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VARIATION OF PHENOLS CONTENT IN WALNUT (*Juglans regia* L.)

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Abstract. *Walnut (Juglans regia L.) cultivars grown in Romania were investigated concerning phenolic compounds. Six phenolic compounds (ferulic acid, vanillic acid, coumaric acid, elagic acid, myricetin and juglone) were quantified in five cultivars. In order to identify and quantify phenols, the method used was HPLC-RP (high performance liquid chromatographic method in reverse phase). Juglone predominates in green husk, while myricetin in leaves. The technique described seems to be useful enough for analyzing phenolic compounds in walnut (leaves and green husk). Analysed polyphenols were present in all genotypes and probes, but differences occurred as regards quantity.*

Keywords*: Juglans regia, phenols, diversity, HPLC-RP*

INTRODUCTION

Walnut (*Juglans regia* L.) has important amounts of phenolic compounds (Verardo et al. 2009). Phenolic acids, such as caffeic, ferulic, and vanillic acids, have been involved repeatedly as natural antioxidants in fruits, vegetables, and other plants (Robards et al. 1999). The content of phenolic compounds in walnut was subject of numerous studies. It is considered that walnut pellicle is the most important source of walnut phenolics; the ratio between the contents in pellicle and kernel varied by at least 14.8-fold for caffeic acid and by up to 752.0-fold for *p*-coumaric acid (Colaric et al. 2005).

In leaves, the highest content of phenolics was found in May and July (Amaral et al.2004). Almeida et al. (2008) have considered that *Juglans regia* leaf extracts can be used as an easily accessible source of natural antioxidants.

As regarding green fruits, Colarič et al. (2005) ascertained that green unripe walnuts are rich in individual phenolic compounds. Regarding juglone,

this is a well-known component of walnut husks (Binder et al. 1989). The juglone concentration in fruit was significantly higher than the contents of other phenols in all cultivars, in studies by Solar et al. (2005). Mahoney et al. (2000) suggested that walnut husk naphthoquinones may be capable of suppressing *A. flavus* growth or aflatoxin biosynthesis providing that concentrations are sufficiently high.

The concentrations of phenols depend on time period (Amaral et al. 2004), time period and geographic origin (Cosmulescu et al. 2010 a,b), ontogenetic stage of the shoots (Solar et al. 2005, 2006), climatic conditions and farming practices (Amaral et al. 2008), cultivar choice and picking date (Jakopic et al. 2007), of some agricultural factors (Areias et al. 2000). Efficiency of phenolics extraction depends on type of solvent (Jakopič et al. 2009; Turkmen et al. 2006).

This paper aims at determining the polyphenol content in leaves and green husk, at the end of vegetation period (beginning of September), in five walnut cultivars (´Germisara´, ´Jupâneşti´, ´Franquette´, ´Vina´, ´Valcor´) of different origins, that are grown under the same climatic, experimental and farming practices.

MATERIALS AND METHODS

Extraction, identification and quantification of free polyphenols have been carried out in walnut leaves and green husk in five different cultivars that are grown under the same experimental area. Probes were taken out from experimental plantation in Râmnicu Vâlcea (Romania) research station (located at 45º07' northern latitude with meridian 24º22'21" eastern longitude), at the beginning of September, and they were preserved by freezing them at the temperature of -40 ^{0}C.

Probes, made of finely chopped vegetal produce, in amount of 500 mg were introduced in conical containers together with 20 mL methanol with 1% BHT(2,6-di-*tert*-butyl-4-methylphenol); they were kept at the temperature of 25 ^{0}C on ultrasound bath for 40 minutes. Extracts were separated by centrifugation at 1200 g. Supernatants were filtered through polyamide membrane with pores diameter of 0.22 μm and were stored at the temperature of -20 ^{0}C. Reverse phase high performance liquid chromatography (HPLC-RP) was used to identify and quantify free polyphenols HPLC-SURVEYOR Plus (Thermo Electron), configured with quaternary pump and degasser of incorporated vacuum SRVYR-LPMPP, thermostatic autosampler Peltier SRVYR-ASP, detector UV-VIS with diodes series and cell within flow of 5 cm, SRVYR-PDA5P, column Chromsep HPLC (250x4.6 mm, Hypersil 5 BDS) and software CHROMQUEST for control of the tool, diagnostic, data acquisition and processing.

The following external standards were used: ferulic acid (Fluka Chemie GmbH), vanillic acid (Sigma-Aldrich Chemie GmbH), coumaric acid (Sigma), elagic acid

(Fluka), myricetin (Sigma) and juglone (5-hydroxy-1,4-naphthoquinone; Aldrich). For mobile phase the following were used: acetonitril (Baker), acetic acid (Merck) and ultrapure water obtained with a SG-Water system. Etalon solutions were obtained by dissolving standards in methanol (Merck) and methanol:acetonitril 50:50 (v/v) for ellagic acid. Mobile phase was filtered through polyamide membrane of 0.2 μm and degassed with the help of ultrasound bath type DK 102p Bandelin. Before injecting, probes were filtered through nylon syringe filters CRS 0.45 μm.

Chromatographic work conditions were set based on method Schieber et al. (2001) in which minor changes were made. A gradient regime was applied, where solvent A is water with 5% (v/v) acetic acid, and solvent B is acetonitril:water 50:50(v/v) with 0.5% acetic acid. Probes and column were thermostated at 25^0C, the eluent flow was set at 1mL/min, while injection volume was 20 μL.

For statistical analysis the programme used was Microsoft Excel and XLSTAT 7.5.2 - Principal Component Analysis (PCA). All data were expressed as means ± standard deviations of triplicate measurements.

RESULTS AND DISCUSSIONS

As testing factor, for the seven probes the content in phenolic acids was studied (ferulic acid, vanillic acid, coumaric acid, elagic acid, myricetin and juglone) in green husk and mature leaves in five cultivars. For identification the scanning was done along with recording of spectrum in the range 230-450 nm; retention time was between 14.4768 min (vanillic acid in green husk) and 48.777 min (juglone in green husk) (Table 1). Differences between analysed probes, as regards retention time, are not significant.

Table 1. Retention Time (mean ± SD, in min)* of phenolic compounds.

No. of Peak	Phenolic compounds	Retention Time (mean ± SD)	
		for leaves	for green husk
1	Vanillic Acid	14.489 ± 0.078	14.476 ± 0.08
2	Syringic Acid	15.649 ± 0.069	15.690 ± 0.10
3	Coumaric Acid	21.825 ± 0.122	21.798 ± 0.04
4	Ferulic Acid	25.529 ± 0.318	25.320 ± 0.05
5	Myricetin	34.903 ± 0.202	34.623 ± 0.06
6	Juglone	48.735 ± 0.257	48.777 ± 0.12

SD = standard deviation;
*Values represents the average (standard deviation) of seven determinations for each compound.

Regarding phenols content in leaves, myricetin has delivered the most of the information (26.78 mg/100g); then followed by (in decreasing order of content) juglone, vanillic acid, coumaric acid, syringic acid, and finally fer-

ulic acid (Table 2). For green husk, juglone has delivered the most of information (31.307 mg/100g); then followed by (in decreasing order of content) vanillic acid, syringic acid, myricetin, ferulic acid, and finally coumaric acid (table 2). Phenolic profiles are enough specific for the five probes (Table 2; Figures 1, 2, 3, 4).

Table 2 Phenolic compositon of walnut (leaves and green husk)* (mg/100 g probe).

Compound / Genotype	Myricetin		Vanillic Acid		Syringic Acid	
	leaves	green husk	leaves	green husk	leaves	green husk
Germisara	25.475	1.642	1.966	1.239	2.342	0.975
Jupanesti	46.119	2.135	3.369	0.724	2.073	0.782
Franquette	16.052	0.068	1.504	2.519	1.379	1.541
Vina	17.066	0.043	1.611	1.355	1.550	1.356
Valcor	29.189	0.038	3.565	1.675	2.382	1.884
Mean	26.780	0.785	2.403	1.502	1.945	1.307
Standard Deviation	12.154	1.022	0.988	0.663	0.458	0.440
Minimum	16.052	0.038	1.504	0.724	1.379	0.782
Maximum	46.119	2.135	3.565	2.519	2.382	1.884
Confidence level (95.0%)	15.092	1.269	1.227	0.823	0.569	0.546

Compound / Genotype	Coumaric Acid		Ferulic Acid		Juglone	
	leaves	green husk	leaves	green husk	leaves	green husk
Germisara	0.271	0.129	0.972	0.277	22.824	27.912
Jupanesti	1.722	0.242	2.333	0.209	5.443	42.780
Franquette	0.620	0.416	0.367	0.304	15.208	40.347
Vina	0.483	0.167	0.629	0.351	12.552	20.569
Valcor	2.442	0.176	1.427	0.295	5.418	24.930
Mean	1.107	0.226	1.145	0.287	12.289	31.307
Standard Deviation	0.933	0.113	0.773	0.051	7.308	9.757
Minimum	0.271	0.129	0.367	0.209	5.418	20.569
Maximum	2.442	0.416	2.333	0.351	22.824	42.78
Confidence level (95.0%)	1.159	0.141	0.960	0.0647	9.074	12.115

SD = standard deviation;
*Values represent the average of three determinations for each compound.

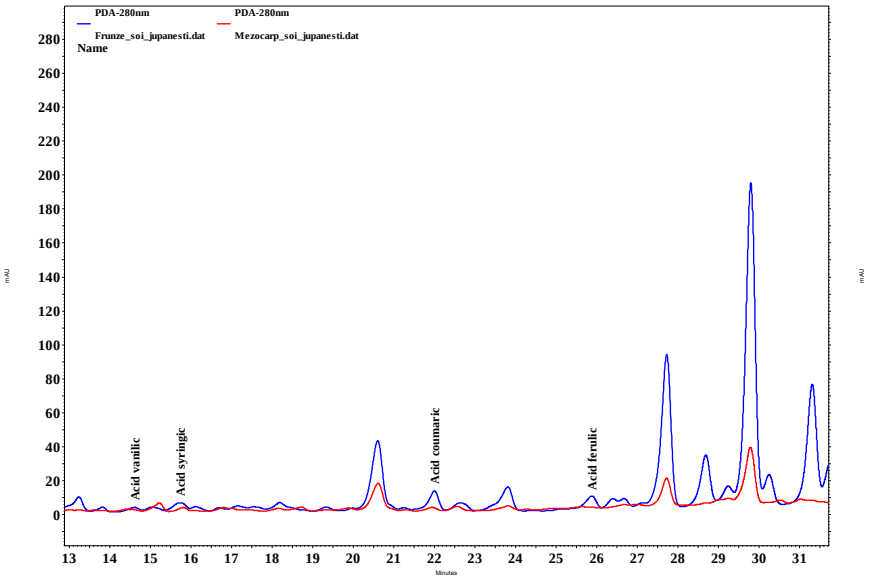

Figure1: HPLC-RP phenolic profile in walnut (red curve- green husk, blue curve - leaves), (cv. Jupanesti). Detection at 280 nm: vanillic acid, syringic acid, coumaric acid, ferulic acid.

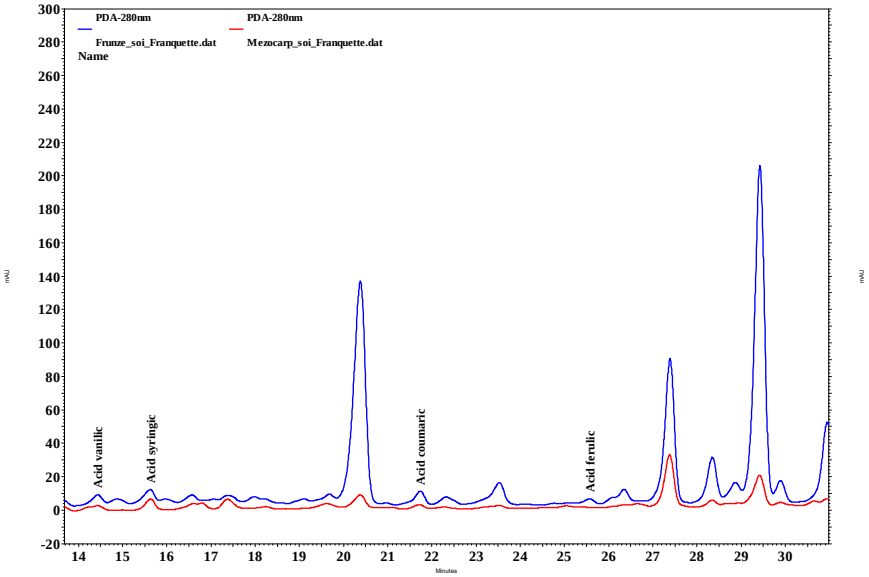

Figure 2: HPLC-RP phenolic profile in walnut (red curve- green husk, blue curve - leaves), (cv.Franquette). Detection at 280 nm: vanillic acid, syringic acid, coumaric acid, ferulic acid.

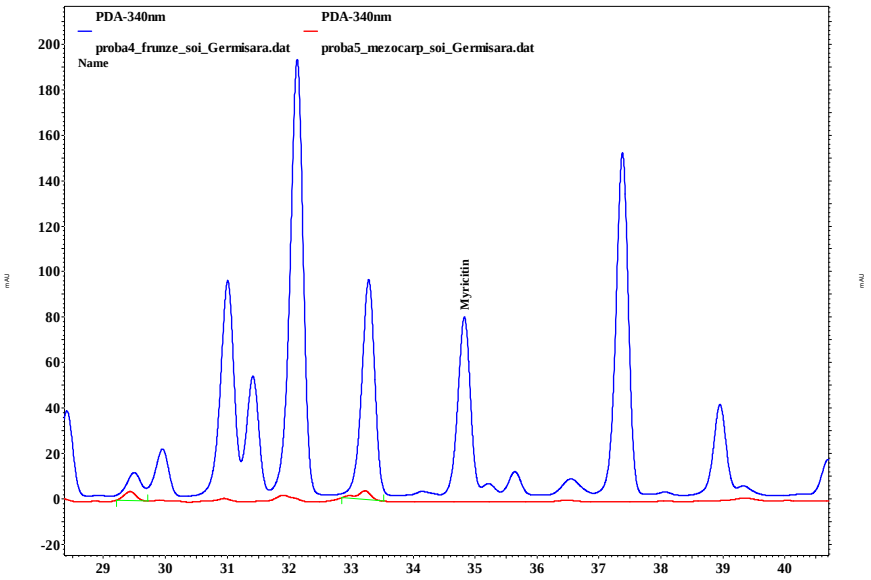

Figure 3: HPLC-RP phenolic profile in walnut (red curve- green husk, blue curve - leaves), (cv.Germisara). Detection at 340 nm: myricetin.

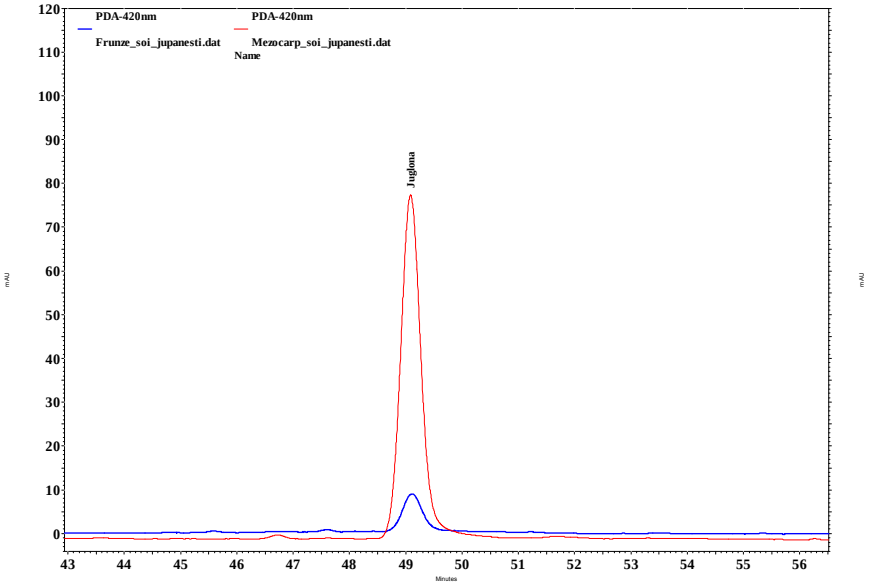

Figure 4: HPLC-RP phenolic profile in walnut (red curve- green husk, blue curve - leaves), (cv. Jupanesti). Detection at 420 nm: juglone.

A first group of phenolic compounds, with retention time between 14.476 and 25.529 min, includes the compounds: vanillic acid, syringic acid, coumaric acid and ferulic acid (Figure 1 and 2). Spectral data were accumulated at 280 nm. At 34.903 min for leaves and 34.623 min for green husk and 340 nm myricetin was identified (Figure 3). Juglone was identified at 420 nm and 48.735 min for leaves, and 48.777 min for green husk (Figure 4). In leaves, myricetin content was found in the highest concentration (16.052-46.119 mg/100g), followed by juglone (5.418-22.824 mg/100g).

Linear decrease in juglone potential in leaves of black walnut, over the growing season, has been reported by Coder (1983). On the other hand, in mature leaves, content of ferulic acid has varied between 0.341 – 2.333 mg/100 g, vanillic acid between 1.504 - 4.395 mg/100 g, syringic acid between 1.379-2.565 mg/100 g, coumaric acid between 0.271-3.210 mg/100g, being in the lowest concentration. In green husk, juglone content was found in highest concentration (20.569-42.780 mg/100g), followed by vanillic acid (0.724-2.519 mg/100g), syringic acid (0.782-1.884 mg/100 g), myricetin (0.038-2.135 mg/100 g), ferulic acid (0.209-0.351 mg/100 g) and coumaric acid (0.129-0.416 mg/100g) (Table 2). In accordance with literature (Solar et al. 2005; Binder et al. 1989), the content in juglone was higher in green husk of fruits. Analysis of PCA (Principal Component Analysis) for green husk has shown that the first two main compounds (juglone and vanillic acid) have represented 57.96% and 34.46%, respectively, of the variant's total (Table 3).

For walnut mature leaves, PCA analysis, has indicated that the main two components (myricetin and juglone) represented 90.814 % of total variation within the set of data that were analysed (74.47 % myricetin and respectively 16.34% juglone), (Table 4).

Table 3. Eigenvalues for four of main components in green husk.

	Juglone	Vanillic Acid	Syringic Acid	Myricetin
Eigenvalue	3.478	2.068	0.345	0.109
% variance	57.959	34.461	5.756	1.824
Cumulative %	57.959	92.420	98.176	100.00

Number of removed trivial eigenvalues: 2

Table 4. Eigenvalues for four of main components in leaves.

	Myricetin	Juglone	Vanillic Acid	Syringic Acid
Eigenvalue	4.468	0.981	0.523	0.028
% variance	74.472	16.343	8.711	0.475
Cumulative %	74.472	90.814	99.525	100.000

Number of removed trivial eigenvalues: 2

The difference represents the contribution of the other components to total variation within the set of data that were analysed.

In conclusion, this study is suggesting that the technique described seems to be useful enough for analyzing phenolic compounds in walnut (leaves and green husk); leaves and green husk in walnut plantations are a source of phenolic compound. Analysed polyphenols were present in all genotypes and probes, but differences occurred as regards quantity.

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