Cobiss Code 1.01

DOI: 10.2478/v10014-009-0002-4

Agrovoc descriptors: *Juglans regia*, walnuts, fruits, phenolic compounds, aromatic compounds, ethanol, methanol, alcohols, tannins, organic acids, catechin

Agris category code: F60

Extraction of phenolic compounds from green walnut fruits in different solvents

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Received September 11, 2006; accepted March 20, 2009
Prispelo 11. septembra 2006; sprejeto 20. marca 2009

ABSTRACT

The extractive efficiency of phenolic compounds from plant material is greatly depended on the solvent. In our research, methanol and ethanol were used for taking out the phenols from green walnut fruits. The total phenolics and some individual phenolics, such as gallic, chlorogenic, ellagic, sinapic and protocatechuic acid, (+)-catechin and juglone were detected. Total phenolic contents were determined spectrofotometric but individual phenols using HPLC. Amounts of total phenolics were higher when for extraction methanol was used compare to ethanol, in both cultivars, Elit and Franquette. Similar effect of the solvents was observed in the case some individual phenols, such as juglone, (+)-catechin, gallic, protocatechuic and chlorogenic acid. On the contrary, for ellagic and sinapic acid the extraction was better when ethanol was used compare to methanol.

Key words: *Juglans regia* L., ethanol, methanol, juglone, gallic acid, catechin

EKSTRAKCIJA FENOLNIH SNOVI IZ ZELENIH PLODOV OREHA Z RAZLIČNIMI TOPILI

IZVLEČEK

Učinkovitost ekstrakcije fenolnih snovi iz rastlinskega tkiva je v veliki meri odvisna od topila. V našem poskusu smo primerjali ekstrakcijo fenolov iz zelenih plodov orehov z etanolom in metanolom. Določali smo skupne in nekatere posamezne fenolne spojine, kot so galna, klorogenska, elagna, sinapinska in protokatehulna kislina, ter (+)-katehin in juglon. Skupne fenole smo določili spektrofotometrično, posamezne pa s pomočjo HPLC sistema. Vsebnost skupnih fenolov je bila pri obeh sortah, 'Elit' in 'Franquette', večja pri ekstrakcji v metanolu kot v etanolu. Podobne rezultate smo dobili pri nekaterih posameznih fenolih, kot so juglon, (+)-katehin, galna, protokatehulna in klorogenska kislina. Nasprotno je bila ekstrakcija pri elagni in sinapinski kislini bolj uspešna v etanolu kot v metanolu.

Ključne besede: *Juglans regia* L., etanol, metanol, juglon, galna kislina, katehin

1 INTRODUCTION

Walnut (*Juglans regia* L.) naturally occurs in Slovenian forests. For many years the trees are also grown in orchards because of the delicious fruits, which can be eaten raw and are excellent for dessert as well as in baking and confectionery (Prasad, 2003). Before the endocarp hardens, the green fruits including green husks can be pickled in vinegar or sliced and steeped into alcohol. The latter case of use is traditional in Slovenia as well as in Italy in preparation of a walnut liqueur (Štampar et al., 2006; Alamprese and Pompei, 2005).

Walnut fruits are rich in phenolic compounds (Prasad, 2003). Their contents depend on many environmental conditions, as well as genotype of different cultivars

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(Colarič et al., 2005; Solar et al., 2006). Furthermore, the presence of phenols changes during the season (Solar et al., 2006). Phenolics are involved in growth and reproduction and provide plants with resistance to pathogens and predators (Bravo, 1998).

For extraction of polyphenols from plant matter different solvent systems have been used (Chavan et al., 2001) and their efficiency varies. Extraction yield depends on the solvent and method of extraction (Goli et al., 2004). Commonly used solvents for extracting various substances from plant material are water, aqueous mixtures of ethanol, methanol and acetone (Sun and Ho, 2005).

We have analyzed the contents of the total and of some of the individual phenolic compounds in green walnut fruits, extracted with methanol or ethanol. Two cultivars, Elit and Franquette were involved in the research. In scientific researches, extraction of the phenolics is often performed in methanol, but when a walnut liqueur is made, green walnuts are steeped in ethanol. The aim of our study was to ascertain the influence of the solvents on the content of total as well as on some individual phenolic compounds.

2 MATERIAL AND METHODS

2.1 Plant material

Material for analyses was taken from two walnut cultivars, Elit and Franquette in an experimental orchard of the Biotechnical Faculty, located in Maribor (Slovenia). In the end of June, green walnut fruits were picked from three trees of each cultivar.

Two fresh fruits were sliced and ground to a fine powder and 100 mg of the sample was measured into a test tube. The sample was extracted with 5 ml methanol or ethanol in ultrasonic bath for 45 minutes. Then the samples were centrifuged for 7 minutes at 4200 rpm. The supernatant was filtered through polyamide filter Chromafil AO-45/25, transferred into vial prior analyses.

2.2 Total phenols content

Total phenolic content was assessed by using the Folin-Ciocalteau phenol reagent method (Singleton and Rossi, 1965). To 100 µl of the samples, 5 ml of bidestilated water and 500 µl of Folin-Ciocalteau reagent were added. After 30 sec to 8 min, 1.5 ml of sodium carbonate (20% w/v) was added. The extracts were stand for 30 min at 40°C. On the basis of measured absorbances at 765 nm, determination from calibration curve and considering dilutions, the total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per gram fresh walnut fruits.

2.3 Individual phenols content

The content of individual phenolics in extracts was analyzed on the Thermo Finningan Surveyor HPLC system equipped with photodiode array detector (PDA detector). A Chromsep HPLC column SS (250 x 4.6 mm, Hypersil 5 ODS) protected with a Chromsep guard column SS (10 x 3 mm) was used. The system was controlled using the ChromQuest^{TM} 4.0 Chromatography workstation software system. The chromatographic conditions (mobile phase, gradient program, temperature of column) were similar to those described by Schieber et al. (2001).

Chromatograms were observed at 280 nm. Identification of individual phenolic was qualitatively achieved using method of external standards and quantitatively comparing peak area on chromatograms of samples with those of diluted standard solutions.

2.4 Chemicals

For quantification of phenolics the following standards were used: juglone, gallic and protocatechuic acid from Merck, chlorogenic and ellagic acid from Sigma, (+)-catechin from Roth and sinapic acid from Fluka.

Following chemicals were used for the determination of total phenolics: Folic-Ciocalteu's phenol reagent and gallic acid from Sigma and sodium carbonate from Riedel-de Haën.

Acetonitrile, methanol and acetic acid were HPLC grade from Merck. The water used was bidistilled, purified with a Milli-Q water purification system (Millipore, Badford, USA).

2.5 Statistical analyses

Data were evaluated by one-way analysis of variance (ANOVA) using Statgraphics Plus 4.0 (Manugistics, Inc.; Rockville, USA) software. T-test was performed to determine significant differences at $P<0.05$.

3 RESULTS AND DISSCUSION

Phenolic compounds were extracted from green walnut fruits, cultivars 'Elit' and 'Franquette'. Total phenolic content as well contents of seven individual phenolics, such as gallic, chlorogenic, ellagic, sinapic and protocatechuic acid, (+)-catechin and juglone, were detected. As extraction solvent, methanol or ethanol were compared.

Colarič et al. (2005) ascertained that green unripe walnuts are rich in individual phenolic compounds. The total phenolics content of green walnut fruits extracted in two solvents are presented in Table 1 for cultivars Elit and Franquette. In the case of ethanol, the total phenolic content ranged from 126.2 mg GAE per g in cultivar Elit to 135.3 mg GAE per g in cultivar Franquette. In the case of methanol more phenolic compounds were extracted in both cultivars. There was significant difference between phenolic contents of the extracts using two mentioned solvents in both cultivars. Also other authors have established that the phenolics content extracts are strongly dependent on the type of the solvent as well as on the different concentrations of solvent (Turkmen et al., 2006; Yilmaz and Toledo, 2006). The extraction yield and extraction efficiency of caffeine and major catechins of green tea were higher with pure methanol comparing to pure ethanol (Perva-Uzunalić et al., 2006). Therefore, methanol proved to have a bit better characteristics as a solvent for polyphenols and anhtocyanins from black currant and grape byproducts than ethanol (Lapornik et al., 2005). On the other hand, Wang and Halliwell (2001) reported that aqueous ethanol was superior to aqueous methanol and acetone for extraction of the flavonoids from tea. In extracting phenolic compounds from peanut skin, ethanol and methanol were more effective than water, with ethanol being the most efficient extraction solvent (Yu et al., 2005). Meanwhile, the methanol was the solvent with best results for phenols from pine sawdust, while in almond hulls ethanol was the best extraction solvent (Pinelo et al., 2004). Jung et al. (2006) also compared the influence of different solvents and they found out that the ethanol extracts contained higher amounts of total phenolics and flavnoids than water and methanol extracts from wild ginseng leaves.

Table 1: The total phenols (mg GAE/g FW) in green walnut fruits, extracted with methanol or ethanol in cultivars Elit in Franquette. Average means and standard errors are presented. *P*-values in last column of the table mark statistically significant differences between the solvents.

	Methanol	Ethanol	*P*-value
Franqette	161.07 ± 7.28	135.27 ± 2.42	0.0041
Elit	148.98 ± 4.74	126.20 ± 5.71	0.0119

In spite of the fact, that higher amounts of total phenolics were extracted in methanol comparing to ethanol, this is not the case for all the individual phenols. The highest values of the analyzed phenols achieved juglone. Its extraction was more effective with methanol than with ethanol. Similar effects of the solvents were observed in the case of (+)-catechin, gallic, protocatechuic and chlorogenic acid (Fig. 1). Kallithara et al. (1995) also indicated that the methanol extraction is best for (+)-catechin, (-)-epicatechin and epigallocatechin, whereas the largest amount of gallic acid were yielded by 75 % of ethanol. In our research 96% ethanol was used, which has proved to be less effective solvent for gallic acid than methanol.

For other analyzed phenols, such as ellagic and sinapic acid, higher contents were gained with ethanol than with methanol. On the Fig.2 their contents are shown for cultivars Elit and Franquette. Also the individual anthocyanin extraction from grape and black currant marc depended on the use of solvent (Lapornik et at al., 2005). Rødtjer et al. (2006) reported that quantification of the total amount of phenolics in the extracts showed that 70% solvent-water mixtures extracted the phenolics more efficiently and contained more complex mixtures of phenolic compounds than the pure solvent extracts did.

Many authors established that the extraction yield of phenols is greatly depending on the solvent polarity (Turkmen et al., 2006; Lapornik et al., 2005). There are some studies, where the influences of the solvent concentration are being studied. Thus, Yilmaz and Toledo (2006) found out that aqueous solutions of ethanol, methanol or acetone were better than a single-compound solvent system for the extraction of the total phenols from Muscadine seed power.

We can conclude that the efficiency of the phenolics extraction depends on the type of the solvent as well on the phenol, which is being isolated. For total phenolics extraction from green walnut fruits methanol was more efficient compare to ethanol. Similar was for some individual phenols, such as juglone, (+)-catechin, gallic, protocatechuic and chlorogenic acid. On the contrary, for ellagic and sinapic acid extraction was better when using ethanol.

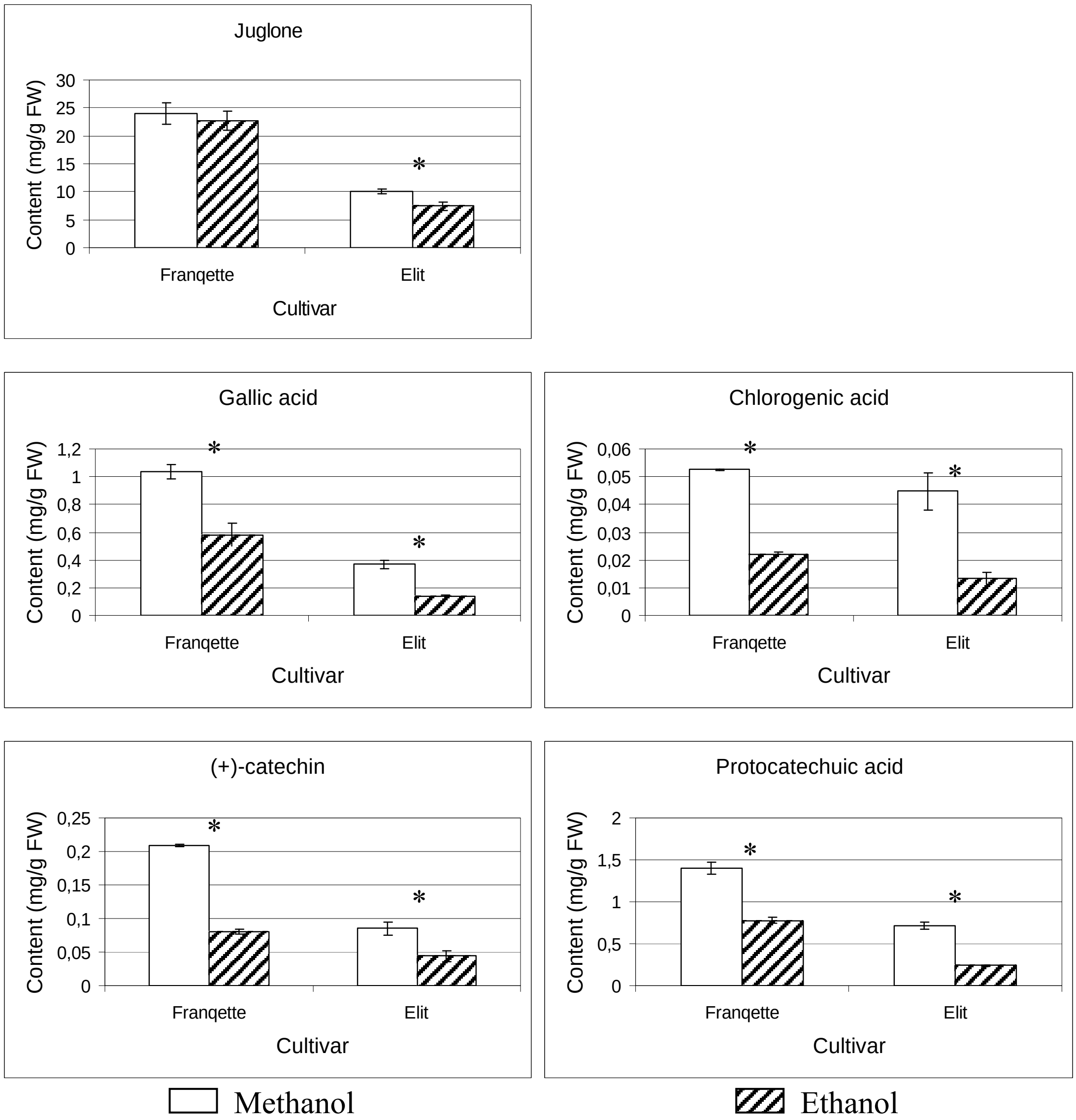

***Figure 1**: The individual phenols (mg/g FW) in green walnut fruits, extracted with methanol or ethanol in cultivars Elit in Franquette. Average means and standard errors are presented. Asterisk marks statistically significant differences between the solvents at P-value < 0.05.*

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