HPLC-MS STUDY OF PHYTOESTROGENS FROM *GLYCYRRHIZA GLABRA*

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Abstract

In this paper we have analyzed the phytoestrogen content of *Glycyrrhiza glabra* roots harvested from four different locations in Syria. The presence of eight phytoestrogen compounds was simultaneously assessed through a LC-MS method, before and after hydrolysis. All tested extracts contain daidzein, daidzin, genistein, formononetin, ononin and coumestrol.

Rezumat

În această lucrare am analizat conținutul de fitoestrogeni din rădăcinile de *Glycyrrhiza glabra,* recoltate din patru locații diferite din Siria. Prezența a opt compuși fitoestrogenici a fost simultan evidențiată printr-o metodă LC-MS, înainte și după hidroliză. Toate extractele testate conțin: daidzeină, daidzină, genisteină, formononetină, ononină și cumestrol.

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

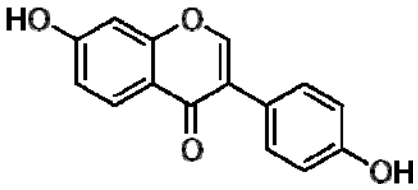
Introduction

Phytoestrogens are naturally occurring substances found in food and are defined as plant compounds that are functionally similar to estradiol. They contain different classes, like isoflavones, coumestans and lignans. The plant lignans and isoflavones glycosides are converted by intestinal bacteria to compounds with estrogenic activity and compared to estradiol are very weak estrogens. One of the many examples of these herbs is licorice root (*Glycyrrhiza glabra). Glycyrrhiza glabra,* as herbal medicine it has an impressive list of well documented uses and is probably one of the most over-looked of all herbal wonders. Hundreds of potentially healing substances have been identified in Liquorice as well, including flavonoids and various plant estrogens (phytoestrogens). Liquorice is useful for many

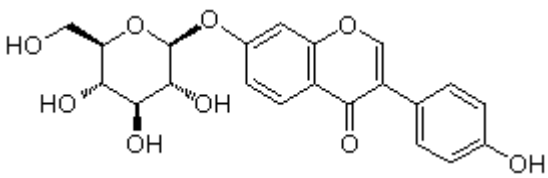
ailments including asthma, athlete's foot, baldness, body odor, bursitis, canker sores, chronic fatigue, depression, colds and flu, coughs, dandruff, emphysema, gingivitis and tooth decay, gout, heartburn, HIV, viral infections, fungal infections, ulcers, liver problems, Lyme disease, menopause, psoriasis, shingles, sore throat, tendinitis, tuberculosis, ulcers, yeast infections, prostate enlargement and arthritis.

The herb's key therapeutic compound, glycyrrhizin (which is 50 times sweeter than sugar) exerts numerous beneficial effects on the body, making Liquorice a valuable herb for treating a lot of ailments. It seems to prevent the breakdown of adrenal hormones such as cortisol (the body's primary stress-fighting adrenal hormone), making these hormones more available to the body [3].

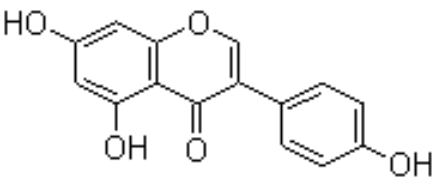
In this study we developed a HPLC-MS method for the analysis of phytoestrogens from roots of *Glycyrrhiza glabra* from Syria. The method can be applied for quantitative analysis of eight compounds with phytoestrogenic activity (seven isoflavones and one coumestan) presented bellow:



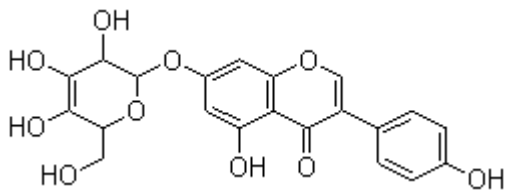
Daidzein (4',7-dihydroxyisoflavone)



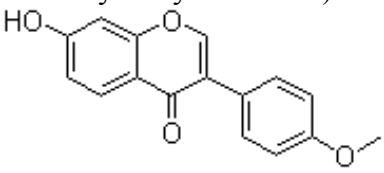
Daidzin (daidzein-7-glucoside)



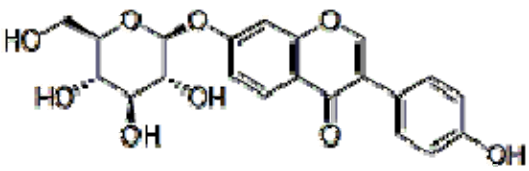
Genistein (4',5,7-trihydroxyisoflavone)



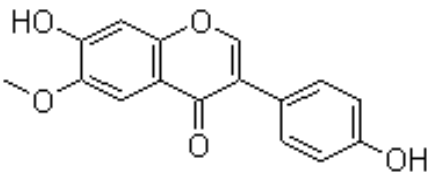
Genistin (genistein-7-glucoside)



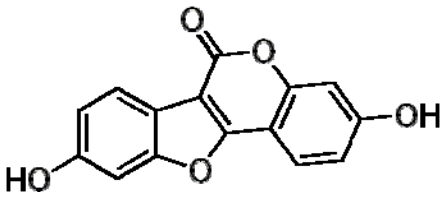
Formononetin(7-hydroxy-4'-methoxyisoflavone)



Ononin (formononetin-7-glucoside)



Glycitein(4',7-dihydroxy-6methoxyisoflavone)



Coumestrol

Materials and methods

Chemicals: Methanol of HPLC analytical-grade, acetic acid and hydrochloric acid of analytical-grade were purchased from Merck (Germany). Standards: daidzin, genistin, ononin, daidzein, genistein, formononetin, glycitein and coumestrol were purchased from Sigma (Germany).

Plant material: The root parts of *Glycyrrhiza glabra* were collected from Syria in four different demurrage (Bilekh, Rakka, Alpo-Azaz, Rass-Aenn) and air-dried at room temperature.

Sample preparation: Samples of 5 g pulverized roots material were extracted by refluxing with 25 ml methanol for 1 hour. In order to study the aglycons that can be obtained by hydrolysis, a portion of each extract was treated with an equal quantity of 6N hydrochloric acid and heated 40 minute at 80 ^{o}C on a water bath. The resulting samples were properly diluted. We noted the samples: G1 (Bilekh), G2 (Rakka), G3 (Alpo-Azaz), G4 (Rass-Aenn).

Apparatus: The experiment was carried out using an Agilent 1100 HPLC Series system equipped with a degasser, binary pump, autosampler, column thermostat, UV detector. The HPLC was coupled with an Agilent Ion Trap 1100 VL mass detector.

Chromatographic conditions: For the separation we used a reversed-phased Zorbax SB-C18 analytical column (100 x 3.0 mm i.d., 5 μm). The column was operated at 50^{o}C. The mobile phase was prepared from methanol:acetic acid 0.1% (v/v), gradient elution (started with 20% methanol 2', until 10' minutes 40%, 0.5" 40% methanol, 1' until 45% methanol and than 1" 45% methanol). The flow rate was 1 ml/min and the injection volume was 5 μl.

MS conditions: The MS was equipped with Turbo-Ionspray (electrospray ionization (ESI)) interface, negative ion mode. ESI settings were: negative ionisation, ion source temperature 360^{o}C, gas: nitrogen, flow rate 12 l/min, nebuliser: nitrogen at 65 psi pressure, capillary voltage 4500 V. The analysis mode was Single Ion Monitoring (SIM)(for aglycones) and Single Reaction Monitoring (SRM) (for glycosides) [1, 2, 4, 5, 6, 7].

Results and discussion

Generally, glycosides ions lose the sugar group thus we can observe the aglycone ion, so all glycosides can be analyzed by SRM mode. Ions of aglycones didn't fragmentize efficiently, so for these compounds we applied a SIM mode analysis.

Table I

Retention time and detection parameters (detection mode, m/z values)

No.	Compound	Retention time (min)	Detection mode	m/z parent ion $[M-H]^-$	m/z monitorized
1	Daidzin	3.9	SRM	415	253
2	Genistin	5.8	SRM	431	268, 269
3	Ononin	9.1	SRM	429	267
4	Daidzein	9.6	SIM	253	253
5	Glycitein	10.4	SIM	283	283
6	Genistein	11.3	SIM	269	269
7	Coumestrol	13.0	SIM	267	267
8	Formononetin	14.8	SIM	267	267

*SRM = single reaction monitoring; SIM = single ion monitoring

Calibration curves were done for the 40-2000 ng/ml range. The parameters are presented in Table II.

Table II

Parameters of calibration curves

No.	Compound	Calibration curves ecuation	R^2
1	Daidzin	y = 3084.8301 x + 26180.5118	0.9981
2	Genistin	y = 2474.7689 x + 1791.9997	0.9984
3	Ononin	y = 616.9387 x + 8605.0743	0.9983
4	Daidzein	y = 6201.2162 x + 11974.6534	0.9998
5	Glycitein	y = 6720.5903 x + 516379.3792	0.9992
6	Genistein	y = 17678.7220 x + 446287.5019	0.9994
7	Coumestrol	y = 19153.8831 x + 731190.3804	0.9992
8	Formononetin	y = 14018.3978 x + 433953.0814	0.9942

The contents of all eight standard compounds in our samples have been summarized in Table III.

Table III

Phytoestrogens content in methanolic extracts of *Glycyrrhiza glabra* (ng/ml)

Phytoestrogen	G1		G2		G3		G4	
	NH	H	NH	H	NH	H	NH	H
Daidzin	233.4	0.0	0.0	0.0	306.9	0.0	114.5	0.0
Genistin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ononin	23120.1	5036.7	507.3	5055.1	13928.8	6605.0	21335.3	4350.7
Daidzein	2681.4	2487.6	0.0	3165.5	2026.6	2590.4	382.6	634.9
Glycitein	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Genistein	285.1	433.6	0.0	193.9	180.3	330.8	69.0	288.3
Coumestrol	1016.1	705.4	0.0	252.6	432.9	320.0	575.5	214.9
Formononetin	7838.7	14505.3	64.2	12032.2	3919.3	14896.6	4789.8	14157.0

NH – non hydrolyzed sample; H – hydrolyzed sample

Daidzein, genistein, formononetin and coumestrol are present in all extracts as glycosides and free aglycones. The glycoside daidzin is found in all non-hydrolyzed extracts, except for G2 sample. None of the tested vegetal product contains genistin or glycitein. Ononin (formononetin-7-glucoside) is present in both hydrolyzed and non-hydrolyzed sample, sometimes in a greater quantity in the hydrolyzed extract. This abnormality can be explained by ineffective hydrolysis in the working conditions. Also, superior glycosides may be hydrolyzed to the monoglycoside and at the same time the later may be partially hydrolyzed. We have to take into account the fact that all compounds are polyphenols and can be degraded during hydrolysis. So, in the hydrolyzed samples the determination is only semi-quantitative.

Conclusions

A HPLC-MS method for the analysis of phytoestrogens was developed. We examined the contents (ng/ml) of daidzin, genistin, ononin, daidzein, glycitein, genistein, coumestrol, formononetin in the methanolic extracts of the four samples.

By comparing the results from the analyzed samples, it can be noticed that Bilekh, Alpo-Azaz and Rass-Aenn extracts are richer in phytoestrogen compounds. Ononin is the most abundant isoflavone glycoside and is found in all samples. Its aglycone, formononetin is present in high quantities in all extracts, except for Rakka sample which contains smaller quantities of the compound. Daidzin and the aglycones genistein and coumestrol are missing from the Rakka extract.

Qualitative and quantitative differences between the tested extracts may be explained by diverse pedo-climate conditions from the four harvesting areas of the vegetal product.

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