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How much do cultivar and preparation time influence on phenolics content in walnut liqueur?

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Abstract

The influence of cultivar and picking date on the phenolic content of walnut liqueur was investigated using HPLC with a PDA detector. Ten phenolic compounds, namely gallic, protocatechuic, ellagic, chlorogenic (5-caffeoylquinic), syringic, *p*-coumaric and sinapic acids, as well as (+)-catechin, 1,4-naphthoquinone and juglone were detected. The walnut liqueur under analysis was made of the cultivars 'Franquette' and 'Elit', on two sampling dates (June 30th and July 7th). A close interaction between cultivar and sampling date was noticed for most of the phenolics analyzed. The content levels of the main phenolic compounds under investigation were highest in 'Franquette' at the end of June and lowest in 'Elit' on the second or both sampling dates, except for syringic acid. A strong influence of cultivar choice and picking date was observed. The content levels of most phenolics were higher in liqueur prepared from the cultivar 'Franquette', than in 'Elit'.

Keywords: Walnut liqueur; Walnut; *Juglans regia* L.; Phenolic compounds; Cultivar; Green walnut fruits; Picking time

1. Introduction

Walnut is a traditionally important and widely spread deciduous tree in all parts of Slovenia (Solar, Ivancic, Stampar, & Hudina, 2002). Its ripe fruit have been used for fresh consumption and in confectionery, while unripe fruit serve to make liqueur. For many years, green unripe walnuts have been picked just before hardening of the endocarp, then sliced and steeped in alcohol; thus the delicious beverage was made. In Italy, a similar alcoholic drink from green walnuts, called *nocino*, is prepared (Alamprese & Pompei, 2005; Alamprese, Pompei, & Scaramuzzi, 2005).

Walnut liqueur, a dark brown, bitter and tasteful beverage, is often served as an aperitif or sometimes taken to treat stomach ache. The astringency and bitterness of foods and beverages depend on their contents of phenolic compounds (Bravo, 1998). Therefore, the bitter taste of walnut liqueur could also be due to the phenolic content, since Stampar, Solar, Hudina, Veberic, and Colaric (2006) reported that the liqueur is a cocktail of phenolics.

Several studies have been carried out to determine total phenol content. Nuts serve as a good source of phenolics with a high antioxidant potential, especially walnuts, pistachios, pecans, almonds with hulls, hazelnuts and peanuts (Kornsteiner, Wagner, & Elmadfa, 2006). Experimental investigations reported by Anderson et al. (2001) support this assumption. Kornsteiner et al. (2006) have analyzed total phenol and total tocopherol content in 10 types of nuts. They have found that both total phenol and total tocopherol content were highest in walnuts, pistachios and pecans. Likewise, Gunduc and El (2003) reported that walnut kernels had the highest total phenol concentration and the highest antioxidant ability among 25 types of commonly consumed foods.

Although phenolic compounds have no known nutritional function, they may be important for human health. It has been reported that phenolic compounds have

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antioxidant, antimutagenic, and free radical-scavenging properties (Bravo, 1998). The phenolic compounds (such as caffeic, ellagic and ferulic acid) also exhibit anticarcinogenic activity and inhibit atherosclerosis (Craig, 1999); (+)-catechin delays oxidation of human plasma and some phenolic compounds, such as *p*-coumaric acid, inhibit LDL oxidation (Lee, Koo, & Min, 2004).

The levels of phenolic compounds are influenced by various factors. They greatly depend on light, they are influenced by genetic factors, environmental conditions and storage, and they vary greatly, even between cultivars of the same species (Bravo, 1998). Colaric, Veberic, Solar, Hudina, and Stampar (2005) have determined the levels of nine phenolic compounds in ripe walnut fruit. They observed considerable differences in phenolic content among 10 walnut cultivars. The influence of cultivar was also confirmed in the phenolic content of apples (Veberic et al., 2005) and in the genus *Prunus* (Veberic & Stampar, 2005). In previous studies, the influence of maturation degree on the phenolic content was proved. Stampar et al. (2006) have studied certain phenolics in green walnut husks, Jay-Allemand et al. (2001) in the young leaves of the walnut and Solar, Colaric, Usenik, and Stampar (2006) in annual walnut shoots.

It has been mentioned that both cultivar and maturation stage of the walnut influence the phenolic content in various parts of the walnut. But how much do they influence the level of certain phenolic compounds in traditionally prepared walnut liqueur? The aim of our study was to answer this question.

2. Materials and methods

2.1. Preparation of samples

Green walnuts from the cultivars 'Franquette' and 'Elit' were picked in the experimental orchard in Maribor (Slovenia) on two sampling dates: June 30th (1st sampling) and July 7th (2nd sampling). Walnut liqueur was prepared according to the traditional method. Six hundred grams of fruits were cut into pieces, put into a glass jar, immersed in 1 l of 40% food-grade ethanol (see also Alamprese et al., 2005) and left to steep for three weeks.

After this time, the liqueur was filtered, diluted with methanol in the ratio liqueur: methanol 1:4 (v/v), and filtered though a 45 μm polyamide filter Chromafil® AO-45/25 (Machery-Nagel) prior to injection into the HPLC system. Four replications were made for each cultivar on each sampling date.

2.2. HPLC analyses

Certain phenolics were detected by the Thermo Finningan Surveyor HPLC system with a photodiode array PDA detector, scanning spectra of wavelength in the range 220–380 nm. Separations were carried out using a Chromsep HPLC Column SS (250 × 4.6 mm, Hypersil 5 ODS), coupled with a Chromsep guard column SS (10 × 3 mm) from Crompack. The system was controlled by the CromQuest™ 4.0 Chromatography workstation software system.

The chromatographic conditions followed the method described by Schieber, Keller, and Carle (2001). The injection volume of a sample was 20 μl, and the flow rate was 1.0 ml per min. The column temperature was 25 °C. Solvent A was 2% acetic acid in bidistilled water, and solvent B was 0.5% acetic acid in bidistilled water and acetonitrile (ratio 1:1, v/v). The gradient used began with 90% of solvent A and introduced a gradient to obtain 45% A at 50 min, 0% at 60 min and again 90% of solvent A at 65 min. The total run time was 65 min, with 15 min of equilibration treatment (90% A) performed between each analysis.

Phenolic compounds were detected at a wavelength of 280 nm. A specimen chromatogram is shown in Fig. 1. The identification of phenolics was achieved through the following: comparison of the retention times of standard solutions with the retention times of compounds in samples, absorption maxima of compounds in the scanned spectrum, and the addition of standards to samples.

The concentrations of certain phenolic compounds were calculated with the help of a corresponding external standard, based on the comparison of peak areas from the samples with those of the standard solution.

2.3. Chemicals

The ethanol used to prepare the liqueur was food-grade 96% (from Merck) diluted with bidistilled water.

The following standards were used to determinate the phenolic compounds: gallic, syringic and protocatechuic acids, 1,4-naphthoquinone and juglone from Merck; ellagic, chlorogenic (5-caffeoylquinic) and sinapic acids from Fluka; and (+)-catechin from Roth.

The chemicals for mobile phases were acetonitrile and methanol from Sigma–Aldrich, and acetic acid from Merck.

The water used in sample preparation, solutions and analyses was bidistilled and purified with a Milli-Q water purification system.

2.4. Statistical evaluation

The results were statistically analyzed with the programme Statgraphics Plus for Windows 4.0, using one-way analysis of variance (ANOVA). The differences in the phenolic contents were estimated with the *t*-test or Duncan's test. *P*-values of less than 0.05 were considered as statistically significant.

3. Results and discussion

In the liqueur prepared from green walnut fruit, the following phenols were detected: gallic, syringic, ellagic, protocatechuic, *p*-coumaric, chlorogenic and sinapic acids, as

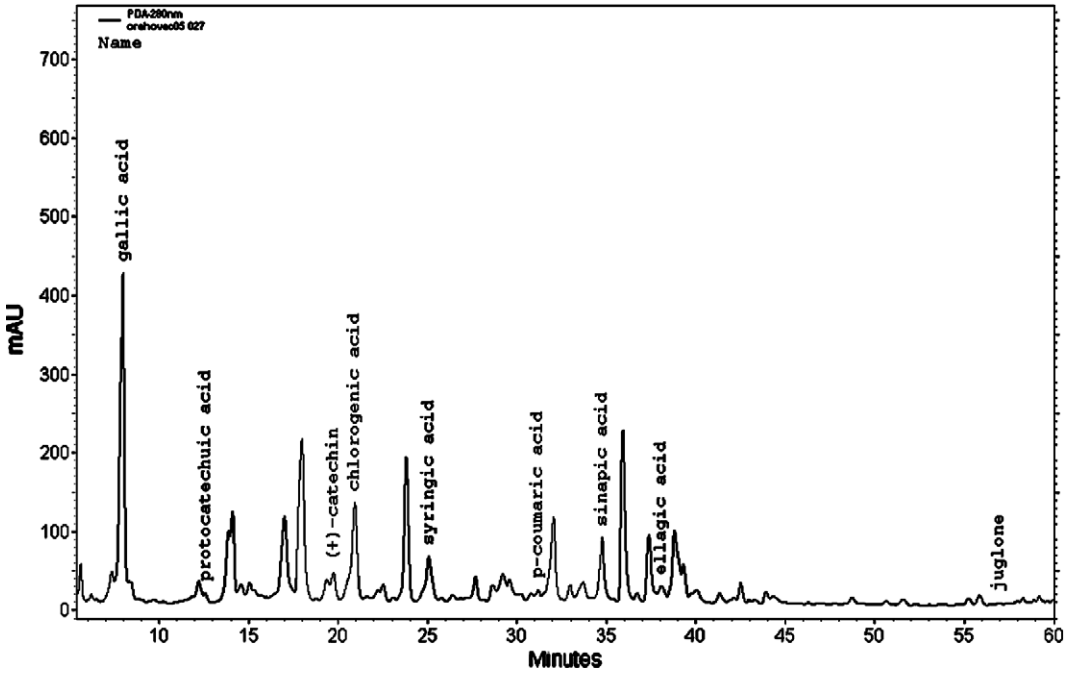

Fig. 1. HPLC chromatogram of walnut liqueur, made in 40% ethanol, from cultivar 'Franquette' at June 30.

well as (+)-catechin, 1,4-naphthoquinone and juglone. Most of these compounds are also present in other parts of the walnut tree. In the leaves, some phenolic acids and quercetins were identified (Amaral et al., 2004); in rejuvenated annual shoots, several flovonoids, phenolic acids and quinones (Solar et al., 2006) were found and, in ripe fruit, some phenolic acids, syringaldehyde and juglone (Colaric et al., 2005) were identified.

Stampar et al. (2006) have detected many phenolic compounds in green walnut husks and have also confirmed their presence in walnut liqueur. They noticed that the overall content level of identified phenolics in walnut husks was highest in June. We compared the quantities of phenolic compounds in walnut liqueur made from unripe walnuts picked at the end of June (1st sampling) and on July 7th (2nd sampling).

Among the detected phenolics in walnut liqueur, the major phenolic was gallic acid, the concentration of which was more than 10 times higher than those of the other phenolic compounds. The interaction between sampling dates and cultivars was statistically significant. Contents were highest in the cultivar 'Franquette' at the first sampling (21.7 mg per 100 ml), lower in the same cultivar at the second sampling and lowest in cultivar 'Elit' on both sampling dates (Table 1). Thus the gallic acid content was higher in the liqueur prepared at the end of June than in the liqueur made a week later (Fig. 2a). Similarly, Stampar et al. (2006) report that the gallic acid content of walnut husks was higher in June than in July and that, likewise, the major phenolic in walnut liqueur was gallic acid but the content was lower (less than 7 mg per 100 ml) than were our results. Differences in gallic acid content were detected, not only

Table 1
The content (mg per 100 ml) of 10 phenolic compounds in walnut liqueur made on June 30th (1st sampling) and on July 7th (2nd sampling) from cultivars 'Franquette' and 'Elit'

	1st sampling		2nd sampling		S	C	C × V
	'Franquette'	'Elit'	'Franquette'	'Elit'			
Gallic acid	21.7 ± 0.43c	11.9 ± 0.620a	15.8 ± 0.520b	11.2 ± 0.060a	*	*	*
Syringic acid	2.98 ± 0.04b	2.36 ± 0.12a	3.56 ± 0.04c	3.58 ± 0.11c	*	*	*
Ellagic acid	1.57 ± 0.33	0.79 ± 0.15	1.60 ± 0.07	0.92 ± 0.41		*	
Protocatechuic acid	0.74 ± 0.01c	0.41 ± 0.02a	0.57 ± 0.03b	0.36 ± 0.01a	*	*	*
p-Coumaric acid	0.33 ± 0.01	0.29 ± 0.01	0.24 ± 0.01	0.19 ± 0.01	*	*	
Sinapic acid	0.10 ± 0.01	0.12 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	*		
Chlorogenic acid	0.32 ± 0.00d	0.23 ± 0.08c	0.18 ± 0.01b	0.15 ± 0.02a	*	*	*
(+)-Catechin	2.07 ± 0.05c	1.54 ± 0.06b	1.42 ± 0.09b	1.18 ± 0.02a	*	*	*
Juglone	0.51 ± 0.02	0.51 ± 0.04	0.22 ± 0.05	0.29 ± 0.02	*		
1,4-Naphthoquinone	0.23 ± 0.03c	0.15 ± 0.01b	0.08 ± 0.01a	0.15 ± 0.01b	*		*

Average means and standard errors are presented. The asterisks (*) mark statistically significant differences between sampling dates (S), cultivars (C) and interaction (C × V) at $P = 0.05$. Values in a horizontal row followed by different letters indicate significant difference in the interaction (C × V) at $P < 0.05$ by Ducan's multiple range test.

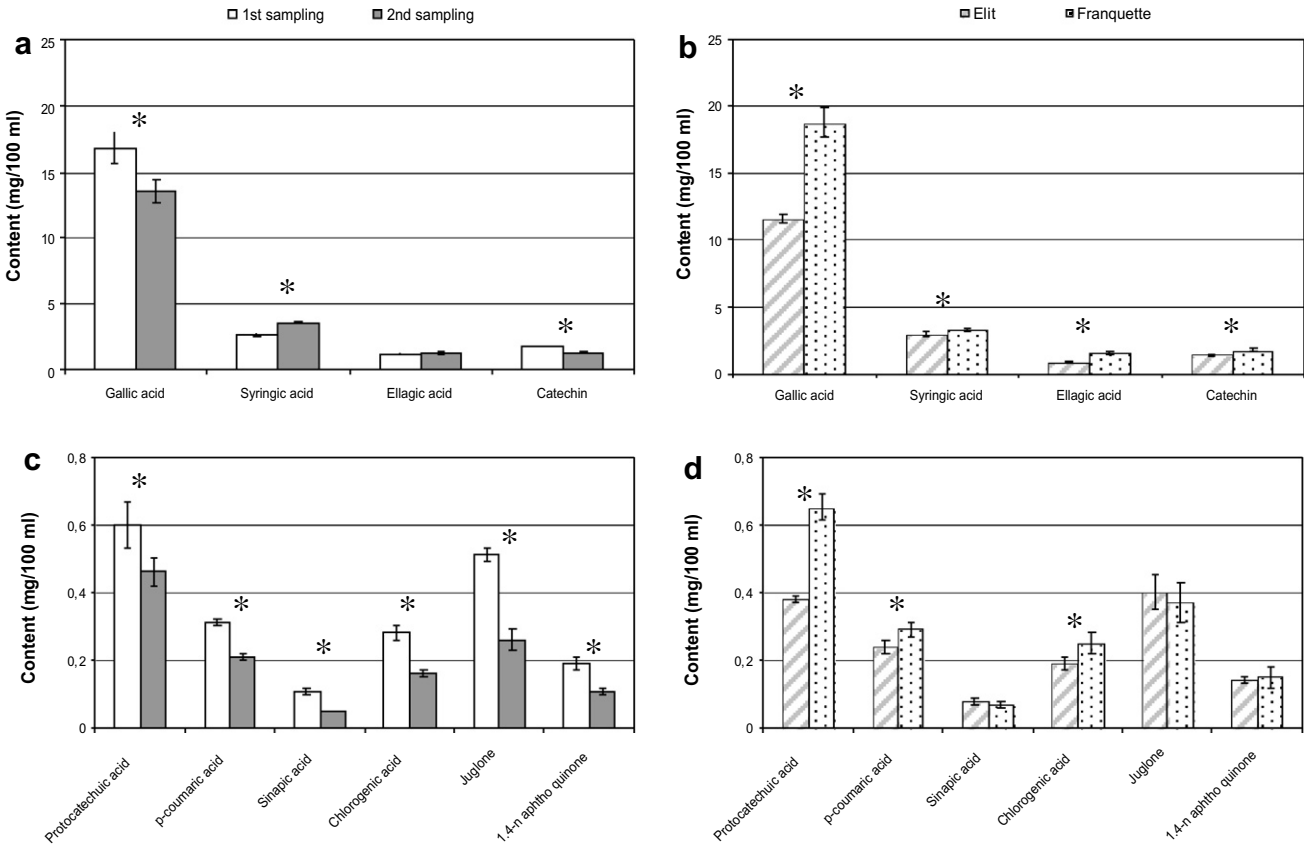

Fig. 2. Contents of certain phenolic compounds in walnut liqueur made from green walnuts on two different sampling dates (a, c) and from two cultivars, 'Elit' and 'Franquette' (b, d). The average values and standard error bars are presented. The asterisks indicate statistical differences at $P < 0.05$.

between sampling dates, but also between cultivars. The concentration in cultivar 'Franquette' was more than a third higher than that in 'Elit' (Fig. 2b).

The concentrations of other phenolic acids observed in walnut liqueur were not as high as gallic acid. Syringic and ellagic acids represented an important share of the phenolic compounds studied in walnut liqueur, although their values were rather low compared to those for gallic acid. The content of syringic acid was around 3 mg per 100 ml; ellagic acid was up to 1.60 mg per 100 ml, and the contents of other phenolic acids, such as protocatechuic, *p*-coumaric, sinapic and chlorogenic, were below 1 mg per 100 ml. The lowest concentrations were detected for sinapic acid (0.05 mg per 100 ml) (Table 1).

One widely distributed single group of phenolic compounds is the flavonoids (Bravo, 1998), which are commonly found in fruit and vegetables (Craig, 1999). In our research, (+)-catechin was detected among flavonoids. We found that walnut liqueur contained 1–2 mg per 100 ml (Table 1). Among flavonoids, Stampar et al. (2006) also reported levels of epicatechin and myricetin in walnut liqueur.

For walnuts, typical phenolic compounds are the quinones. In our study, we have confirmed the present of two quinones: 1,4-naphthoquinone and juglone (5-hydroxy-1,4-naphthoquinone). The content of 1,4-naphthoquinone was below 0.52 mg per 100 ml of walnut liqueur (Table 1). Binder, Benson, and Flath (1989) detected the presence of eight volatile types of 1,4-naphthoquinones in unripe fruit of English walnut (*Juglans regia* L.). Solar et al. (2006) reported that quinones (juglone and 1,4-naphthoquinone) represented the largest portion of eight investigated phenolics contained in annual shoots of the walnut.

Juglone, a well-known component of walnut, is present in considerable amounts in all green and growing parts of the trees and unripe hulls of the nuts, but the juglone level in the kernels was either very low or nil (Prasad, 2003). In our study, we also noticed that, in walnut liqueur prepared from green walnuts, the content of juglone was higher in June than on the later sampling date (Fig. 2c), but the amounts were very low (up to 0.51 mg per 100 ml). The investigation reported by Stampar et al. (2006) confirms our findings that in green walnut husks, the major phenolic compound is juglone, with the highest content (up to 1000 mg per 100 g dry weight) in June and that the juglone levels were much lower for the walnut liqueur than for the husks. We also found that juglone levels were almost the same in walnut liqueur made from the walnut cultivar 'Franquette' as in liqueur made from cultivar 'Elit' (Fig. 2d).

And how much did the cultivars and sampling dates influence the contents of phenolic compounds in walnut liqueur? The interacting influence of picking time and

cultivar was detectable in the levels of most phenolic compounds. That has been mentioned above for gallic acid, as well as for protocatechuic and chlorogenic acid, (+)-catechin and 1,4-naphthoquinone (Table 1). The highest concentrations of phenolics were detected on the first sampling date in the cultivar 'Franquette'. Similarly, the levels of most other phenolic compounds, such as ellagic, *p*-coumaric acid and juglone, were highest in the same treatment, although the interaction between cultivar and sampling date was not statistically significant. The lowest phenolic content was detected in the cultivar 'Elit', mostly on the second sampling date. Among the phenolic compounds under investigation, the only exception was syringic acid, where the values were not highest in the cultivar 'Franquette' on the first sampling date but on the later sampling date, in both cultivars, and they were lowest on the first sampling date in the cultivar 'Elit'.

The walnut liqueur made from the cultivar 'Franquette' showed higher levels of the major phenolic compounds than did the liqueur made from the cultivar 'Elit' (Fig. 2b and d). Only the contents of sinapic acid and juglone were slightly higher in the cultivar 'Elit', but the difference was not statistically significant. Colaric et al. (2005) observed cultivar variations in the phenolic content, and they noticed many differences in walnut kernels as well as in walnut pellicles. They determined that the cultivar 'Franquette' had higher concentrations of most of the nine identified phenolic compounds in walnut kernels and pellicle, except for chlorogenic acid in the pellicles, where the level was higher in the cultivar 'Elit' and caffeic acid, the level of which was similar in the walnut kernels as well as in the pellicles of both cultivars. The difference in phenolic content could also be a consequence of phenological properties. Although the buds of cultivars 'Elit' and 'Franquette' break at almost the same time, full blooming of the female flowers and harvest time come a week later in 'Franquette' (Table 2). The first sampling date was 48 days after full bloom (DAFB) for the cultivar 'Elit' and 40 days after full bloom for 'Franquette'; the second sampling date was 55 DAFB for the cultivar 'Elit' and 47 DAFB for the cultivar 'Franquette'. Thus the 'Franquette' was picked at an earlier maturation stage than the 'Elit'. But the DAFB on the first sampling date in the cultivar 'Elit' and on the second in the cultivar 'Franquette' were almost the same; nevertheless, a difference in phenolic content was detected.

Research by Alamprese and Pompei (2005) highlighted the fact that the relative ripeness of the walnuts is an important parameter of the production process for nocino liqueur. They found the total level of phenol in nocino liqueur made from green walnut fruit decreased over the course of walnut ripening. We prepared our walnut liqueurs from green fruit, picked at the end of June and from those picked a week later. The contents of all the investigated phenolics in the liqueur were higher on the first sampling date, except for the level of syringic acid, which was lower on the same sampling date (Fig. 2a and c). Similarly, the content of ellagic acid was slightly lower on the first sampling date, but the difference was not statistically significant. Stampar et al. (2006) observed variation of certain phenolic compounds in green walnut husks during the growing season, and they ascertained that the majority of the phenolics under investigation decreased during maturation.

Table 2
The average dates of bud breaking, full blooming of female flowers and fruit ripening for cultivars 'Elit' and 'Franquette' for the period 1991–2001

Cultivar	Bud break	Full bloom ♀	Harvest
'Elit'	30.4	13.5	23.9
'Franquette'	2.5	21.5	1.10

4. Conclusions

The results of our study have highlighted the fact that the levels of 10 particular phenolic compounds in walnut liqueur depend highly on the cultivar and the picking time of walnut fruit. An interaction between the above parameters was shown. Contents of most phenolics were highest in walnut liqueur made at the end of June from the walnut cultivar 'Franquette', and lowest in liqueur prepared from the cultivar 'Elit' a week later. Only in the case of syringic acid did the values increase during the ripening, but they were still higher in the cultivar 'Franquette' than in the 'Elit'.

On the basis of our results, we suggest use of the cultivar 'Franquette' for making walnut liqueur, since it is proved to be a better choice than 'Elit', and recommend that it is better to pick green walnut fruit at the end of June rather than a week later.

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