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LC/MS ANALYSIS OF STEROLIC COMPOUNDS FROM *GLYCYRRHIZA GLABRA*

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ABSTRACT. In this study we have attempted to identify and quantify phytosterols from four samples of *Glycyrrhiza glabra* harvested from four different areas (Bilekh, Rakka, Alpo-Azaz, Rass-Aenn) of Syria. Beta-sitosterol and stigmasterol are predominant in all samples. The highest amount of beta-sitosterol and campesterol was found in Alpo-Azaz sample and the biggest quantity of stigmasterol and ergosterol was found in Bilekh sample. This investigation offers an approach to rapidly characterization of phytosterols in herbal products.

Keywords: *HPLC-MS-MS, phytosterols, Glycyrrhiza glabra*

INTRODUCTION

Phytosterols are naturally occurring substances found in greater quantity in higher plants, especially in oily seeds, legumes and cereals. The most common is sitosterol which is sometimes accompanied by a smaller percentage of stigmasterol. In nature, plant sterols are found in free or esterified form, as well as glycosides. They are used in the semisynthesis of steroid hormones and vitamin D. They seem to have an important role in the body by keeping cholesterol level low. The main biologically active components of *Glycyrrhiza glabra* licorice are triterpenoid saponins, flavonoids, chalcones and isoflavones. Numerous studies have been focused on researching these compounds. Beside those, a series of minor constituents, such as coumarins, stilbenoids, gamma-lactones and sterols, are also present. The literature mentions the presence of beta-sitosterol, stigmasterol and dihydrostigmasterol [1-4], but to the best of our knowledge, the sterol composition of licorice root has not been investigated yet.

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Analysis of sterols from four samples of *Glycyrrhiza glabra* harvested from Syria has been carried out by using a HPLC-APCI-MS method. The applied method of analysis is based on a published HPLC method from the literature [5], to which we've made some changes. The main modification was to change the chromatographic column and mobile phase. As a result, compared to the method published in literature, the analysis time decreased from 30 minutes to 5 minutes without affecting the separation and peak resolution of sterols [6-11].

For quantitative determination, four standards were used: beta-sitosterol, stigmasterol, campesterol and ergosterol, purchased from Sigma Company (Germany).

RESULTS AND DISCUSSION

According to chromatographic conditions, retention times of the four analyzed sterols are: 2.4 min for ergosterol, 3.7 min for both stigmasterol and campesterol (co elution) and 4.2 min for beta-sitosterol. Ions monitored by MS method are presented in Table 1. Since in the ionization conditions, all sterols lose a molecule of water, ions detected by the spectrometer are always of the form $[M\text{-}H_2O+H]^+$.

Table 1. Sterol-specific ions monitored in the screening method, in order of their retention times

Sterol	Retention time (min)	M	$M\text{-}H_2O$	$M\text{-}H_2O+H^+$
Ergosterol	2.4	396	378	379
Stigmasterol	3.7	412	394	395
Campesterol	3.7	400	382	383
Beta-Sitosterol	4.2	414	396	397

Total ion chromatogram of a mixture of four sterols is shown in Figure 1. The sum of all ions scanned by spectrometer was plotted, regardless of their mass.

Specific ions of the four sterol standards (379 for ergosterol, 395 for stigmasterol, 383 for campesterol and 397 for beta-sitosterol) were fragmented and based on fragments of the spectrum MS chromatograms of each compound extracted were drawn.

This method of analysis (also called MS/MS) is highly specific compared to the screening method, where is recorded only the intensity of the main ion and an isomer compound - with the same molecular weight - can give a false positive signal.

Based on analysis of fragments of the MS spectrum, which are specific to each structure separately and are not the same for different

isomers, the MS/MS method will detect only the compound of interest without interference from others.

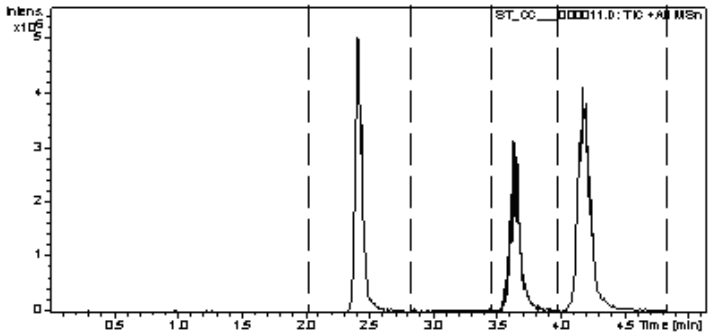

Figure. 1 Chromatogram of standard solution of sterols. Elution order: ergosterol (2.4 min), stigmasterol and campesterol (both at 3.7 min), beta-sitosterol (4.2 min)

Moreover, the intensity of ions in the mass spectrum is proportional to the concentration of the substance in the sample, so the method can also be applied for quantitative determination.

In order to quantify the four sterols from plant extracts, we have constructed the extracted chromatograms for each of them, taking into account the intensity of major ions in the mass spectrum. Ions used for quantification are listed in Table 2.

Table 2. Ions from MS spectra of the analyzed sterols, used in quantification

No.	Compound	Specific ions for identification
		$[M-H_2O+H^+]$ ion > ions from spectra
1	Ergosterol	379> 158.9; 184.9; 199; 213; 225; 239; 253; 295; 309; 323
2	Stigmasterol	395> 255; 297; 283; 311; 241; 201
3	Campesterol	383> 147; 149; 161; 175; 189; 203; 215; 229; 243; 257
4	Beta-Sitosterol	397> 160.9; 174.9; 188.9; 202.9; 214.9; 243; 257; 287.1; 315.2

In order to create calibration curves, the four sterol standards were dissolved in chloroform (concentration 1 mg/mL), after which successive dilutions were made in acetonitrile, at different concentration levels, selected as representative of the range of concentration in sample. Regression analyses of various concentrations of standard solutions (0.08-8 µg/ml) have good

correlation coefficients for the calibration curves of sterols in all cases. In Table 3 are presented the calibration curves for each standard.

Table 3. Parameters of calibration curve for each standard

STANDARD	CALIBRATION CURVE ECUATION	R^2
Ergosterol	y = 2006.6073 x + 14988.2649	0.9994
Stigmasterol	y = 656.1371 x + 6552.3729	0.9957
Campesterol	y = 2514.6804 x + 56452.9510	0.9987
Beta-Sitosterol	y = 2085.1047 x + 16268.9043	0.9937

The limit of quantification was set to 0.08 µg/ml for each analyte. The accuracy and precision were 97.2 % - 104.7 % and 8.1 % for ergosterol, 89.1 -110.5 % and 9.2 % for stigmasterol, 93.5 - 109.6 % and 11.7 % for campesterol and 92.9 - 112.7 % and 9.9 % for beta-sitosterol, respectively. For each analyte, the limit of detection was 0.02 µg/ml, based on a signal-to-noise ratio of 3.

Concentrations of sterols found in the four *Glycyrrhiza glabra* extracts are presented in Table 4. As expected, beta-sitosterol and stigmasterol are found in higher amounts than campesterol in all samples. The quantity of campesterol is very small in all extracts. The sample of Alpo-Azaz G3 has the largest amount of campesterol.

Of the four analyzed samples, the G1 sample of Bilek has a greater amount of stigmasterol than beta-sitosterol, which is less common in the plant world. Usually, in plants beta-sitosterol is predominant, as for samples G2-G4.

Table 4. Content of sterols in samples (concentration are registrated in HPLC sample injected)

Sample	**G1**	**G2**	**G3**	**G4**
Compound	**Content (ng/ml)**			
Ergosterol	3117.8	2053.1	2977.2	1894.3
Stigmasterol	7185.5	6346.0	5784.1	3241.9
Campesterol	1484.0	1411.5	2285.8	1074.2
Beta-Sitosterol	6624.8	7408.6	12448.7	5562.0

It's interesting to note that ergosterol quantity in all samples is appreciable, even higher than campesterol, although this compound is not specific to higher plants. Its presence in root extracts of *Glycyrrhiza glabra* is probably due to the mycorrhizal fungi. They can contribute to the biological activity of the vegetal product by the metabolites synthesized and also by increasing the glycyrrhizin concentration in roots [11].

Comparative sterols content in all four samples is presented in figure 2.

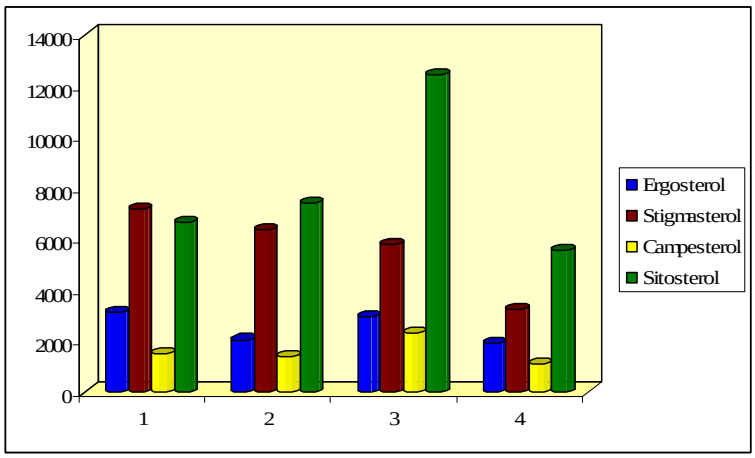

Figure. 2 Comparative sterols content in four extracts of *Glycyrrhiza glabra*

CONCLUSIONS

In the present study we have analyzed, for the first time, the phytosterols from roots of *Glycyrrhiza glabra* collected from four different areas of Syria. The presence of beta-sitosterol, stigmasterol, campesterol and ergosterol was assessed through a HPLC/APCI/MS method. This method permitted us the identification and quantification of plant sterols in the four extracts. In all samples beta-sitosterol and stigmasterol are predominant; the G3 sample of Alpo-Azaz has the highest amount of beta-sitosterol and campesterol and the G1 sample of Bilekh has the largest amount of stigmasterol and ergosterol.

EXPERIMENTAL SECTION

Plant material: The root parts of *Glycyrrhiza glabra* were collected from Syria in four different areas (Bilekh, Rakka, Alpo-Azaz, Rass-Aenn) and air-dried at room temperature. Plants were identified and a voucher specimen of each was deposited at the herbarium of Pharmaceutical Botany Department, Faculty of Pharmacy, University of Medicine and Pharmacy Iasi.

Sample preparation: Samples of 5 g pulverized roots material were extracted by refluxing with 25 mL methanol, in a Soxhlet extractor, for 1 hour. The resulting samples were appropriately diluted before injection in chromatograph. We marked the samples as follows: G1 (Bilekh), G2 (Rakka), G3 (Alpo-Azaz), G4 (Rass-Aenn).

Stock solutions: Stock solutions (1 mg/mL) were prepared from standards, kept at 4^0C and protected from day light. Before being used as working solutions, they were appropriately diluted with acetonitrile.

Chemicals: Methanol and acetonitrile of HPLC analytical-grade, and chloroform were purchased from Merck

Apparatus and chromatographic conditions. The analysis was carried out using an Agilent 1100 HPLC Series system equipped with G1322A degasser, G1311A binary pump and G1313A autosampler. For the separation we used a reversed-phased Zorbax SB-C18 analytical column (100 mm x 3.0 mm i.d., 5 µm particles) fitted with precolumn Zorbax SB-C18, both operated at 40^0C. The mobile phase was prepared from methanol and acetonitrile 30:70 (v/v), isocratic elution. The flow rate was 1 mL/min and the injection volume was 4 µL.

All solvents used were filtered through 0.5 ml Sartorius filters and degassed with ultrasounds. MS/MS detection using multiple reaction monitoring (MRM) of specific daughter ions was used for each sterol.

The HPLC was coupled with an Agilent Ion Trap 1100 VL mass detector, equipped with an atmospheric pressure chemical ionization (APCI) interface, working in positive ion mode. Operating conditions were: gas – nitrogen, flow rate 7 L/min, ion source temperature 250^0C, nebuliser - nitrogen at 50 psi pressure, capillary voltage -4000 V.

All chromatographic data were processed using ChemStation (vA09.03) software and Data Analysis (v 5.3) from Agilent,USA.

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