Total phenolics content and Phenolic profile of Walnut (*Juglans regia* L.) Leaves in different Cultivars Grown In Iran

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Walnut leaves constitute a good source of healthy compounds, namely phenolics, suggesting that it could be useful in the prevention of diseases in which free radicals are implicated. In this study walnut (*Juglans regia* L.) leaves from eleven different cultivars (Mayette, Fernor, Mellanaise, Elit, Orientis, Lara, Hartley, Franquette, Parisienne, Arco, Marbot) were studied for their phenolic compounds. The evolution of major phenolic compounds was monitored from May to September. Two extractive procedures were assayed and the best results were obtained using acidified water (pH 2) and a solid phase extraction column purification step. Qualitative analysis was performed by HPLC-DAD/MS and, in all samples; nine phenolic compounds were identified (3-caffeoylquinic, 3-p-coumaroylquinic and 4-p-coumaroylquinic acids, quercetin 3-galactoside, quercetin 3-arabinoside, quercetin 3-xyloside, quercetin 3-rhamnoside, quercetin 3-pentoside and kaempferol 3-pentoside). Quantification of phenolic compounds was performed by HPLC-DAD, which revealed that the highest content of phenolics was found in May and July the quercetin 3-galactoside was always the major compound while 4-p-coumaroylquinic acid was the minor one. In addition, Franquette cultivars total phenolic content was the highest among all cultivars.

Keywords: *Juglans regia* L., total phenolics content, Phenolic profile, Walnut leaf, HPLC-DAD, HPLC/DAD/ESI-MS

INTRODUCTION

The genus *Juglans* (family Juglandaceae) comprises several species and is widely distributed throughout the world. Green walnuts, shells, kernels and seeds, bark, and leaves are used in the pharmaceutical and cosmetic industries (Sharafati-Chaleshtori et al., 2011). Since ancient times, traditional medicinal plants have played important roles in public health, being a source of health care and disease prevention, especially in un-developed and developing countries. Thirteen phenolic compounds were identified in walnut hulls: chlorogenic acid, caffeic acid, ferulic acid, sinapic acid, gallic acid, ellagic acid, protocatechuic acid, syringic acid, vanillic acid, catechin,epicatechin, myricetin, and juglone (Stampar et al., 2006). Recently, considerable attention has been focused on dietary antioxidants that are able to scavenge reactive oxygen species (ROS), thereby offering protection against oxidative stress.

Walnuts are rich in components that have anti-oxidant and anti-inflammatory properties (Muthaiyah et al., 2011). Walnut (*Juglans regia* L.) leaf has been widely used in folk medicine for treatment of venous insufficiency, replace with haemorrhoidal symptomatology, and for its antidiarrheic, antihelmintic, depurative and astringent properties (Oliveira et al., 2008; Pereira et al., 2008; Bruneton, 1993; Van Hellemont, 1986; Wichtl and Anton, 1999). Keratolytic, antifungal, hypoglycaemic, hypotensive, anti-scrofulous and sedative activities have also been described (Gırzu et al., 1998; Valnet, 1992). Juglone (5-hydroxy-1, 4-naphthoquinone) is the

characteristic compound of *Juglans spp*., which is reported to occur in fresh walnut leaves (Bruneton, 1993; Gırzu et al., 1998; Wichtl and Anton, 1999). Nevertheless, because of polymerization phenomena, juglone is reported to occur in the drug (dry leaves) only in vestigial amounts (Wichtl and Anton, 1999), which means that the compound is not suitable for use in the quality control of the dry plant. Besides these, other phenolics, namely phenolic acids and flavonoids, have been reported in walnut leaves (Wichtl and Anton, 1999). However, due to their ubiquity in nature, these flavonoids do not guarantee the plant authenticity and, more identified compounds would be more useful for characterisation. In some European countries, dry walnut leaves are still largely used as an infusion. Because flavonoids and phenolic acids have already been successfully applied in the quality control of several foodstuffs (Andrade et al, 1997; Andrade et al, 1997; Areias et al, 2001; Ramos et al., 1999; Silva et al., 2000). In the present work, phenolics of walnut leaves have been studied by HPLC/ DAD/ESI MSMS. A useful methodology for routine quality control, based on HPLC-DAD quantification of major phenolics was developed and applied to eleven different cultivars growing under the same agricultural, geographical and climatic conditions. The evolution of phenolic compounds from May to September was monitored and total phenolics content for all cultivars were evaluated.

MATERIALS AND METHODS

Samples

Walnut leaves were collected from eleven cultivars (Mayette, Fernor, Mellanaise, Elit, Orientis, Lara, Hartley, Franquette, Parisienne, Arco, Marbot) grown in Iran. Fresh leaves were collected from ‘‘Saatloo’’, an orchard in Urmia, in the northewest of Iran (37°44 N, 45°10 Altitude 1338 m). The orchard has a planting density of 8×8 m, with all trees being more than eighteen years old. They are pruned when necessary and receive organic fertilization, but no phytosanitary treatments are applied. Fresh samples of all cultivars were collected on the same day, from May to September of 2010, at the end of each month. For each sample, about 100 g of leaves were manually collected from the middle third of branches exposed to sunlight, dried in a stove at 30 °C for five days and stored in paper bags in order to protect them from light. Just before phenolic extraction, each sample was powdered to a maximum particle size of 500 µm.

Chemicals

The standards were purchased from Sigma (St. Louis, MO, USA) and Extrasynthese (Genay, France). Methanol and hydrochloric and formic acids were obtained from Merck (Darmstadt, Germany). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Extraction of phenolic compounds

For analytical purposes, the sample (0.2 g) was thoroughly mixed with methanol until complete extraction of phenols. The extract was then filtered, evaporated to dryness under reduced pressure (40 °C), and redissolved in 3 ml of methanol. A chloroform extract was also prepared with the same sample: ca. 0.5 g of plant material was extracted three times with 100 ml of chloroform, with agitation, for 10 min. The extracts were pooled, taken to dryness under reduced pressure (40 °C) and the residue dissolved in 3 ml of methanol. For quantification purposes, each sample (ca. 0.2 g) was thoroughly mixed with acidified water (pH 2 with HCl) until complete extraction of phenolic compounds and filtered. The filtrate was passed through an ISOLUTE C18 (NEC) column (50-µm particle size, 60 A porosity; 10 g sorbent mass/70 ml reservoir volume), previously preconditioned with 60 ml of methanol, followed by 140 ml of water (pH 2 with HCl). The retained phenolic fraction was eluted with methanol (ca. 75 ml) and the methanolic extract obtained was filtered, evaporated to dryness under reduced pressure (40 °C) and redissolved in methanol (3 ml).

HPLC/DAD/MS/MS for qualitative analysis

Chromatographic separation was carried out on a reversed-phase LiChroCART column (250×4 mm, RP-18, 5 µm particle size; Merck, Darmstadt, Germany) using two solvents: trifluoroacetic acid (1%) (A) and methanol (B), starting with 30% methanol and installing a gradient to obtain 50%B at 30 min, 70%B at 32 min, 80%B at 33 min and 80%B at 35 min. The flow rate was 1 ml min^{-1}, and the injection volume was 5 µl. The HPLC system was equipped with a diode array detector (DAD) and mass detector in series (Agilent 1100 Series LC/MSD Trap). It consisted of an Agilent G1312A HPLC binary pump, an Agilent G1313A autosampler, an Agilent G1322A degasser and an Agilent G1315B photo-diode array detector controlled by Agilent software v. A.08.03 (Agilent Technologies, Waldbronn, Germany). Chromatograms were recorded at 280, 320 and 350 nm. The mass detector was an Agilent G2445A Ion-Trap Mass Spectrometer (Agilent Technologies, Waldbronn, Germany) equipped with an electrospray ionisation (ESI) system and controlled by Agilent Software v. 4.0.25. Nitrogen was used as nebulizing gas at a pressure of 65 psi and the flow was adjusted to 11 l min^{-1}. The heated capillary and voltage were maintained at 350 °C and 4 kV, respectively. The full scan mass spectra of the

phenolic compounds were measured from m/z 60 up to m/z 800. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, with a voltage ramping to 0.3 up to 2 V. Mass spectrometry data were acquired in the negative ionisation mode. MS^2 data were acquired in the automatic mode.

HPLC/DAD for quantitative analysis and evaluation of total phenolics:

Chromatographic separation was achieved with an analytical HPLC unit (Gilson), using a reversed-phase Spherisorb ODS2 (250×4.6 mm, 5 µm particle size, Merck, Darmstadt, Germany) column. The solvent system used was a gradient of water/formic acid (19:1) (A) and methanol (B), starting with 5% methanol and installing a gradient to obtain 15%B at 3 min, 20%B at 5 min, 25%B at 12 min, 30%B at 15 min, 40%B at 20 min, 45%B at 30 min, 50%B at 40 min, 70%B 45 min and 0%B at 46 min. The flow rate was 1 ml min^{-1}, and the injection volume was 20 µl. Detection was accomplished with a DAD (Gilson), and chromatograms were recorded at 320 and 350 nm. The retention times for the different identified compounds are shown in Table 1. Spectral data from all peaks were accumulated in the 200–400 nm range. Data were processed on Unipoint system software (Gilson Medical Electronics, Villiers le Bel, France). Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to external standards, with detection at 320 nm for phenolic acids and at 350 nm for flavonoids. 3-O-Caffeoylquinic acid was quantified as 5-O-caffeoylquinic acid, 3-p-coumaroylquinic and 4-p-coumaroylquinic acids were quantified as p-coumaric acid; the quercetin 3-pentoside derivative and quercetin 3-xyloside were quantified as quercetin 3-arabinoside. The other compounds were quantified as themselves and total phenolics were evaluated.

RESULTS AND DISCUSSION

As dried leaves were used, juglone was not detected in any extract, which is in accordance with Wichtl and Anton (1999). Gırzu et al. (1998) have reported the isolation of juglone from a chloroform extract of fresh walnut leaves. In the present work it was possible to detect this compound only in the chloroform extract from a fresh sample (data not shown). Bearing in mind that infusion is traditionally prepared with dry plant and that juglone is not detected in the water extract, a methodology based on phenolic compounds determination seemed to be useful for the quality control of walnut leaves. With the development of electrospray ionisation mass spectrometry (ESI/MS), it has become technically and economically feasible to analyse polar compounds by liquid chromatography coupled with ESI/MS. As several authors have successfully used HPLC/DAD/ESI MS in the identification of phenolic compounds in foodstuffs (Llorach et al., 2003; Zafrilla et al., 2001), this technique was applied to walnut leaf in order to identify the highest possible number of compounds.

The UV spectra of the compounds obtained by HPLC/DAD analysis revealed that phenolic acids and flavonoids were the two main groups of compounds in walnut leaf extract. The first group, corresponding to peaks A, B, C (Figure 1), presented spectral characteristics of cinnamic acids, with two absorption maxima at 250 and 320 nm. HPLC-MS data provided some interesting information about those compounds. Fragmentation of pseudomolecular ion $[M-H]^-$ at m/z 353.70, found for compound A, yielded the ion at m/z 191.47 ($[M-H]^-$-162), base peak corresponding to quinic acid by the loss of a caffeoyl radical from the pseudomolecular ion. Besides, in the MS^2 study, the ion at m/z 179.63 was also obtained with an abundance of 35% which, in accordance to Clifford et al (2003), characterizes 3- caffeoylquinic acid. A pseudomolecular ion $[M-H]^-$ at m/z 333.92 was found for compound B. Fragmentation of this ion yielded a base peak at m/z 163.2, corresponding to the loss of quinic acid radical, which is in accordance with literature data found for 3-p-coumaroylquinic acid (Clifford et al., 2003). Compound C also had a pseudomolecular ion at an identical m/z found for compound B; in the MS^2 study, the base peak was at m/z 173.2. According to Clifford et al. (2003) the compound was identified as 4-p-coumaroylquinic acid. The second group of compounds, corresponding to peaks D-I, showed UV spectra characteristic of flavonoids. Pseudomolecular ions $[M-H]^-$ at m=z 464.61, 434.39, 434.37 and 448.58 were found for peaks D, F, G and H, respectively (Figure 1). Fragmentation of these ions provided a characteristic m=z at 300.87, a typical mass in the negative mode of the quercetin aglycone. Injection of authentic standards of quercetin 3-galactoside, quercetin 3-arabinoside, quercetin 3-xyloside and quercetin 3- rhamnoside confirmed the occurrence of these compounds in walnut leaf extract. Compound E had a pseudomolecular ion $[M-H]^-$ at m/z 432.94 and fragmentation of this also provided a characteristic m/z at 300.9, suggesting the presence of a pentosyl quercetin derivative. In peak I, three compounds with pseudomolecular ions at m/z 417.4, 475.4 and 489.4 were co-eluting in the same order. An Extracted ion chromatogram (EIC), and MS^2 study were done for these ions. The MS of 417.4 yielded a main ion at m/z 284.9, characteristic of kaempferol, suggesting the presence of a pentosyl kaempferol derivative. MS data from the all cultivars showed a common qualitative pattern, presenting nine identified phenolic compounds (Figure 1): 3-caffeoylquinic, 3-p-coumaroylquinic and 4-p-coumaroylquinic acids, quercetin 3- galactoside, quercetin 3-arabinoside, quercetin 3-xyloside, quercetin 3-rhamnoside, quercetin 3-pentoside and kaempferol 3-pentoside. As far as we know, 3-p-coumaroylquinic and

Table 1. Phenolic composition (g/kg, dry basis) of walnut leaf samples.

Cultivar	Phenolic compound [b]									
	3Cqa (g/kg)	3pCo (g/kg)	4pCo (g/kg)	Q3Gal (g/kg)	Q3Pen (g/kg)	Q3Ara (g/kg)	Q3Xyl (g/kg)	Q3Ram (g/kg)	K3Pen (g/kg)	Total (g/kg)
May										
Mayette,	5.12 (0.04)	2.69 (0.01)	0.24 (0.01)	12.6 (0.06)	0.76 (0.01)	3.81 (0.07)	4.53 (0.05)	3.03 (0.09)	1.69 (0.08)	34.4
Fernor,	4.82 (0.19)	1.77 (0.00)	nq	12.06 (0.15)	1.27 (0.04)	3.41 (0.07)	4.80 (0.34)	3.02 (0.47)	1.97 (0.11)	33.1
Mellanaise,	6.82 (0.26)	1.75 (0.10)	0.42 (0.02)	10.8 (0.04)	0.80 (0.04)	3.47 (0.23)	5.27 (0.13)	3.68 (0.09)	1.29 (0.08)	34.3
Elit,	3.71 (0.19)	0.86 (0.01)	0.12 (0.01)	12.86 (0.34)	1.02 (0.06)	3.58 (0.05)	3.23 (0.10)	3.20 (0.06)	1.66 (0.08)	30.2
Orientis,	3.17 (0.37)	1.98 (0.14)	0.83 (0.05)	12.36 (0.70)	1.21 (0.02)	3.77 (0.24)	4.82 (0.50)	2.99 (0.03)	1.79 (0.10)	32.9
Lara,	5.82 (0.03)	1.51 (0.01)	nq	12.2 (0.43)	0.88 (0.07)	3.53 (0.23)	6.12 (0.09)	3.82 (0.01)	1.01 (0.08)	35.9
Hartley,	5.71 (0.30)	1.50 (0.09)	nq	8.74 (0.18)	0.63 (0.03)	2.56 (0.03)	3.69 (0.46)	2.70 (0.10)	1.82 (0.13)	27.4
Franquette,	6.30 (0.28)	1.31 (0.12)	nq	9.66 (0.03)	0.39 (0.07)	2.90 (0.25)	4.71 (0.31)	3.05 (0.08)	1.79 (0.08)	30.1
Parisienne,	3.85 (0.42)	1.84 (0.03)	nq	11.9 (0.22)	2.12 (0.06)	4.47 (0.11)	5.33 (0.36)	2.13 (0.14)	1.47 (0.09)	32.2
Arco,	2.48 (0.19)	1.41 (0.05)	nq	15.24 (0.88)	1.04 (0.11)	4.60 (0.39)	4.30 (0.28)	3.74 (0.27)	1.51 (0.15)	34.3
Marbot	5.16 (0.16)	2.19 (0.01)	nq	10.8 (0.26)	0.57 (0.01)	2.86 (0.01)	4.55 (0.06)	2.18 (0.17)	1.61	29.4
June										
Mayette,	2.81 (0.02)	0.77 (0.01)	0.33 (0.00)	6.81 (0.09)	0.55 (0.01)	2.26 (0.01)	2.16 (0.01)	1.44 (0.14)	0.31 (0.01)	17.5
Fernor,	1.45 (0.06)	0.97 (0.04)	0.38 (0.01)	4.07 (0.01)	0.07 (0.00)	1.75 (0.04)	0.43 (0.04)	2.23 (0.14)	0.04 (0.00)	11.4
Mellanaise,	2.29 (0.07)	0.58 (0.02)	nq	5.19 (0.01)	0.22 (0.00)	2.60 (0.01)	1.92 (0.01)	1.59 (0.02)	0.08 (0.01)	14.5
Elit,	1.59 (0.04)	0.27 (0.02)	0.17 (0.03)	2.85 (0.03)	0.44 (0.01)	0.41 (0.04)	0.46 (0.04)	1.65 (0.21)	nq	7.8
Orientis,	2.01 (0.06)	0.86 (0.08)	0.37 (0.01)	6.12 (0.01)	1.11 (0.01)	1.10 (0.08)	0.28 (0.02)	2.58 (0.15)	0.18 (0.01)	14.6
Lara,	1.78 (0.00)	0.73 (0.00)	nq	10.4 (0.03)	0.97 (0.01)	2.32 (0.03)	3.42 (0.01)	2.17 (0.01)	0.19 (0.01)	22.0
Hartley,	0.70 (0.03)	0.33 (0.03)	nq	2.30 (0.00)	nq	0.43 (0.01)	0.24 (0.03)	2.12 (0.02)	0.07 (0.00)	6.2
Franquette,	3.60 (0.07)	0.74 (0.01)	nq	11.3 (0.13)	0.93 (0.05)	3.46 (0.01)	3.20 (0.03)	2.74 (0.03)	0.08 (0.01)	26.7
Parisienne,	1.90 (0.00)	0.85 (0.00)	0.30 (0.01)	10.5 (0.04)	0.87 (0.01)	3.19 (0.01)	1.96 (0.00)	2.55 (0.02)	0.09 (0.00)	23.1
Arco,	0.74 (0.01)	0.53 (0.00)	0.32 (0.00)	4.11 (0.00)	0.22 (0.00)	0.62 (0.05)	0.51 (0.01)	1.36 (0.10)	0.37 (0.00)	8.8
Marbot	2.20 (0.05)	0.81 (0.02)	0.25 (0.01)	8.55 (0.01)	0.74 (0.01)	2.16 (0.00)	1.86 (0.02)	2.42 (0.01)	0.12 (0.01)	19.1
July										
Mayette,	4.92 (0.51)	1.56 (0.20)	0.42 (0.07)	12.1 (0.76)	1.32 (0.09)	3.57 (0.30)	4.92 (0.18)	2.89 (0.05)	0.59 (0.06)	32.3
Fernor,	3.64 (0.09)	0.99 (0.12)	0.12 (0.00)	10.64 (0.22)	0.68 (0.02)	3.60 (0.09)	3.91 (0.15)	4.63 (0.01)	1.18 (0.06)	29.4
Mellanaise,	5.48 (0.05)	1.05 (0.01)	nq	8.55 (0.03)	0.63 (0.04)	2.76 (0.04)	3.90 (0.01)	2.76 (0.04)	0.41 (0.02)	25.6

Table 1 continue

Elit,	2.75 (0.08)	0.82 (0.07)	0.71 (0.00)	10.31 (0.45)	0.61 (0.02)	2.40 (0.13)	1.35 (0.10)	4.56 (0.07)	0.19 (0.00)	23.7
Orientis,	1.64 (0.11)	0.86 (0.08)	0.77 (0.11)	7.51 (0.69)	0.11 (0.01)	1.34 (0.12)	0.51 (0.03)	2.93 (0.29)	0.18 (0.01)	15.9
Lara,	4.13 (0.08)	0.85 (0.03)	0.32 (0.01)	12.3 (0.61)	1.13 (0.11)	3.67 (0.07)	3.74 (0.14)	3.30 (0.09)	1.01 (0.02)	30.5
Hartley,	1.68 (0.09)	0.45 (0.03)	0.25 (0.01)	5.68 (0.44)	0.21 (0.03)	1.44 (0.13)	0.42 (0.01)	2.65 (0.23)	0.03 (0.00)	12.9
Franquette,	3.99 (0.12)	0.95 (0.03)	nq	12.1 (0.04)	1.50 (0.01)	4.34 (0.01)	4.73 (0.25)	2.78 (0.16)	0.40 (0.02)	30.8
Parisienne,	2.59 (0.27)	1.12 (0.07)	nq	14.58 (0.41)	2.50 (0.04)	4.47 (0.07)	4.04 (0.12)	2.50 (0.15)	0.38 (0.03)	32.2
Arco,	2.17 (0.09)	0.93 (0.06)	0.44 (0.01)	11.22 (0.72)	0.92 (0.01)	2.73 (0.17)	0.50 (0.03)	2.86 (0.14)	0.14 (0.01)	21.9
Marbot	2.79 (0.00)	1.01 (0.00)	nq	9.85 (0.00)	0.85 (0.01)	2.85 (0.03)	4.02 (0.02)	2.92 (0.00)	0.88 (0.04)	25.2
August										
Mayette,	2.76 (0.06)	1.27 (0.05)	0.32 (0.00)	7.97 (0.16)	0.75 (0.03)	1.96 (0.05)	2.52 (0.05)	2.06 (0.04)	0.21 (0.03)	19.8
Fernor,	3.53 (0.17)	0.69 (0.04)	0.38 (0.01)	6.46 (0.31)	0.34 (0.05)	1.91 (0.17)	1.38 (0.06)	3.25 (0.18)	0.77 (0.01)	18.7
Mellanaise,	3.37 (0.33)	0.58 (0.04)	nq	6.18 (0.02)	0.28 (0.01)	1.71 (0.04)	2.20 (0.08)	2.31 (0.05)	0.19 (0.03)	17.1
Elit,	1.84 (0.05)	0.60 (0.02)	0.71 (0.01)	6.42 (0.19)	0.28 (0.01)	1.76 (0.08)	0.78 (0.03)	2.80 (0.02)	0.15 (0.00)	15.3
Orientis,	2.74 (0.04)	1.40 (0.03)	0.41 (0.01)	8.01 (0.13)	0.68 (0.05)	2.91 (0.06)	3.37 (0.19)	2.57 (0.18)	1.23 (0.02)	23.3
Lara,	2.45 (0.01)	0.88(0.05)	nq	10.72(0.00)	0.87 (0.01)	3.25 (0.00)	4.16 (0.01)	2.46 (0.02)	0.81 (0.03)	26.6
Hartley,	3.56 (0.17)	0.57 (0.03)	0.27 (0.02)	3.37 (0.26)	0.36 (0.01)	1.33 (0.06)	1.62 (0.02)	2.56 (0.06)	0.38 (0.03)	14.0
Franquette,	4.63 (0.02)	0.93 (0.01)	0.11 (0.01)	11.9 (0.05)	0.85 (0.02)	1.83 (0.03)	5.19 (0.15)	2.95 (0.15)	0.88 (0.01)	29.3
Parisienne,	2.90 (0.09)	1.21 (0.01)	0.24 (0.12)	9.86 (0.16)	0.87 (0.02)	3.63 (0.08)	4.63 (0.23)	2.17 (0.11)	1.00 (0.09)	26.5
Arco,	2.30 (0.12)	0.85 (0.08)	0.13 (0.01)	8.33 (0.23)	0.43 (0.03)	2.44 (0.01)	1.63 (0.15)	2.46 (0.02)	0.68 (0.04)	19.2
Marbot	2.55 (0.04)	0.90 (0.01)	0.41 (0.03)	10.55(0.21)	0.78 (0.03)	2.89 (0.08)	3.20 (0.04)	2.88 (0.08)	0.47 (0.03)	24.6
September										
Mayette,	2.43 (0.04)	0.92 (0.00)	0.85 (0.00)	8.97 (0.12)	0.72 (0.00)	2.57 (0.05)	3.97 (0.03)	2.69 (0.02)	0.40 (0.04)	23.4
Fernor,	1.36 (0.06)	0.63 (0.01)	0.21 (0.01)	6.18 (0.08)	0.30 (0.01)	1.55 (0.08)	0.85 (0.04)	2.90 (0.09)	0.50 (0.01)	14.5
Mellanaise,	2.57 (0.00)	0.88 (0.00)	0.30 (0.01)	6.76 (0.06)	0.53 (0.01)	2.00 (0.02)	2.42 (0.10)	2.04 (0.01)	0.38 (0.02)	17.9
Elit,	2.11 (0.09)	0.39 (0.03)	0.06 (0.00)	6.65 (0.58)	0.43 (0.03)	1.89 (0.22)	3.09 (0.30)	2.46 (0.08)	1.38 (0.09)	18.5
Orientis,	1.53 (0.09)	0.96 (0.07)	0.79 (0.01)	8.97 (0.49)	0.52 (0.03)	2.46 (0.19)	1.98 (0.19)	2.53 (0.02)	0.60 (0.03)	20.3
Lara,	1.39 (0.00)	0.38 (0.07)	0.12 (0.01)	6.54 (0.04)	0.35 (0.01)	2.08 (0.02)	1.61 (0.03)	1.38 (0.11)	0.81 (0.04)	13.7
Hartley,	1.65 (0.11)	0.41 (0.04)	0.23 (0.03)	6.28 (0.09)	0.41 (0.14)	1.96 (0.20)	0.85 (0.10)	2.86 (0.14)	0.18 (0.01)	14.8
Franquette,	3.19 (0.05)	1.36 (0.04)	0.35 (0.01)	8.43 (0.01)	0.65 (0.01)	2.55 (0.11)	2.40 (0.01)	1.89 (0.02)	1.02 (0.01)	20.9

Table 1 continue 1

Parisienne,	2.14 (0.11)	0.77 (0.00)	0.24 (0.03)	7.94 (0.32)	0.59 (0.01)	2.51 (0.07)	2.84 (0.05)	1.48 (0.05)	0.42 (0.04)	18.9
Arco,	1.22 (0.06)	0.89 (0.04)	0.17 (0.01)	7.34 (0.36)	0.47 (0.08)	2.17 (0.13)	1.99 (0.04)	2.22 (0.20)	0.70 (0.01)	17.2
Marbot	2.04 (0.01)	0.83 (0.01)	0.26 (0.04)	5.80 (0.00)	0.49 (0.01)	2.03 (0.08)	2.26 (0.02)	1.83 (0.01)	0.29 (0.02)	15.8

[a] Values are expressed as mean (standard deviation) of three determinations for each sample. nq: notquantified. [b] Identity of compounds as in Figure 1.

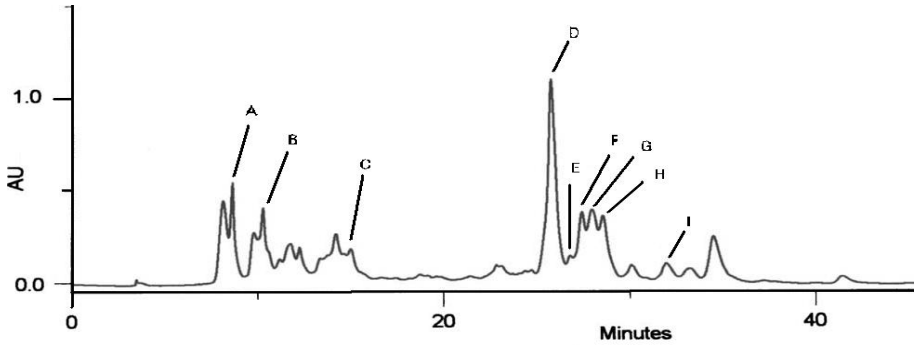

Figure 1. HPLC/DAD walnut leaf phenolic profile. Detection at 320 nm. A: 3-caffeoylquinic; B: 3-p-coumaroylquinic acid; C: 4-p-coumaroylquinic acid; D: quercetin 3-galactoside; E: quercetin 3-pentoside derivative; F: quercetin 3-arabinoside; G: quercetin 3-xyloside; H: quercetin 3-rhamnoside; I: kaempferol 3-pentoside.

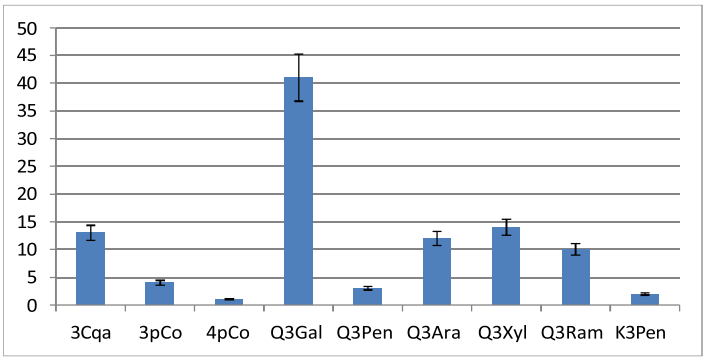

Figure 2. Phenolic fingerprint of Mayette, Fernor, Mellanaise, Elit, Orientis, Lara, Hartley, Franquette, Parisienne, Arco and Marbot cultivars. Results are the means of five samples collected for each cultivar. Identities of compounds are in Figure. 1.

4-p-coumaroylquinic acids are reported in this species for the first time. Wichtl and Anton (1999) described the existence of other phenolic acids in walnut leaves, namely caffeic, ferulic, p-coumaric, p-hydroxyphenylacetic, gallic, salicylic, chlorogenic and neochlorogenic acids but, with the exception of 3-caffeoylquinic acid, those compounds were not detected in the present study.

As no data concerning the cultivar and geographical origin of those samples were found, no correlation between the absence of those compounds and a given cultivar or origin can be inferred. Chloroform was the solvent that extracted a small number of phenolic compounds. Besides, when comparing methanolic extraction and extraction with acidified water and purification via the SPE columns, it was possible to observe that both led to the same qualitative phenolic profile but, as a general rule, the extraction with acidified water, leads to an extract with a higher amount of phenolic compounds. This last technique has the advantage of eliminating chlorophylls and allows the concentration of the extract in a shorter period of time. For quantification purposes all samples were subjected to this procedure. As with the qualitative profile, all the analysed samples showed a common quantitative pattern (Figure 2). In this profile, quercetin 3-galactoside was the

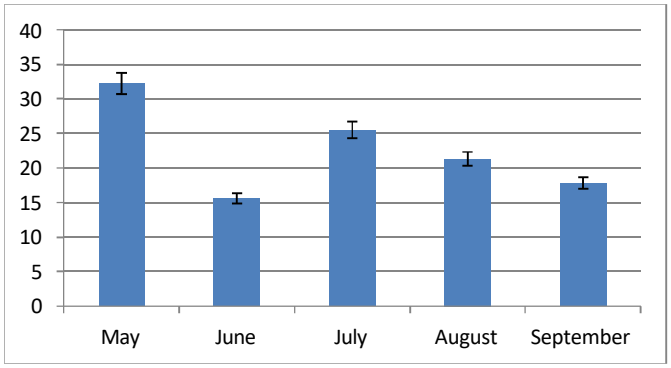

Figure 3. Total phenolics percentage, between May and September 2010, of the walnut leaf samples. Results are the means of the six analysed cultivars and standard error bars are on the top of each column.

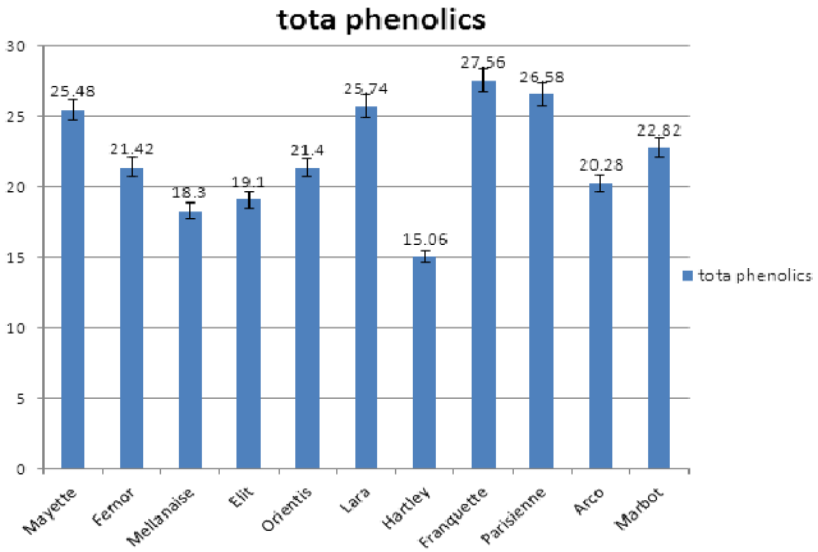

Figure 4. Total phenolics content of walnut leaves in eleven cultivars.

major flavonoidic compound (42±2.18%) and 3-caffeoylquinic acid was the major phenolic acid (14±0.73%).

A systematic analysis was carried out and samples were collected from May to September, in order to evaluate changes in the phenolic composition of the studied cultivars. All cultivars presented slightly higher values of total phenolic compounds in May and July (Table 1). Mean values of phenolic acids, flavonoids and total phenolic contents seem to point to a decrease of compounds from May to June, an increase in July and a new decrease until September. The first decrease might be related to the rapid development of the fruit in June, when most of the nutrients and photoassimilates are employed for fruit growth (Charlot and Germain, 1988). The hypothesis that flavonoid content is related to sun exposure, because of their function as sun filters, may possibly explain their rise in July, since this was the month with a higher value of solar radiation (Figure 3).

The average of total phenolic content in 11 walnut leaves phenolic extracts was 22.16 ± 0.66 (g/kg). The maximum total phenolics content in leaves extract was 27.56 ± 0.83 (g/kg) for Franquette and minimum total phenolics content was 15.06 ± 0.45 (g/kg) for Hartley cultivar (Figure 4).

CONCLUSION

This study suggests that the technique herein described seems to be quite useful for analysis of walnut leaf phenolic compounds. This set of compounds, when examined qualitatively and quantitatively, defines a fingerprint that may be suitable for assessing identity and quality. The nature of the cultivar and the month of collection do not seem to influence the mentioned phenolic fingerprint of walnut leaves. Keeping in mind that flavonoids and phenolic acids have been the subject

of several studies because of their antioxidant potential (Aquino et al., 2001; Sakakibara et al., 2003; Valentao, 2002), the results obtained suggest that, for this purpose, walnut leaves should preferentially be collected in May or July, when phenolic content is higher. Besides, the phenolics present, mainly caffeic acid and quercetin derivatives, are excellent antioxidants since they bear the required structural characteristics for that, namely an ortho-dihydroxy group on an aromatic ring.

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