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Phenolics of Green Husk in Mature Walnut Fruits

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Abstract

Qualitative and quantitative determinations of phenolic compounds were carried out on green husk (mesocarp) of mature walnut fruits collected in September of five walnut (*Juglans regia* L.) cultivars ('Germisara', 'Jupanesti', 'Franquette', 'Vina', 'Valcor') grown in an uniform agroclimatic condition. Six compounds (ferulic acid, vanillic acid, coumaric acid, syringic acid, myricetin and juglone) were identified in all the cultivars by using reverse phase-high performance liquid chromatography (HPLC-RP).

Keywords: walnut fruit, phenolic compounds, HPLC-RP

Introduction

Walnut fruits are rich in phenolic compounds (Prasad, 2003). Thirty-seven compounds (including four new hydrolysable tannins and two new dicarboxylic acid derivatives were isolated from walnut extracts and their structures) were characterized (Fukuda *et al*., 2006; Stampar *et al.,* 2006) identified thirteen phenolic compounds in walnut husks: chlorogenic acid, caffeic acid, ferulic acid, sinapic acid, gallic acid, ellagic acid, protocatechuic acid, syringic acid, vanillic acid, catechin, epicatechin, myricetin and juglone. Determinations carried out (Colaric *et al*., 2005) on the content of phenolic acids (chlorogenic, caffeic, *p*-coumaric, ferulic, synapic, ellagic, and syringic acid) in kernel and thin skin of walnut in ten walnut cultivars, have shown that the thin skin of walnut is a main source of phenols in walnut.

The content of phenolic compounds depend on many environmental conditions, as well as genotype of different cultivars (Solar *et al*., 2006) and on the geographical location, on climatic conditions (Amaral *et al*., 2008). The concentrations of phenols depend on developmental stage of nuts (Solar *et al*., 2006). As regards correlation between content and time period, the highest content of phenolic compounds was found in May and July (Amaral *et al*., 2004). Higher content of juglone in fruit skin was revealed by Stampar *et al*. (2006) on the 21st of June (1404 mg/100 g).

Polyphenols are classified as antioxidant matter whose main action is preventing the formation of free radicals. It was demonstrated that walnut polyphenols are effective inhibitors of *in vitro* plasma and LDL oxidation (Koren *et al*., 2001). The kernel pellicles of walnut are rich in ellagitannins with antioxidative activity (Shimoda *et al.,* 2009). Phenolic compounds extracted from walnut skin into 95% ethanol contained the highest amount of total phenolics and exhibited the highest antioxidative capacity as evaluated by the trolox equivalent antioxidant capacity assay (Samaranayaka *et al*., 2008). All walnut leaf cultivars have high antioxidant activity (EC50 values lower than 1 mg/ml) (Pereira *et al*., 2007).

As far as the content of total phenols is concerned, Oliveira *et al*. (2008) considered that walnut green husks is a significant source in obtaining compounds with protective activity for health, having antimicrobial potential. The total phenols content was determined by colorimetric assay and their amount ranged from 32.61 mg/g of GAE ('Mellanaise') to 74.08 mg/g of GAE ('Franquette'). By analysing the content of phenolic compounds in cv. 'Chandler', Verardo *et al*. (2009) have shown that total phenol content was higher than in most foodstuffs. More than 96% of phenolic fraction belonged to the tannin class.

Setting out from the idea of using the walnut green husk resulted from mature fruit crop as secondary raw material, this paper aims at determining phenols content in mature fruits green husk in five walnut cultivars that are cultivated in Romania and harvested at the beginning of September. Previous researches showed that Romanian walnut cultivars proved to be important sources of nutritive elements, and walnut kernel consumption can contribute to a well balanced diet (Cosmulescu *et al*., 2009).

Materials and methods

Extraction, identification and quantifying of free polyphenols were carried out in mature walnut green husks in five different cultivars ('Germisara', 'Jupanesti', 'Franquette', 'Vina', 'Valcor'). Green husks of mature fruits were collected from the experimental plantation of Ramnicu Valcea research station (45°07' northern latitude with me-

ridian 24°22'21" eastern longitude), at the beginning of September and they were preserved by freezing at a temperature of -40°C.

Green husks of mature fruits were finely chopped and phenolics were extracted from 500 mg husks in an ultrasonic bath with 1% BHT (2,6-di-*tert*-butyl-4-methylphenol) in 20 ml methanol, at 25°C for 40 minutes. Extracts were centrifuged at 1200 x g and the supernatants were filtered through 0.22 µm polyamide membrane and were stored at -20°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.

Chemicals

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 the mobile phase the following were used: acetonitril (Baker), acetic acid (Merck) and ultrapure water obtained with a SG-Water system. Control 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 by the help of ultrasound bath type DK 102p Bandelin. Before injecting, probes were filtered through nylon syringe filters CRS 0.45 µm.

Work conditions

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, solvent B acetonitril: water 50:50(v/v) with 0.5% acetic acid. Probes and column were thermostated at 25°C, the eluent flow was set at 1ml/min, while the injection volume was 20 µl

Statistical analysis

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 discussion

Composition in phenols was studied in mature walnut fruits' green husk in five walnut cultivars. Methanol was used to extract phenols, setting out from the fact that Jakopič *et al.* (2009) considered that extraction efficiency of phenolic compounds in plant material highly depends on solvent and the amounts of total phenolics were higher when methanol was used for extraction compared to ethanol.

Tab. 1. Retention time (mean ± SD, in min)* of phenolic compounds and spectral characteristics

No. of Peak	Phenolic compound	Retention Time (mean ± SD)	Spectral characteristics λ (nm); average
1	Vanillic acid	14.476 ± 0.08	280
2	Syringic acid	15.690 ± 0.10	280
3	Coumaric Acid	21.798 ± 0.04	280
4	Ferulic Acid	25.320 ± 0.05	280
5	Myricetin	34.623 ± 0.06	340
6	Juglone	48.777 ± 0.12	420

SD = standard deviation; *values are expressed as mean (standard derivation) of five determinations for each sample

The method of High Performance Liquid Chromatography in Reverse Phase (HPLC-RP) was used in determinations and external standards, has enabled the identification of 6 phenolic compounds. The retention time of different compounds that are identified is presented in Tab. 1.

The first group of phenolic compounds, with retention time comprised between 14.476 min and 25.320 min, includes the following compounds: vanillic acid, syringic acid, acid coumaric and ferulic acid (Fig .1). The retention time for myricetin was 34.623 minutes, while for juglone it was 48.777 min. Spectral data for all peaks were accumulated during the interval 280 nm (vanillic acid, syringic acid, coumaric acid and ferulic acid) and 420 nm (juglone).

Tab. 2 presents phenolic composition of green husk in walnut mature fruits for the five cultivars analyzed. The amount of phenols has varied within quite wide ranges.

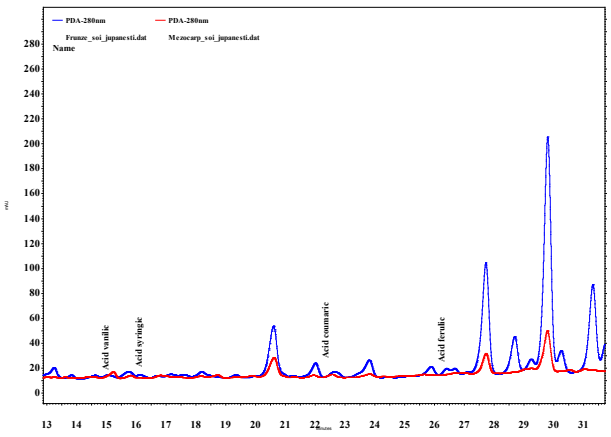

Fig. 1. HPLC-RP walnut green husk (red curve) and leaf (blue curve) phenolic profile (cv. 'Jupanesti'); detection at 280 nm: vanillic acid, syringic acid, coumaric acid, ferulic acid

Tab. 2. Phenolic composition (mean ± SD, mg/100 g probe)* of green husk in mature walnut fruits

Compound/ Genotype	Myricetin	Vanillic acid	Syringic acid	Coumaric acid	Ferulic acid	Juglone
'Germisara'	1.642±0.05	1.239±0.059	0.975±0.076	0.129±0.015	0.277±0.007	27.912±1.11
'Jupanesti'	2.135±0.12	0.724±0.073	0.782±0.068	0.242±0.006	0.209±0.006	42.780±0.95
'Franquette'	0.068±0.006	2.519±0.173	1.541±0.067	0.416±0.008	0.304±0.007	40.347±1.23
'Vina'	0.043±0.032	1.355±0.062	1.356±0.061	0.167±0.002	0.351±0.006	20.569±0.87
'Valcor'	0.038±0.026	1.675±0.073	1.884±0.073	0.176±0.04	0.295±0.11	24.930±0.92
Mean±SD	0.785± 0.914	1.502 ±0.593	1.308 ± 0.394	0.226±0.102	0.287± 0.046	31.308±8.72

Confidence level (95.0%); * values are expressed as mean (standard derivation) of three determinations for each sample

Among the identified phenols, juglone content was found in the highest concentrations (20.56-42.78 mg/100g) in all cultivars, followed by vanillic acid (0.72-2.52 mg/100g). According to data in the literature, juglone, a well-known component of walnut, is found in considerable amounts in all green and growing parts of trees and unripe hulls of nut (Prasad, 2003). Also, Stampar *et al.* (2006) has shown that within walnut green husk, juglone is the major phenolic compound.

In accordance with literature (Solar *et al*., 2006), the content in phenols varied from a cultivar to another. Differences between cultivars, in terms of juglone were 22.21 mg/100g. Cultivars 'Jupanesti' and 'Franquette' had concentrations close to juglone (two times higher than in cultivar 'Vina'); similar results were recorded also in cultivars 'Germisara' and 'Valcor'.

The content in vanillic acid has varied according to cultivar, with differences between cultivars of 1.79 mg (3.5 times higher in cultivar 'Franquette' compared to cultivar 'Jupanesti'; 0.72-2.52 mg/100g) and in ferulic acid the difference between cultivars was 0.142 mg/100 g (0.20-0.35 mg/100g). Variations of vanillic and ferulic acid depending on cultivar were also revealed by Solar *et al*. (2006) in the green husks of six commercial walnut cultivars, 3-4 weeks after blooming (vanillic acid range 3-48 mg 100 g^{-1} of DW, ferulic acid range 11-55 mg 100 g^{-1} of DW). According to data collected and the ones in literature, it was shown that the content of vanillic acid and ferulic acid in green husk is significantly decreasing during the maturation period of fruits.

As regards the sum of identified phenolic compounds, the highest value was obtained in cultivar 'Jupanesti', 46.87 mg/100 g. Similar results were obtained in cultivar 'Franquette' (45.19 mg/100g). The lowest total content among identified phenolic compounds was obtained in cultivar 'Vina' (23.84 mg), the differences between cultivars were double.

Analysis of PCA (Principal Component Analysis) 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 (Tab. 3).

The plot established according to the first two PCA axes (Fig. 2) suggests the existence of four groups of varieties. Group I, formed by the cv. 'Valcor' and 'Vina' with the lowest percentage of juglone (12.64% and 9.68% respectively) and lowest vanillic acid content (3.90% and 21.82% respectively). Group II, encompasses cv. 'Franquette' characterized by the highest percentage (59.36%) of vanillic acid and the lowest juglone content (13.57%). Group III, encompasses cv. 'Germisara' characterized by the lowest vanillic acid content (10.28%) and juglone (8.31%). Group IV encompass cv. 'Jupanesti' with higher percentages of juglone (55.78%) and lowest vanillic acid content (4.62%).

Tab. 3. Eigenvalues for four of main components

	F1	F2	F3	F4
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	100.000

Number of removed trivial eigenvalues: 2

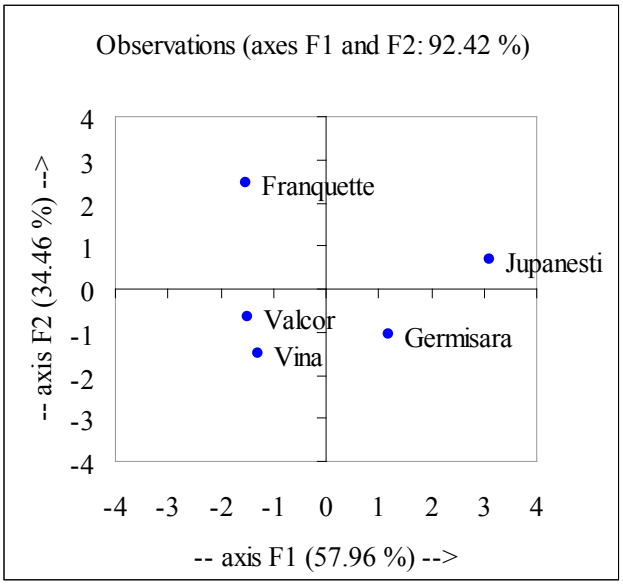

Fig. 2. Plot of the first two principal components (F1 and F2). Eigenvalues for each two principal component are listed in parentheses

Conclusions

In conclusion, the results proved that the cultivar has an influence on the content of phenolic compounds. In

making comparison with specialty literature (Solar *et al*., 2006; Amaral *et al*., 2004), the content of phenolic compounds within the green husk of fruits is significantly decreasing during maturation time. The green husk of walnuts is a by-product in walnut plantations, having in view its limited use. The study suggests that the green husk in walnut, as a by-product, can become the raw material for phenols extraction.

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