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HPLC analysis of flavonoids from *Scutellaria altissima*

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High-performance liquid chromatographic method with gradient elution and diode-array detection was developed to quantify flavonoids (scutellarin, baicalin, baicalein, wogonin, wogonoside, luteolin, chrysin) and verbascoside. The separation was performed on a Hitachi C18 AQ (250 mm × 4.6 mm, 5 μm) column with detection at 330 nm for verbascoside, scutellarin and 275 nm for baicalin, baicalein, wogonin, wogonoside, luteolin, chrysin. Validation procedures were conducted and the method was proven to be precise, accurate and sensitive. The limits of detection were from 0.067 to 0.229 μg/ml. The relative standard deviations ranged from 3.78 % to 7.78 %. Recoveries were between 96.5% and 103.2%. The method was applied to quantification of mentioned above substances in extracts of *Scutellaria altissima* from the area of Mezek. This is the first study in Bulgaria on the flavonoid composition of *Scutellaria altissima*

Keywords: HPLC-DAD, validation, flavonoids, verbascoside, *Scutellaria altissima*

INTRODUCTION

The plants of the genus *Scutellaria* belong to the Lamiaceae family and, thanks to the biologically active compounds contained therein, are used in the treatment of a number of diseases [1]. In 1910, the first flavonoid scutellarein was isolated from *Scutellaria altissima* [2]. To date, about 35 species of *Scutellaria* have been studied and more than 295 compounds have been identified, including flavonoids and diterpenes as major biologically active substances. One of the most famous species of this genus is *Scutellaria baicalensis*, also known as "Baical Scullcap", which is widely used in the traditional medicine of Russia and several East Asian countries [1, 3] and is listed in various Pharmacopoeias. According to European Pharmacopoeia 8.0, the content of the baicalin in *Scutellariae Baicalensis* Radix should be not less than 9% (dried drug) [4].

There are eight species in Bulgaria, one of which is *Scutellaria altissima*, from which only terpene compounds have been isolated and identified and their cytotoxic and antifeedant activity has been proved [5]. So far, no studies of polyphenol compounds or of the biological activity of their aqueous and ethanol extracts have been carried out. This gives grounds for conducting a study in this direction which will enrich the information about the composition of the Bulgarian *Scutellaria altissima*.

Chromatographic methods are some of the most commonly used methods for analyzing biologically active substances [6]. Gao *et al.* (2008) offer an HPLC method for the determination of the flavonoids baicalin, baicalein and wogonin in tinctures of two species of *Scutellaria* - aerial part of *Scutellaria lateriflora* and root of *Scutellaria baicalensis* [7]. A number of flavonoids, the most important of which are scutellarin, baicaline and chrysin, are identified in *Scutellaria galericulata* from 17 regions in eastern Poland by an HPLC method [8].

In the present work, a high-performance liquid chromatographic method with gradient elution and diode-array detection was developed and validated to quantify flavonoids (scutellarin, baicalin, baicalein, wogonin, wogonoside, luteolin, chrysin) and verbascoside. The method was applied to the quantification of the above mentioned substances in extracts of *Sculellaria altissima* from the area of Mezek, Bulgaria.

EXPERIMENTAL

Chemicals

Standards of flavonoids (scutellarin, baicalin, baicalein, wogonin, wogonoside, luteolin, chrysin), and verbascoside were purchased from Sigma-Aldrich (Germany). Methanol and acetonitrile (HPLC gradient grade) were purchased from

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Sigma-Aldrich (Germany). Water was obtained from a Milli-Q Gradient water purification system (Milipore, Barnstead).

Plant material

Aerial parts of *Scutellaria altissima* were collected during flowering in June 2017, from the area of Mezek, Bulgaria. Collected raw material was dried at 25°C. A voucher specimen (n.17494) was deposited in the Herbarium of the University of Agriculture, Plovdiv, Bulgaria.

Samples

Dried plant material was powdered and 0.2 g of each of it were extracted with 10 ml of distilled water, 70% ethanol, 96% ethanol and methanol, respectively. The extraction was performed at room temperature 25 °C for 24 hours. The obtained extracts were filtered through a microfilter (0.25 μm) and injected into the HPLC system. For each sample, the complete procedure was carried out in triplicate and the standard deviation was calculated.

HPLC analysis

The HPLC system was composed of a ProStar 230 solvent delivery module and photodiode array detector model 335 and Hitachi C18 AQ (250 mm × 4.6 mm, 5 μm) column. A solvent system including deionized water (A) adjusted to pH 3.0 with phosphoric acid and acetonitrile:methanol 40:60 (B) was used in the following gradient mode: 0-25 min 75A / 25B - 10A / 90B; 25-27 min - 10A / 90B; 27-30 min 10A / 90B - 75A / 25B. The flow rate was 0.9 ml/min and detection was at 330 nm for verbascoside, scutellarin and 275 nm for baicalin, baicalein, wogonin, wogonoside, luteolin, chrysin. The analysis was performed at the same temperature 25 °C. The compounds of interest in the extracts were identified through their retention times, as well as by comparing their absorption spectra with those of standard substances. They were quantified using a calibration curve. Star Chromatography Workstation Version 6.30 (build 5) software was used.

Validation of the HPLC method

Linearity. A linear range was established by using six mixed calibration solutions with concentrations from 2 to 25 μg/ml, each of which was injected three times into the HPLC system. Chromatographic data were then used to build the calibration curve of peak area to the concentration of each standard substance. The coefficients of determination were used as the measure of linearity.

Limit of detection (LOD) and limit of quantification (LOQ). LOD was defined as the lowest concentration of sample determined by the analytical method to obtain the ratio of signal to noise (3:1). LOQ as the lowest concentration of compounds was determined by injecting a known concentration of the diluted standards until the signal-to-noise ratio reached the ratio of 10:1

Precision. The repeatability of the retention times was determined from all injections of the six mixed calibration solutions made for linearity and were expressed by relative standard deviation.

Accuracy. The accuracy was reported in two ways. The accuracy of the method was assessed by performing a recovery study. Three samples with known concentrations (low, medium and high, different from those analyzed for the calibration) of the investigated compounds were analysed [9]. Results of five replicates of the same sample were averaged and recovery (R^a) was calculated as follows:

$$R^a\ (\%) = 100 \times \text{found concentration / true concentration}$$

The evaluation of the matrix effect through the recovery (R^b) was studied too [9,10]. A known amount of standards was added to a certain amount (0.5 g) of plant material, and then extracted and analyzed using the method described above. Five replicates were performed for the test. R^b was determined using the formula:

$$R^b\ (\%) = 100 \times (C_f - C_u) / C_a$$

where C_f is the concentration detected in the spiked sample, C_u is the concentration detected in the sample before the spiking, and C_a is the true added concentration.

RESULTS AND DISCUSSION

According to the literature, reversed phase columns C18 are some of the most commonly used ones in the separation of polyphenol compounds [8, 11] in combination with mobile phases containing water and acetonitrile, water and methanol or water/acetonitrile/methanol and various values of the pH of the medium [7]. The authors' team has experience in the determination of polyphenol compounds in plant substances and combined extracts [12] and has developed this method on the basis of this experience.

In the present experiment, the Hitachi C18 AQ columns (250 mm × 4.6 mm, 5 μm) and Microsorb-MV C18 column (150×4.6 mm, 5 μm) were used to find the most suitable conditions for the best possible separation of flavonoids (scutellarin, baicalin, baicalein, wogonin, wogonoside, luteolin, chrysin) and the phases: water and acetonitrile and water/acetonitrile/methanol in different gradient modes were used as well. To obtain symmetrical peaks the ratio of acetonitrile to methanol in the organic phase was varied, as well as the pH of the

aqueous phase - 3.0; 3.5 and 3.8. Best results were obtained using a Hitachi C18 AQ column, mobile phase containing water at pH 3 (A) and organic phase 40 acetonitrile / 60 methanol (B). The glycosides scutellarin, baicalin and wogonoside are more polar compounds compared to the aglycones baicalein, wogonin, and chrysin, due to their shorter retention time under the specified process conditions. Verbascoside is retained for an even shorter time compared to them due to its specific structure of caffeoyl phenylethanoid glycoside. Figure 1 shows a chromatogram of a model mixture with a concentration of 10 μg/ml.

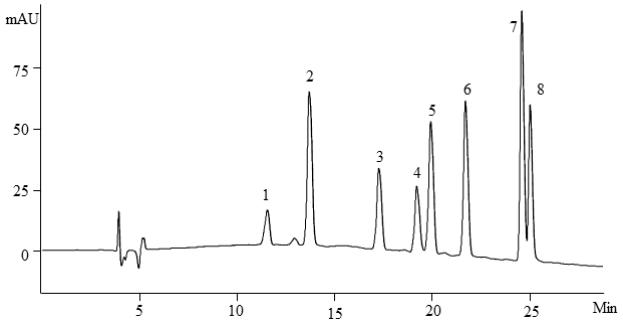

Figure 1. Chromatogram of a model mix (10 μg/ml) of verbascoside-1 and flavonoids (scutellarin-2, baicalin-3, luteolin-4, wogonoside-5, baicalein-6, wogonin-7, chrysin-8)

The validation procedure for the HPLC method includes the determination of the following parameters: linearity, LOD, LOQ, accuracy and precision. Three series of standard solutions of the pure substances were analyzed under the specified conditions, and it was established that in the chosen concentration ranges (Table 1, column 3) there is a linear relationship between the concentration and the area of the chromatographic peak (r^2 = 0.9924 ÷ 0.9985). This means that the method can be used to quantify the test substances. Regression equations of their standard curve coefficients of determination (r^2) and LOD and LOQ are listed in Table 1.

Recovery experiments were performed to study the reliability and suitability of the method. Three samples with known concentrations of the eight investigated compounds and a spiked sample were analysed (n=5). Recoveries between 96.6% and 102.7% (R^a) were obtained through the first experiment. Good recoveries in the range were obtained by the fortification of the samples at a concentration of 12 μg/ml for verbascoside, scutellarin, baicalin, luteolin, wogonoside, baicalein, wogonin and chrysin through the second experiment. It was evident from the results that the percent recoveries for all the eight analytes of interest were in the range of 96.5%-103.2% (R^b) which is a confirmation for the accuracy of the method [13]. The data are reported in Table 2.

Table 1. Parameters of calibration curves, LOD and LOQ for HPLC method validation

Analyte	λ (nm)	Concentrations (μg/ml)						RT (min)	Regression equations	r^2	LOD (μg/ml)	LOQ (μg/ml)
		S_1	S_2	S_3	S_4	S_5	S_6					
Verbascoside	330	2	5	10	15	20	25	11.81	y=2.9511e+005x	0.9937	0.229	0.735
Scutellarin	330	2	5	10	15	20	25	13.98	y=1.0303e+006x	0.9924	0.088	0.281
Baicalin	275	2	5	10	15	20	25	17.52	y=6.4478e+005x	0.9938	0.102	0.368
Luteolin	275	2	5	10	15	20	25	19.41	y=4.8185e+005x	0.9984	0.133	0.409
Wogonoside	275	2	5	10	15	20	25	20.16	y=9.7821e+005x	0.9955	0.079	0.269
Baicalein	275	2	5	10	15	20	25	21.90	y=1.0883e+006x	0.9974	0.096	0.284
Wogonin	275	2	5	10	15	20	25	24.76	y=1.4736e+006x	0.9985	0.067	0.210
Chrysin	275	2	5	10	15	20	25	25.26	y=1.0024e+006x	0.9963	0.084	0.278

Table 2. Parameters related to precision and accuracy for HPLC method validation

Parameters / Analyte	RSD(%)	Real concentration, (μg/ml)			Concentration found, (μg/ml)			Recovery[a], (%±0.1)			Recovery[b], (%±0.1)
Verbascoside	7.78	4	12	22	4.05	11.52	21.90	101.3	96.1	99.5	97.0
Scutellarin	7.01	4	12	22	3.87	12.32	21.79	96.8	102.7	99.0	101.7
Baicalin	6.76	4	12	22	4.11	11.76	21.97	99.9	98.0	100.1	98.3
Luteolin	3.78	4	12	22	3.86	11.87	21.66	96.6	98.9	98.5	99.2
Wogonoside	3.85	4	12	22	3.97	11.76	22.29	99.3	98.0	101.3	103.2
Baicalein	7.39	4	12	22	4.09	11.82	22.34	102.4	98.5	101.5	102.3
Wogonin	5.96	4	12	22	3.97	11.94	21.70	99.3	99.5	98.6	99.0
Chrysin	6.13	4	12	22	3.94	11.99	22.06	98.6	99.9	100.2	96.5

Analysis of extracts

The relative standard deviation ranged from 3.78 % to 7.78 %. This indicates that the proposed method is accurate and precise. By analyzing the results, presented in Tables 1 and 2, it can be concluded that the developed method is precise, accurate and sensitive enough for the simultaneous quantitative evaluation of eight investigated compounds in dry extracts, plant material or food supplements.

A number of authors have studied the flavonoid composition of various species of the genus *Scutellaria* and have demonstrated the presence of baicalin, baicalein, scutellarin and chrysin in wild growing *Scutellaria galericulata* [8], baicalin, baicalein and wogonin in tinctures derived from *Scutellaria lateriflora* and *Scutellaria baicalensis* [7] as well as baicalin and wogonoside in wild type root culture of *Scutellaria barbata* [11]. Grzegorczyk-Karolak *et al*. have also identified luteolin and verbascoside in *Scutellaria altissima* callus cultures [14, 15] in addition to the baicalin and wogonoside which are characteristic of the species. The developed method was applied to the quantification of flavonoids (scutellarin, baicalin, baicalein, wogonin, wogonoside, luteolin, chrysin) and verbascoside in *Scutellaria altissima* extracts. For this purpose, aqueous, methanolic and ethanol extracts of dry plant material were prepared. The results we obtained show that they contain the specific to the genus *Scutellaria* scutellarin, baicalin, baicalein, as well as wogonoside, wogonin, chrysin and verbascoside. Chromatograms of aqueous and methanolic extract are shown in Figs. 2 and 3, respectively.

Under the chosen operating conditions, 70% ethanol extracts the highest degree of baicalin, baicalein, scutellarin and wogonoside, 31250 ± 291, 288 ± 11, 4131 ± 29 and 2827 ± 102 μg/g, respectively. The amounts obtained are twice as much compared to the extraction with methanol and six times more than the extraction with water (Table 3). On the other hand, methanol also extracts verbascoside, wogonin and chryzin, but in quantities less than the limit of quantification of the method. This is a prerequisite for conducting experiments to optimize extraction conditions to increase the yield.

In the available literature, data on the flavonoid composition of *Scutellaria altissima* are only found in shot cultures and the amounts of baicalin and wogonoside shown are commensurate with those obtained by us [14, 15].

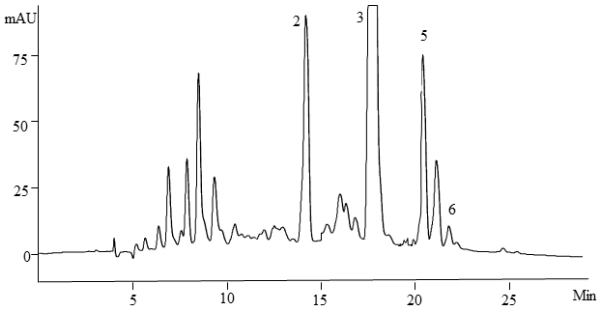

Figure 2. Chromatogram of *Scutellaria altissima* aqueous extract

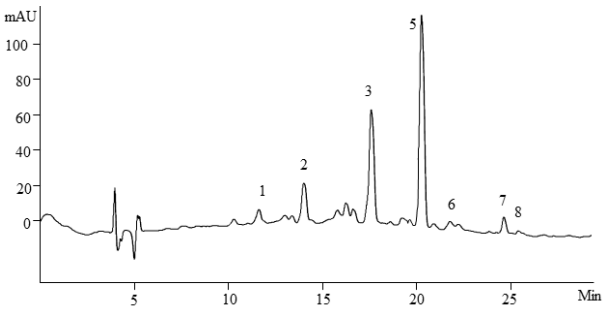

Figure 3. Chromatogram of *Scutellaria altissima* methanolic extract.

Table 3. Quantities of scutellarin, baicalin, baicalein, wogonin, wogonoside, luteolin, chrysin and verbascoside, determined in μg/g dry plant material

Analyte, μg/g / Extraction solvent	Verbascoside	Scutellarin	Baicalin	Luteolin	Wogonoside	Baicalein	Wogonin	Chrysin
H_2O	-	687±53	2469±127	-	497±26	144±9	-	-
70% EtOH	-	4131±29	31250±291	-	2827±102	288±11	-	-
96% EtOH	-	590±33	5992±203	-	1412±61	82±5	-	-
MeOH	traces	2165±98	17230±342	-	2210±95	85±5	traces	traces

All values are mean ± SD (n=3)

CONCLUSIONS

An HPLC method for the determination of polyphenol compounds: the flavonoids - scutellarin, baicalin, baicalein, wogonin, wogonoside, luteolin, chrysin and the caffeoyl phenylethanoid glycoside - verbascoside was developed. The proposed HPLC assay showed good separation of the compounds and proved to be efficient, precise and accurate, therefore, it could be used for the simultaneous

determination of biologically active compounds in plant material and dry extracts or phyto products. The method can also be used to prove the authenticity of plants of the genus *Scutellaria*. This is the first study in Bulgaria on the flavonoid composition of *Scutellaria altissima*, which implies the continuation of the experiments to determine the composition and the comparison of the quantities of the tested compounds in the other *Scutellaria* species, which occur on the territory of the country.

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