Quantitative Determination of Caffeic Acid, Chlorogenic Acid and Luteolin-7-glucoside from *Scilla bifolia* by HPLC-MS

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Scilla bifolia is an indigenous specie highly widespread in the flora of our country, and once investigated in terms of therapeutic principles, may be considered a cheap and easily accessible resource. However, studies performed on this species, are extremely limited. The aim of this work was to identify and accurately quantify three polyphenols known for their beneficial effects on human health of Scilla bifolia bulbs and herba. Analyses were performed by HPLC-MS. The proposed method is selective, precise and has a high accuracy.

Keywords: HPLC-MS, Scilla bifolia, caffeic acid, chlorogenic acid, luteolin-7-glucoside

Scilla bifolia (*Liliaceae*) is an herbaceous plant found mainly in deciduous forests, meadows and bushes from the plain area and hills of the flora of our country. Among its popular names are: blue snowdrops, alpine squill, field-hyacinth, etc. In some countries, *Scilla bifolia* is grown for supply raw materials for the pharmaceutical industry. In parks and gardens it is cultivated as ornamental plant.

Traditionally, the roots are administrated as tea against cough. Leaves, similarly to those of other related species are used in the treatment of wounds and sores [1-2].

Compared to other species belonging to the genus *Scilla* (*Scilla maritime, Scilla ingridiae, Scilla autumnalis*), *Scilla bifolia* is a species that has not been studied so far and, with the exception of traditional medicine, very little information is known about the chemical composition and its uses [3-5].

The first of the studied polyphenols is caffeic acid, a hydroxycinnamic acid common in the plant world. It has a proved chemopreventive, antioxidant and antibacterial activity [6], but there are contradictory informations regarding its anticancer effects [7].

Both chlorogenic acid and luteolin-7-glucoside regulate vascular endothelial function by increasing the release of nitric oxide from plant extracts containing them, having vasodilatory and cardioprotective action. Chlorogenic acid also presents hypoglycemic, antiviral and anticancer activity;

Their determination raises enough problems as the concentration of these compounds in medicinal herbs is low and other plant constituents interfere with their detection, putting the issue of sensitivity and specificity of the adopted analysis method.

MS methods have been developed for rapid screening of similar compounds in plants. The HPLC coupled with mass spectrometry provides the selectivity and sensitivity necessary to quantify polyphenols in a complex mixture such as plant extracts [8-10]. This method has been extensively used in the separation of secondary metabolites from different classes provided by indigenous plants from spontaneous flora [11].

Experimental part

Plant material

The raw material (aerial parts- *herba* and bulbs of *Scilla bifolia*) was collected at the flowering stage from the spontaneous flora in two consecutive years. The plant was identified and marked with two voucher specimens: first year: *Scilla bifolia* bulbs - SBB1 and *Scilla bifolia herba* - SBH1 and second year *Scilla bifolia bulbs* – SBB2 and *Scilla bifolia herba* – SBH2.

After harvesting, samples were cleaned of soil and dust particles or damaged portions that may have influenced the analytical results and were air-dried (25-27°C) in a dark room at constant humidity. After drying, the collected samples were ground to fine powder.

Reagents

All chemicals were of analytical grade.

Extraction

About 10 g of powdered aerial parts or bulbs were refluxed separately with 100 mL volumes of ethanol 70 %, 3 times successively at room temperature for 60 min . Combined extracts were brought to 10 mL by rotary evaporation under reduced pressure. All samples analyzed were diluted with distilled water (1/15 for caffeic acid determination, 1/3 for chlorogenic acid and 1/10 for luteolin-7-glucoside) and then injected as a preliminary centrifugation at 12,000 rpm for 1 min.

Equipment

HPLC coupled with mass spectrometer: HP 1100 Series system equipped with binary pump, autosampler (HP 1100 Series) and thermostat (HP 1100 Series) was used.

Detectors: mass spectrometer: Agilent Ion Trap 1100 V;

Chromatographic conditions for determination of caffeic acid and chlorogenic acid

For the separations, a Gemini NX (50 mm x 2.0 mm i.d., 3 μm from Phenomenex, SUA) column was used with an

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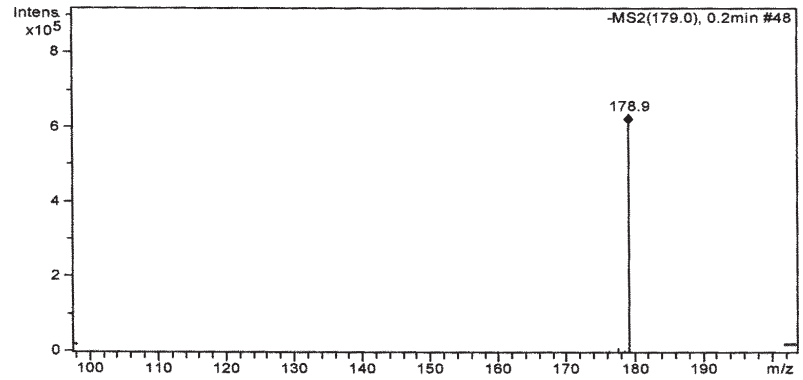

Fig. 1. Full-scan spectra of caffeic acid in the mobile phase

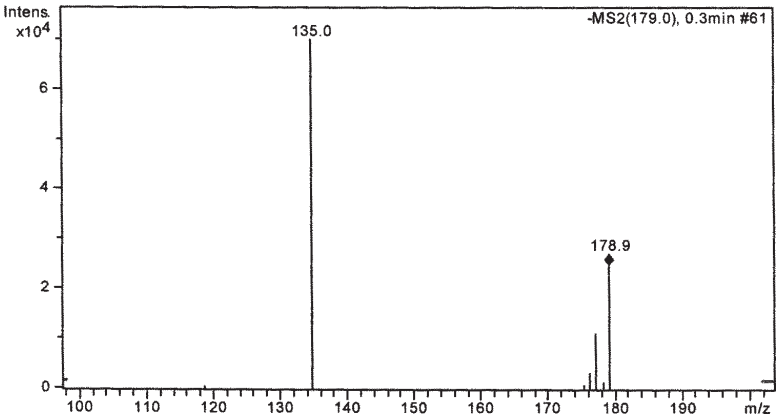

Fig. 2. MS/MS spectrum of caffeic acid in the mobile phase

on-line 0.2 micron filter (Agilent). The mobile phase was prepared from mixed solution of acetic acid 0.1% (v/v) and acetonitrile 92/8 (v/v), isocratic elution. The flow rate was: 0.6 mL/min at 40 ^{0}C; injection volume was 5 μL;

MS working conditions

Ion source: ESI (electrospray ionization interface); ionization mode: negative; nebuliser: nitrogen, pressure: 55 psi; drying gas: nitrogen, flow: 10 L/min, temperature: 350 ^{0}C; capillary potential: +4000 V;

Analysis mode caffeic acid: isolation and fragmentation of the ion with m/z 179, corresponding to deprotonated caffeic acid molecule, and monitoring ions with m/z 135 in the MS/MS spectrum of the analyte.

Analysis mode chlorogenic acid: isolation and fragmentation of the ion with m/z 353, corresponding to deprotonated caffeic acid molecule, and monitoring ions with m/z 190 in the MS / MS spectrum of the analyte.

Chromatographic conditions for determination of luteolin-7-glucoside

A Zorbax SB-C18 reversed-phase (100 mm x 3.0 mm i.d., 3.5 μm) column was used, with an on-line 0.2 micron filter (Agilent). The mobile phase was prepared from mixed solution of acetic acid 0.1% (v/v) and methanol, 65/35 (v/v), isocratic elution. The flow rate was: 1 mL/min at 45 ^{0}C; injection volume was 10 μL

MS working conditions

Ion source: ESI (electrospray ionization interface); ionization mode: negative; nebuliser: nitrogen, pressure: 65 psi; drying gas: nitrogen, flow: 12 L/min, temperature: 360 ^{0}C; capillary potential: +4000 V;

- Analysis mode: isolation and fragmentation of the ion with m/z 447, corresponding to deprotonated molecule of luteolin-7-glucoside, and monitoring ions with m/z 285 in the MS /MS spectrum of the analyte.

Results and discussions

Determination of caffeic acid

Mass spectrum (full-scan) recorded for the caffeic acid solution under the conditions described above is shown in figure 1. Isolation and fragmentation of the ion with m/z 179, corresponding to deprotonated molecule of caffeic acid was performed. It was noted that by fragmentation, caffeic acid is split into a main fragment with m/z 135, which was selected for quantification (fig. 2).

Caffeic acid calibration curve was achieved in the concentration range 20-640 ng/mL. All solutions were prepared in distilled water. The accuracy of the analytical method, calculated for each concentration (n = 6) is shown in table 1.

Figure 3 shows typical chromatograms for caffeic acid resulted in the four analyzed samples.

Determination of chlorogenic acid

The expected ion in accordance with the molecular weight of the analyte (M = 354) and with ionization mode (negative) was the ion with m/z 353, corresponding to the deprotonated molecule. Chlorogenic acid calibration curve was achieved in the concentration range 21.6-346 ng/mL (fig. 4). All solutions were prepared in distilled water.

Figure 5 presents typical chromatograms of chlorogenic acid resulted in the four analyzed samples.

Determination of luteolin-7-glucoside

The expected ion in accordance with the molecular weight of the analyte (M=448) and with ionization mode (negative) was the ion with m/z 447, corresponding to the deprotonated molecule (fig. 6). In order to increase the selectivity of HPLC / MS method, the characteristic ion was fragmentated, and the MS/MS spectrum was recorded (fig. 7).

For the concentration range 20-640 ng/mL a quadratic calibration curve was obtained ($y= ax^2+bx+c$) as shown in figure 8. The accuracy of the analytical method ranged from 97-101% for each concentration separately. Figure 9

	Calibration curve	
Concentraţie-teoretică (ng/ml)	Recovered concentration (ng/ml)	Accuracy
20.00	21.73	108.6
40.00	40.05	100.1
80.00	77.06	96.3
160.00	145.06	90.7
320.00	333.92	104.4
640.00	644.66	100.7
Coeficient X	2506.5	
Intercept	-8794.4	
Correlation coefficient r^2	0.9992	

Table 1
ACCURACY OF THE ANALYTICAL METHOD FOR QUANTITATIVE DETERMINATION OF CAFFEIC ACID

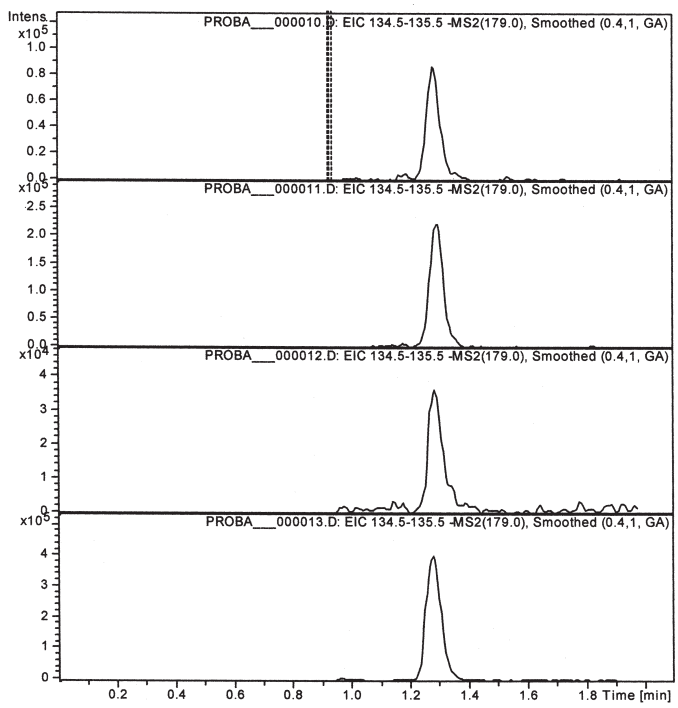

Fig. 3. Caffeic acid chromatograms of the four analyzed samples (from top to bottom: SBH1, SBB1, SBH2, SBB2); analyte retention time: 1.3 min

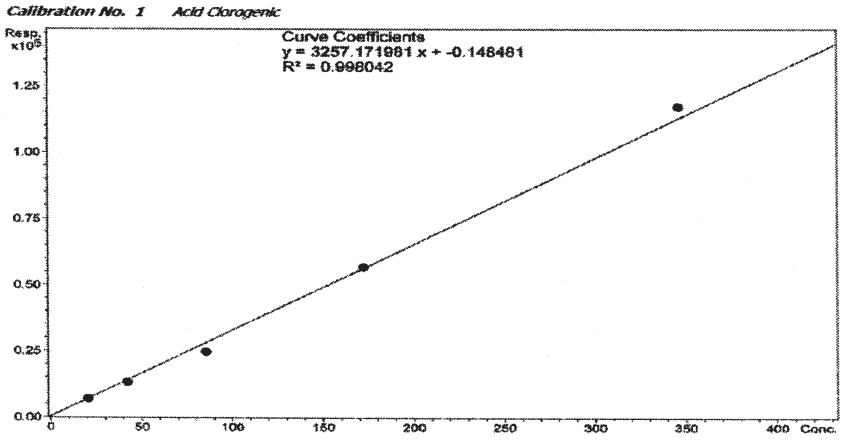

Fig. 4. Chlorogenic acid calibration curve, detection MS/MS

presents typical chromatograms of luteolin-7-glucoside determined in the four analyzed samples.

Quite small amounts of chlorogenic acid were found in *Scilla bifolia* herba compared to luteolin-7-glucoside and caffeic acid (table 2). Luteolin-7-glucoside is also available in much larger quantities in *Scilla bifolia* herba than in bulbs while caffeic acid is found in large amounts in bulbs than herba. Differences between the same samples harvested in successive years were observed, suggesting that the medium factors have an influence on the accumulation of

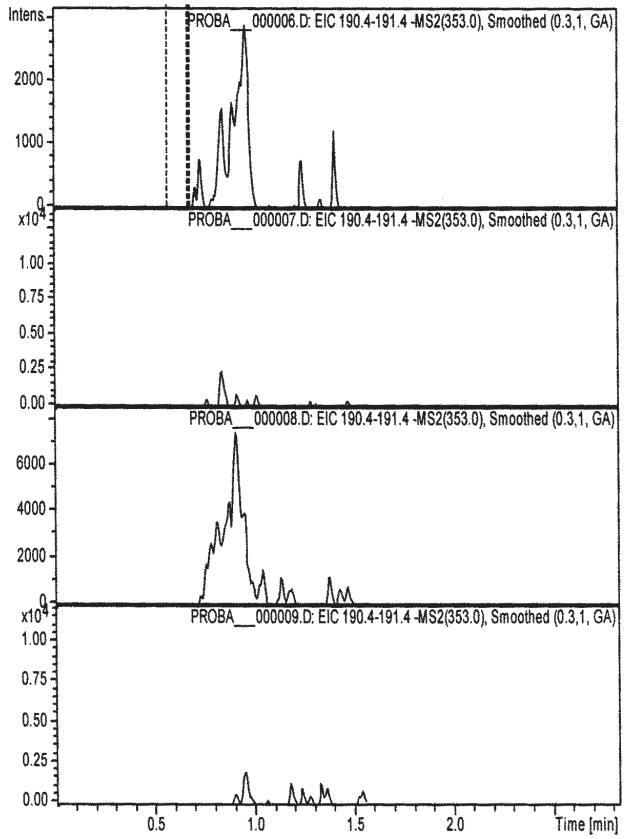

Fig. 5. Chlorogenic acid chromatograms of the four analyzed samples (from top to bottom: SBH1, SBB1, SBH2, SBB2); analyte retention time: 0.95 min

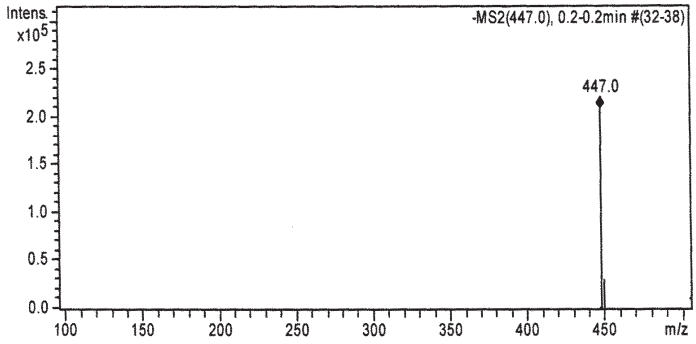

Fig. 6. Full-scan spectra of luteolin-7-glucoside in the mobile phase

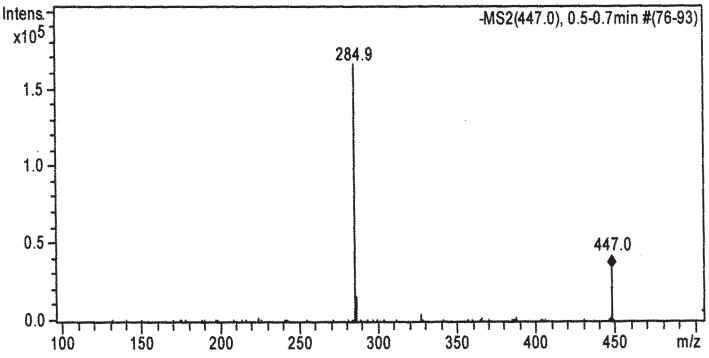

Fig. 7. MS/MS spectrum of luteolin-7-glucoside, in the mobile phase

Sample	Caffeic acid ng/ml	Chlorogenic acid ng/ml	Luteolin-7-glucoside ng/ml
SBH1	1722.1	15.74	4542.6
SBB1	4368.0	0.00	100.27
SBH2	786.1	43.91	4513.6
SBB2	8803.9	0.00	0.0

Table 2
RESULTS OF QUANTITATIVE DETERMINATIONS: CAFFEIC ACID, CHLOROGENIC ACID AND LUTEOLIN-7-GLUCOSIDE

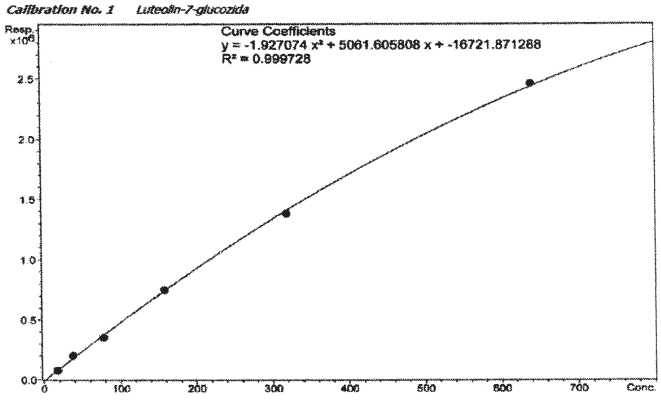

Fig. 8. The calibration curve of luteolin-7-glucoside, detection MS / MS

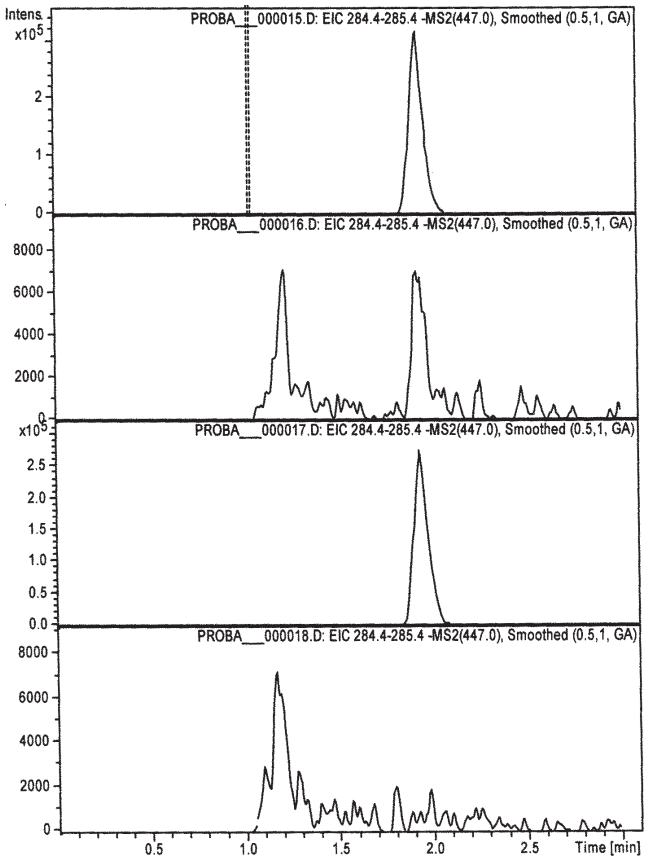

Fig. 9. Luteolin-7-glucoside chromatograms of the four analyzed samples (from top to bottom: SBH1, SBB1, SBH2, SBB2); analyte retention time: 2 min

these polyphenols. *Scilla bifolia* species also contains along with these polyphenols other compounds from the same class [12].

Conclusions

In this study three polyphenols known for their beneficial effects on human health were quantified in *Scilla bifolia* bulbs and herba by HPLC-MS. The used method provides good selectivity and sensitivity and can be successfully applied in the analysis of medicinal herbs.

References

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