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Food Chemistry 95 (2006) 627–631
Food Chemistry
www.elsevier.com/locate/foodchem

Traditional walnut liqueur – cocktail of phenolics

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Received 1 November 2004; received in revised form 18 January 2005; accepted 18 January 2005

Abstract

The phenolic composition in walnut husks of the Slovenian cultivar Elit, which is a basic material for the traditional making of walnut liqueur, was investigated by HPLC with a PDA detector. Four different samplings of green husks were performed on different dates in order to compare the contents of phenolic compounds. The 2nd sampling coincided with the picking time of walnut fruits intended for the making of liqueur. Phenolic composition of walnut liqueur was investigated as well. Thirteen phenolic compounds were identified 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. In walnut liqueur, 1,4-naphthoquinone was also identified. The major phenolic in the husks was juglone with the highest content in the 2nd sampling; its content in walnut liqueur was low. The concentrations of individual phenolics in the liqueur were quite low compared with the contents in the green husks, due to the traditional way of making the liqueur.

Keywords: Green husks; *Juglans regia* L.; Liqueur; Phenolic compounds; Walnut

1. Introduction

Walnut is an old, traditionally important and widely spread species in Slovenia mainly grown in natural, artificial (plantation) and rural populations (Solar, Hudina, & Stampar, 2001; Solar, Ivancic, Stampar, & Hudina, 2002; Solar, Stampar, & Smole, 1997). Green walnuts, shells, kernels and seeds, even bark and leaves, have been used in the pharmaceutical and cosmetic industries. Walnuts are used in human nutrition; also the young green walnuts are much appreciated in traditional folk medicine for the making of an alcoholic wholesome drink – walnut liqueur, which is riched in phenolic compounds and vitamins. This liqueur is made out of walnuts with green husk and just before the hardening of the endocarp. These fruits are left to steep in food-grade ethanol (see also Alamprese, Pompei, & Scaramuzzi, 2005).

Walnut fruits and many walnut products, such as walnut liqueur, are rich in phenolic compounds (Prasad, 2003). Phenolic compounds play a number of crucial roles in the complex metabolism of plants and also of fruit trees. They are involved in physiological processes of fruit tree growth and development and affect different aspects of fruit pre- and post-harvest life (Usenik et al., 2004). Consumption of certain phenolics in the food is considered beneficial for human nutrition (Golding, McGlasson, Wyllie, & Leach, 2001). Epidemiological evidence shows that foods rich in phenolics derived from fruits is associated with lower risks of cancer and coronary heart disease, as well as cataracts, brain and immune dysfunction and stroke (Lattanzio, 2003). Phenolics also contribute to astringent but pleasant taste of the liqueur.

Halvorsen et al. (2002) reported that walnuts have one of the highest contents of antioxidants among all

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0308-8146/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2005.01.035

analysed nuts and seeds. Alamprese et al. (2005) studied the antioxidative potential of walnut liqueur and proved that antioxidant activity was directly correlated with the total phenol content and this characteristic did not change during storage, even for many years. The phenolic content was influenced by ripeness of fruits. Temperature and length of steeping of the fruits in ethanol have little effect on the phenolic composition of the liqueur (Alamprese & Pompei, 2005).

Juglone, an important phenolic compound of walnut is known for its antimicrobial effect and it was also reported to decrease the incidence of tumours of the small intestine of rats (Sugie et al., 1998). Kris-Etherton et al. (1999) argue that ellagic acid and flavonoids in walnuts have potential serum cholesterol-modulating effects, and one group of flavonoids has cardioprotective effects. Lee, Koo, and Min (2004) reported that catechin inhibits low-density lipoprotein (LDL) oxidation and protects lymphoid cells against cytotoxic effects of oxidized LDL.

The aim of our study was to investigate the phenolic content of the Slovenian traditional cultivar Elit, which is a basic material in the making of walnut liqueur. The analyses were performed on four different dates in order for us to compare the traditional picking date for making walnut liqueur with earlier and later ones. We also analysed the phenolic content of the liqueur.

2. Materials and methods

2.1. Preparation of samples

Walnut husks of the traditional cultivar Elit were collected in the experimental orchard in Maribor (Slovenia). Sampling dates were: May 30th (1st sampling), June 21st (2nd sampling), July 18th (3rd sampling) and August 19th (4th sampling) in the year 2003. Immediately after sampling, the husks were immersed in liquid nitrogen and stored at −20 °C. Before preparation, samples were lyophilised. The walnut fruits for liqueur production were gathered at the 2nd sampling. The liqueur was made according to the traditional method. The walnuts were cut into pieces and left to steep in 40% ethanol (1/3 volume was filled with walnuts and the other 2/3 was filled with ethanol) for 3 weeks. After that time, the mix was filtered and sucrose was added up to 30° Brix.

The husk of each sample was ground with liquid nitrogen to a fine powder and 50 mg were transferred into a test tube. The sample was extracted with 5 ml methanol containing 1% of 2,6-di-*tert*-butyl-4-methylphenol (BHT) in an ultrasonic bath for 45 min at ambient temperature. After extraction, the samples were centrifuged for 5 min at 4200 rpm. The supernatant was evaporated to dryness under reduced pressure at 40 °C, using a Büchi rotoevaporator system (R-114 with vacobox B-171). The residue was dissolved in 2 ml of methanol and filtered through 45 μm polyamide filter Chromafil® AO-45/25 (Macherey-Nagel) prior to injection into the HPLC system.

Walnut liqueur was diluted with methanol in a ratio of liqueur:methanol of 1:5 (v/v) and filtered through a 45-μm polyamide filter prior to injection into the HPLC.

2.2. HPLC analyses

The samples were analysed on the Thermo Finnigan Surveyor HPLC system with photodiode array detector (PDA detector). The system was controlled with the ChromQuest™ 4.0 Chromatography workstation software system. A Chromsep HPLC column SS (250 × 4.6 mm, Hypersil 5 ODS), coupled with a Chromsep guard column SS (10 × 3 mm) from Chrompack, was used. The chromatographic conditions were identical to the conditions previously described by Schieber, Keller, and Carle (2001). The column temperature was 25 °C. Solvent A was 2% acetic acid in aqueous solution and solvent B was 0.5% acetic acid in aqueous solution and acetonitrile (ratio 1:1). The flow rate was 1.0 ml/min and the volume of injected sample was 20 μl. For the analysis of phenolic compounds, the gradient used was as follows: 90% A to 45% A (50 min), 45% A to 0% A (10 min), 0% A to 90% A (5 min). The total run time was 65 min. Between two samples a run of 15 min of equilibration treatment was performed.

Phenolic compounds were detected at a wavelength of 280 nm. Also, the spectrum of compounds was recorded between 220 and 360 nm. The identification of compounds was achieved by comparing the retention times, spectra as well as by addition of standards. The concentrations of phenolic compounds were calculated according to the concentrations of a corresponding external standard.

Phenolic compounds investigated in the husks were chlorogenic acid, caffeic acid, ferulic acid, sinapic acid, gallic acid, ellagic acid, protocatechuic acid, syringic acid, vanillic acid, catechin, epicatechin, myricetin and juglone. In walnut liqueur, 1,4-naphthoquinone was also determined.

2.3. Chemicals

All the chemicals used were of HPLC or analytical grade. The following standards were used for determination of phenolic compounds: gallic acid, protocatechuic acid, syringic acid and 1,4-naphthoquinone from Merck; chlorogenic acid, sinapic acid, ellagic acid and myricetin from Sigma; vanillic acid, caffeic acid, ferulic acid and (−)-epicatechin from Fluka; (+)-catechin from Carl Roth; juglone from Aldrich.

BHT, used in the extraction solution, was obtained from Sigma and methanol was from Merck. Acetonitrile

of HPLC grade, used as elutant, was purchased from Merck. Water, used for sample preparation, solutions and analyses, was bi-distilled, and purified with a Milli-Q water purification system.

2.4. Statistical analyses

The significant difference of phenolic contents from walnut husks among different sampling dates were analysed with the programme Statgraphics plus 4.0 using one-way analysis of variance (ANOVA). The differences between the treatments were estimated using Duncans multiple range test at $P < 0.05$. Four replications were done for each data point.

3. Results and discussion

3.1. Phenolic composition of walnut husks

The phenolic compounds determined in the husks were chlorogenic acid, caffeic acid, ferulic acid, sinapic acid, gallic acid, ellagic acid, protocatechuic acid, syringic acid, vanillic acid, catechin, epicatechin, myricetin, and juglone (Table 1). Also, some other phenolic compounds were identified (1,4-naphthoquinone) but the data are not shown due to some difficulties with proper integration as a consequence of sub-optimal separation of these compounds. Most of those compounds were also reported in small amounts (ranging from 0.02 to 0.20 μg/g) in walnut kernels by Prasad (2003). Some of these compounds were also identified in other walnut organs, for instance in leaves (Amaral et al., 2004; Radix, Bastien, Jay-Allemand, Charlot, & Seigle-Murandi, 1998), shoots (Mehmet Sen, Karadeniz, Balta, Tanrisever, & Ekmel Tekintas, 1997) and hardwood (Bianco, Handaji, & Savolainen, 1998).

In our study, all determined compounds in the husks, with the exception of syringic acid, showed statistically significant differences in contents on different sampling dates. Many phenolic compounds had higher contents on the 1st and 2nd sampling date; shortly afterwards their concentrations decreased. The decrease in phenolics during the vegetative period was noticed also in other fruit species, for instance apple (Mayr, Treutter, Santos-Buelga, Bauer, & Feucht, 1995).

Most of the phenolic compounds in apple fruits are concentrated in fruit peel rather than fruit pulp, as reported by Veberic et al. (2005). We assume that, also in walnuts, the highest content of phenolics is in the green husks. Juglone was the major phenolic compound in the husks with the highest content all sampling dates. Because of the high juglone content, there was the highest content of total investigated phenolics on the 2nd sampling date. This finding is interesting because the 2nd sampling coincided with the traditional picking time of walnut fruits used in the making of walnut liqueur. When juglone was not considered, the highest value of most analysed phenolics was on the 1st sampling date. Compared with our results Radix et al. (1998) found an even higher juglone content in walnut husk tissues in cultivar Franquette (up to 2200 mg/100 g of dry weight) sampled on trees located in permeable soils in June and July. Moreover, the content of caffeic acid was also higher (720 mg/100 g of DW in 'Parisienne' fruits and was 900 mg/100 g of DW in 'Franquette' fruits) in comparison to the results with 'Elit' fruits. Also Jay-Allemand et al. (2001) and Girzu, Fraisse, Carnat, Carnat, and Lamaison (1998) reported a high juglone content in young walnut leaves (up to 100 mg and up to 498 mg/100 g of DW, respectively).

Table 1
Phenolic composition in walnut husks of cultivar Elit on different sampling dates

Phenolics	1st Sampling		2nd Sampling		3rd Sampling		4th Sampling	
Chlorogenic acid	15.2 ± 2.55	b	7.98 ± 2.82	a	7.56 ± 0.65	a	3.89 ± 2.14	a
Caffeic acid	1.87 ± 0.10	b	1.45 ± 0.12	ab	1.00 ± 0.13	a	1.81 ± 0.28	b
Ferulic acid	21.3 ± 3.69	b	1.91 ± 0.53	a	0.91 ± 0.21	a	0.99 ± 0.31	a
Sinapic acid	99.6 ± 22.3	b	5.14 ± 2.03	a	5.76 ± 1.71	a	1.92 ± 0.53	a
Gallic acid	122 ± 10.0	c	28.3 ± 4.65	b	13.8 ± 1.54	ab	9.16 ± 0.94	a
Ellagic acid	98.3 ± 5.56	b	7.31 ± 3.05	a	3.90 ± 0.84	a	5.48 ± 1.61	a
Protocatechuic acid	23.0 ± 4.78	c	11.3 ± 1.41	b	3.21 ± 0.52	a	2.92 ± 0.93	a
Syringic acid	15.3 ± 2.20		17.3 ± 2.29		13.1 ± 2.85		17.1 ± 2.20	
Vanillic acid	21.0 ± 2.45	b	2.59 ± 0.43	a	1.18 ± 0.26	a	3.36 ± 1.09	a
(+)-Catechin	47.5 ± 6.77	c	4.51 ± 0.49	a	20.4 ± 7.68	ab	27.4 ± 6.88	b
(−)-Epicatechin	23.9 ± 3.02	b	16.4 ± 2.29	ab	7.25 ± 0.89	a	18.8 ± 8.69	ab
Myricetin	25.0 ± 10.0	b	17.5 ± 4.38	ab	2.88 ± 0.38	a	4.49 ± 1.25	a
Juglone	288 ± 83.7	a	1404 ± 96.8	b	412 ± 42.2	a	218 ± 42.4	a
$\sum$ of phenolics	802 ± 102	b	1526 ± 111	c	498 ± 55.2	a	315 ± 51.9	a

The average content ± standard error, in mg/100 g of dry weight, is presented, and also the sum of investigated phenolics.
Values in a horizontal row followed by a different letter are significantly different at $P < 0.05$ by Duncans multiple range test.

3.2. Phenolic composition of walnut liqueur

Beside the phenolic compounds determined in the husks, 1,4-naphthoquinone in walnut liqueur was also identified (Fig. 1). Gallic acid was the major phenolic compound, and epicatechin, syringic acid and ellagic acid were also in higher quantities than the others. Despite the fact that juglone was the major phenolic compound in walnut husks at the 2nd sampling, its content was very low in the liqueur.

It was noticed that, with the classical extraction method, commonly used in the traditional making of the walnut liqueur, the content of phenolics was considerably lower. Despite this fact, its beneficial effects against stomach trouble and digestion inconveniences are well known. Recently, walnut extracts containing ellagic acid, gallic acid and flavonoids were reported to inhibit the oxidation of human plasma and low-density lipoproteins in vitro (Anderson et al., 2001). Juglone was also studied as a cancer therapy drug (Sugie et al., 1998). The phenolics in walnuts can positively influence human health because of their multiple health-beneficial components (Feldman, 2002). All the above-mentioned compounds were found in the walnut liqueur analysed in our study.

Alamprese et al. (2005) and Alamprese and Pompei (2005), in their studies, reported that the total content of phenolics in liqueur decreased with ripening of the walnut fruits. The liqueur that was made from more ripened fruits therefore showed a lower antioxidant power. Also, in our study, we noticed that the contents of the most investigated phenolics in walnut husks decreased on the 3rd and 4th sampling dates. Therefore, we can assume that the walnut liqueur made from walnuts of later sampling dates would also have a lower content of phenolics.

4. Conclusions

The study revealed the concentrations of 13 individual phenolics in walnut husks. In previous research, only total phenolic content or just a few individual phenolics had been determined. The phenolic composition in the walnut liqueur was investigated as well and similar compounds as in the husks were detected. On the 2nd sampling date the juglone content in the husks was the highest; the concentration of other phenolics was somewhat higher on the 1st sampling date. Picking time of fruits for the making of the liqueur coincided with the 2nd sampling date. We can conclude that the traditional way of making walnut liqueur from green fruits is appropriate. With the improved extraction method used in the making of traditional medicine – walnut liqueur, the content of phenolics could be increased and that may be a basis for future research. It would be of interest to collect walnuts for making liqueur on different sampling dates and perform additional research into

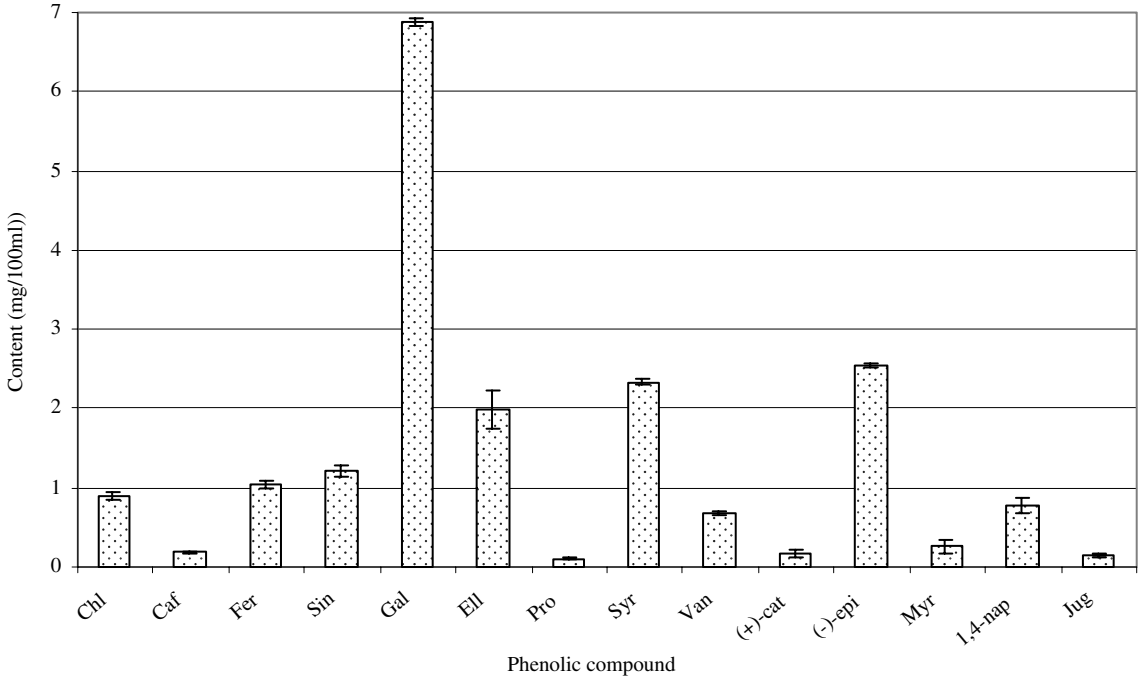

Fig. 1. Phenolic composition of walnut liqueur. The average content and standard error bars in mg/100 ml of liqueur are shown. Legend: Chl, chlorogenic acid; Caf, caffeic acid; Fer, ferulic acid; Sin, sinapic acid; Gal, gallic acid; Ell, ellagic acid; Pro, protocatechuic acid; Syr, syringic acid; Van, vanillic acid; (+)-cat, (+)-catechin; (−)-epi, (−)-epicatechin; Myr, myricetin; 1,4-nap, 1,4-naphthoquinone; Jug, juglone.

walnut liqueur – for instance, to investigate antioxidant capacity of phenolics and their role in human health.

Acknowledgement

This work is part of a programme Horticulture No P4-0013-0481, granted by the Slovenian Ministry of Higher Education, Science and Technology.

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