phenolic acids and flavonoids have many bioactivities. In plants the existence of compounds with antioxidant activity is important because such compounds have crucial roles in terms of free radicals inhibition. For this pupose it is great importance to search and investigate such compounds with bioactivity. Table 3 summarizes the phenolic acid content of the *Salvia poculata* extracts in µg/g extract with the associated uncertainties that are determined by LC-MS/MS.

In current study the phenolic acid composition of the methanol extract of *Salvia poculata* is determined to have fumaric acid in highest level. Five phenolic acid compounds were identified in the methanol extract of *Salvia poculata* caffeic acid (88.69±6.48), fumaric acid (1095.16±101.53), *p*-coumaric acid (57.43±4.25), syringic acid (10.23±2.35) and *t*-ferulic acid (169.34±15.44). The results of the this study showed that *Salvia poculata* methanol might be used as a source of nutritional supplement due to the high levels of phenolic acid content and its possible antioxidant properties.

This developed and validated method with LC-MS/MS technique indicates that novel methodologies could be developed for phenolic acid determination which could be an alternative to the current methods to analyze the phenolic acids in plant matrices.

Table 3. Amount of phenolic acids in methanol extract of *Salvia poculata* in µg/g

	S. poculata	U_{95}
Caffeic acid	88.69±6.48	7,31
Fumaric acid	1095.16±101.53	9.27
p-Coumaric acid	57.43±4.25	7.34
Syringic acid	10.23±2.35	6.78
t-Ferulic acid	169.34±15.44	9.11

The chemical complexity of *Salvia* methanol extract, such as having different functional groups with differingpolarity and chemical behaviour, could lead to unrelated results, depending on the type of the technique employed. Therefore, a strategy using multiple confirmative assays to asses the antioxidant potential of the extract would be more informative and will be addressed in future studies.

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