

Aqueous Extracts of Walnut (*Juglans regia* L.) leaves: quantitative analyses of hydroxycinnamic and chlorogenic acids

Anggy Lusanna Gutiérrez Ortiz,^a Federico Berti,^a Luciano Navarini^{b,*} Paola Crisafulli^c, Silvia Colomban^c and Cristina Forzato^{a,1*}

^aDipartimento di Scienze Chimiche e Farmaceutiche, Università di Trieste, via L. Giorgieri 1, 34127 Trieste, Italy

^billycaffè S.p.A., via Flavia 143, 34100, Trieste, Italy

^cAromalab, illycaffè S.p.A., AREA Science Park, Padriciano 99, 34012 Trieste, Italy

Abstract

Identification of both hydroxycinnamic and chlorogenic acids present in aqueous extracts of walnut leaves (*Juglans regia* L.) were carried out by using, for the first time, standard compounds not commercially available for qualitative identification. In particular, in addition to caffeic, ferulic, *p*-coumaric and sinapic acids, *cis* and *trans* mono-caffeoylquinic, di-caffeoylquinic, mono-feruloylquinic and *cis* and *trans* mono-*p*-coumaroylquinic acid isomers were detected and quantified by UHPLC and the seasonal variations of these secondary metabolites were investigated.

Keywords: chlorogenic acids, walnut leaves, UHPLC, quantification, *Juglans regia*

Introduction

Polyphenols are very important compounds in human diet since many health benefits have been recognized with their intake such as antioxidant (1–3) and free radical scavenging properties (4), human chronic degenerative disease protection (5), cancer and cardiovascular disease protection (6) and others (7). It has been estimated that the dietary intake of phenolics is one gram per day, a

* Corresponding authors: e-mail address: cforzato@units.it (C. Forzato) and Luciano.Navarini@illy.com (L. Navarini)

higher amount with respect to other antioxidants present in our common diet (8). Among them, the chlorogenic acids family is widely distributed in the plant kingdom. Chlorogenic acids (CGAs) are esters of (-)-quinic acid at the OH groups in positions 3, 4 or 5 of the quinic ring with different hydroxycinnamic acids (caffeic acid, ferulic acid, *p*-coumaric acid and sinapic acid) and the most abundant ones are monoesters of caffeic acid, usually present as a mixture of different isomers but small amounts of diesters or triesters are also found in the plant. Esters at position C-1 of the quinic acid core are also present in the plant kingdom although to a much minor extent. Cynarin, present for example in artichoke (*Cynara* species) and in *Echinacea* species, is a 1,3-dicaffeoylquinic acid (9). Furthermore, plants are known to synthesize the *trans*-isomers over the *cis*-isomers of CGAs. The latter have been reported to be formed in tissue or extracts previously exposed to UV light (10).

The composition of CGAs is directly related to the plant species although other parameters such as climate, soil of cultivation, part of the plant, seasonality and others can influence their distribution. In addition, plants growing in an urban context, could develop a tolerance to the environmental stress, as the air pollution, that could be partially explained with a major production of secondary metabolites in tissue leaves, thanks to their antioxidant activity (11). Coffee, apples, ciders, blueberries, spinach and so on, are rich in CGAs but they differ in the type of hydroxycinnamic acids involved. Caffeoylquinic acids (CQAs) are the most abundant in coffee and in other species but minor compounds, such as CGAs of *p*-coumaric, ferulic or sinapic acids contribute to define the specific profile and uniqueness of the plant. Unfortunately, due to a lack of commercially available standard compounds, the qualitative identification of different isomers is not always reliable without an ion-trap-LC-MS method. Very recently, Craig et al. (12) designed a rapid quantification of chlorogenic acids in green coffee extracts and seven main CGAs isomers were quantified by HPLC using standard compounds.

Moreover, it is important to note that the numbering system of these compounds is not always coherent in literature since many authors adopted the IUPAC numbering while others used the non-

IUPAC numbering. This can create confusion in the identification of the different regioisomers and it is thus important to specify the numbering system adopted as well as to show in figures the correct stereochemistry of all isomers. As recently reported by Clifford, (13) the IUPAC numbering for the quinic acid moiety, which was introduced in 1976, defines C-5 the carbon atom with the OH group in *cis* configuration with the COOH group as indicated in figure 1 for chlorogenic acids analyzed in the present work. When comparing results with literature data, numbering system is crucial to avoid mistakes. To note that Reguiero et al. (14) used the non IUPAC numbering while Solar et al. (15) and Amaral et al. adopted the IUPAC numbering (16). Pereira et al. (17) did not specify the stereochemistry of the chemical structures depicted and the numbering system considered was not defined.

The establishment of a reliable, rapid and cost-effective method to extract, identify and quantify these compounds in plants via UHPLC would help to better understand CGAs role in defensive mechanisms of the plant and beneficial effects to the human being, as reported for those vegetables and fruits which are particularly rich in chlorogenic acids. In particular, walnut leaves, which are frequently used as traditional remedy and its aqueous tea infusion already demonstrated to possess biological activity (18-20), have the characteristic to be particularly rich in *p*-coumaroylquinic acids (*p*CoQA) as illustrated in the literature by several authors (14-22).

In the present work we optimized a suitable UHPLC method in order to quantify and evaluate seasonal variation of chlorogenic acids derivatives in walnut leaves as well as the presence of *cis* isomers, thanks to UV irradiation ad hoc experiment on standard solutions of CQAs and *p*CoQAs (23). Qualitative identification was performed using synthesized standards of FQAs and *p*CoQAs, not commercially available (24-25). Quantitative analyses are expressed as 5-caffeoylquinic acid equivalents in order to assure reliable results (26).

Experimental

Chemicals

3-Caffeoylquinic acid (3-CQA), 4-caffeoylquinic acid (4-CQA), 5-caffeoylquinic acid (5-CQA), 3,4-dicaffeoylquinic acid (3,4-diCQA), 3,5-dicaffeoylquinic acid (3,5-diCQA), 4,5-dicaffeoylquinic acid (4,5-diCQA) were purchased from Phytolab. Hydroxycinnamic acids standards and acetonitrile (HPLC grade) were purchased from Sigma –Aldrich while formic acid was obtained from Carlo Erba reagent. Not commercially available standards such as feruloylquinic acids (FQAs) and *p*-coumaroylquinic acids (*p*-CoQA) were obtained by carrying out their chemical synthesis from condensation reactions between a quinic acid derivative and the corresponding acyl chloride. The identity of these CGAs was confirmed by ¹H and ¹³C NMR spectroscopy (24, 25). Water was treated in a Milli-Q water purification system (Millipore Academic).

Samples

Fresh leaves from different branches were collected from a single *Juglans regia* L. tree, in an urban context (Trieste, Italy), in four different period of growth, from spring to late summer 2016 (April 21th, May 3rd, July 21th and September 9th). After sampling, leaves were dried on an absorbent paper and the ones with similar size were chosen and weighed. Then, they were immediately put in plastic bags and stored in a freezer at -20 °C. In a second time, samples were freeze dried (lyophilizer Christ Alpha 1-2) for subsequent analysis.

Extraction of Phenolic Compounds and Sample preparation

Extraction was performed in duplicate by decoction preparation, in order to simulate home preparation for medicinal uses. For this purpose, 1g of lyophilized leaves for each collection time was added to 200 mL of boiling water (27). The mixture was stirred for 5 min at 200 rpm on a heated plate (Arex Velp Scientifica) and filtered through qualitative filter paper n. 302 (VWR Europe). The aqueous extract was frozen with liquid nitrogen and freeze dried for 3 days.

For quantification purposes, lyophilized decoction material, around 235 mg/g of dry material, was dissolved in water to afford concentrations of 30 mg/mL. In order to analyze each compound

accurately, diluted solutions in ratios of 1:2, 1:4 and 1:10 were prepared in water and filtered across a nylon filter (pore size 0.2 μm), transferred to a vial and immediately analyzed by Ultra High Pressure Liquid Chromatography (UHPLC).

Instrumentation

Analysis of caffeoylquinic acids (CQAs), dicaffeoylquinic acids (diCQAs), feruloylquinic acids (FQAs) and *p*-coumaroylquinic acids (*p*CoQAs) along with the hydroxycinnamic acids (caffeic acid, *p*-coumaric acid, ferulic acid and sinapic acid) were performed using a 1290 UHPLC system (Agilent, Germany), consisting of degasser, quaternary pump, column thermostat and diode array detector (DAD) operating at 305 nm (specific for *p*CoQAs and *p*-coumaric acid) and 324 nm. A Kinetex XB-C18 column 2.6 μm 100 x 2.1 mm (Phenomenex, USA) was used at 25°C. Solvents were delivered at a total flow rate of 0.5 mL/min and the volume of injection was 2.0 μL . Solvent A was water/formic acid (1000:1 v/v) and solvent B acetonitrile. The gradient profile was from initial 97% of solvent A to 85% of A in 8 min, then 60% of A at 11min, and a return to 97% A at 12 min to re-equilibrate.

Qualitative analyses were carried out using the following standards: caffeic acid (CA) **1**; *trans* 3-caffeoylquinic acid (*trans* 3-CQA) *t*-**1a**; *trans* 4-caffeoylquinic acid (*trans* 4-CQA) *t*-**1b**; *trans* 5-caffeoylquinic acid (*trans* 5-CQA) *t*-**1c**; *p*-coumaric acid (*p*CoA) **2**; *trans* 3-*p*-coumaroylquinic acid (*trans* 3-*p*CoQA) *t*-**2a**; *trans* 4-*p*-coumaroylquinic acid (*trans* 4-*p*CoQA) *t*-**2b**; *trans* 5-*p*-coumaroylquinic acid (*trans* 5-*p*CoQA) *t*-**2c**; ferulic acid (FA) **3**; *trans* 3-feruloylquinic acid (*trans* 3-FQA) *t*-**3a**; 4-feruloylquinic acid (*trans* 4-FQA) *t*-**3b**; 5-feruloylquinic acid (*trans* 5-FQA) *t*-**3c**; sinapic acid (SA) **4**; 3,4-dicaffeoylquinic acid (3,4-diCQA) **5**; 3,5-dicaffeoylquinic acid (3,5-diCQA) **6**; 4,5-dicaffeoylquinic acid (4,5-diCQA) **7** (Figure 1).

Qualitatively identification of CGAs was achieved by comparison of specific retention times of diluted standard solutions and by spiking samples with small amounts of each respective standard.

Stereoisomers *cis* 3-caffeoylquinic acid (*cis* 3-CQA) *c-1a*, *cis* 4-caffeoylquinic acid (*cis* 4-CQA) *c-1b*, *cis* 5-caffeoylquinic acid (*cis* 5-CQA) *c-1c*, *cis* 3-*p*-coumaroylquinic acid (*cis* 3-*p*CoQA) *c-2a*, *cis* 4-*p*-coumaroylquinic acid (*cis* 4-*p*CoQA) *c-2b* and *cis* 5-*p*-coumaroylquinic acid (*cis* 5-*p*CoQA) *c-2c* were clearly identified using a 1290 UHPLC system (Agilent Technologies) equipped with a Triple Quad 4500 (Sciex) with an electrospray ionization source. In order to discriminate the isomers a Monitoring Reaction Mode (MRM) acquisition method was used in negative ionization, as previously reported (27).

Quantitative determination was performed by UHPLC using calibration curve of *trans* 5-CQA. Standard stock solution was prepared in MeOH:H₂O (1:1) at appropriate concentration and different diluted solutions were prepared from stock solution.

Identification and Characterization of Chlorogenic Acids

Fresh leaves from different branches were collected from a single *Juglans regia* L. tree, in four different period of growth, from spring to late summer 2016. The dimensions of the leaves were determined and their mean values (on a sample of 15 leaves) are reported in Table I.

The percentage of water loss (%WL) was calculated using the following equation:

$$\%WL = 100 - \frac{W_{AF} * 100}{W_{BF}}$$

W_{BF} corresponds to the weight before freeze dried and W_{AF} to the weight after freeze dried. The %WL in the different months was as follows: April (71% WL), May (74% WL), July (72% WL), September (67% WL).

UHPLC analyses were performed at different dilutions in order to have a better identification of all chlorogenic acids. Quantification was performed on peak areas obtained with OpenLab software (Agilent, Germany).

At the beginning, aqueous diluted samples in a 1:10 ratio were analyzed and three different classes of chlorogenic acids could be unequivocally detected with comparison of authentic samples retention times (Figure 2). All three *trans* isomers *trans* 3-, 4- and 5-CQA and the three *trans* 3-, 4- and 5-*p*CoQA isomers were clearly identified in all collection times while the only *trans* 3-FQA isomer was detected (Figure 2). 3- and 5-CQA as well as 3- and 4-*p*CoQA have already been identified by Pereira et. al (17) while Santos et al. in 2013 identified 4-caffeoylquinic acid (27).

Since *cis* isomers show the same fragmentation pattern of the corresponding *trans* isomers, as reported in the literature (10), the presence of possible *cis* isomers was confirmed by analyses of the specific fragmentation of the UV treated standard solutions: *trans* 3-CQA and *trans* 5-CQA (m/z 353.6) has the same fragmentation pattern (28-29) as well as the corresponding *cis* isomers, giving a base peak at m/z 191.5 while *trans* and *cis* 4-CQA (m/z 353.6) give a base peak at m/z 173.5. Fragmentation of pseudomolecular ion $[M-H]^-$ at m/z 337.1 were found for *p*CoQAs, yielding a base peak at m/z 163 for *trans* and *cis* 3*p*CoQA, 174 m/z for *trans* and *cis* 4*p*CoQA and 191 m/z for *trans* and *cis* 5*p*CoQA (22). UHPLC analyses clearly identified *cis* 3-CQA and *cis* 3-*p*CoQA in all collection times for the first time (Figure 2) while the presence of *cis* 4- and 5-CQA and *cis* 4- and 5- *p*CoQA was detected via LC-MS/MS method but not fully confirmed via UHPLC, probably due to low concentrations of these regioisomers.

Calibration curve of *trans* 5-CQA showed a good response linearity with a coefficient of correlation (r^2) of 0.999. Limit of quantification (LOQ) and limit of detection (LOD) were calculated as 3 times lower concentration of analyte on signal to noise ratio (LOD) or 10 times lowest concentration of analyte on signal to noise ratio (LOQ) resulting 0.88 $\mu\text{g/mL}$ for LOQ and 0.26 $\mu\text{g/mL}$ for LOD.

The concentrations of all identified phenolic compounds, in the different periods, are reported in Table II.

In a second time the analyses were performed on the aqueous extracts both without any dilution and in 1:2 diluted solutions and the results are reported in Figure 3.

Discussion

In the analyses of seasonal variations, in aqueous diluted samples in a 1:10 ratio, we observed that the most concentrated chlorogenic acid is the *trans* 3-caffeoylquinic acid with a higher concentration in April (35.85 mg/g, table II). All data reported are expressed as mg/g dry weight. All *trans* isomers at position 3 showed a considerable decrease from July to September; however, both *trans* 3-caffeoylquinic acid and *trans* 3-feruoylquinic acid showed a similar behavior with a constant concentration from April to May, while, *trans* 3-*p*-coumaroylquinic acid showed a slight increase of the concentration from April to May (from 7.15 mg/g to 8.30 mg/g). In general, it was observed that *trans* isomers concentrations achieved values of half of the initial concentration at the end of Summer. For *cis* isomers a different behavior was observed, since *cis* 3-caffeoylquinic acid did not show significant variations from April (1.27 mg/g) to July (1.29 mg/g) and then a decrease of approximately half of the concentration until September, while *cis* 3-*p*CoQA showed the highest concentration in May (2.18 mg/g) to continue with a gradual decrease until September.

The *trans/cis* ratio decreases from April to September (Table III) and this is in accordance with what already observed by Clifford et al. in 2008 (10) and Kuhnert et al. in 2015 (30) indicating that during summer a photochemical *trans-cis* isomerization under ultraviolet (UV) irradiation is occurring. Furthermore, it is evident that *trans* 3-*p*CoQA is more easily transformed by UV irradiation in the corresponding *cis* isomer with respect to 3-CQA as observed in 1967 by Kahnt for the corresponding hydroxycinnamic acids (31).

The highest concentration values of isomers at position 5 (*trans* 5-CQA and *trans* 5-*p*CoQA) were found in April (7.98 mg/g and 1.74 mg/g, respectively) and then a gradual decrease was observed until September. *Trans* 4-CQA also showed a constant decrease during summer time (from 9.34

mg/g in April to 2.55 mg/g in September) while *trans* 4-*p*CoQA showed a slight decrease from April (1.58 mg/g) to May (1.27 mg/g) and then it remained quite constant (Table II).

In the aqueous extracts, on 1:2 diluted solutions, minor constituents were found: three different dicaffeoylquinic acids (3,4-dicaffeoylquinic acid **5**, 3,5-dicaffeoylquinic acid **6** and 4,5-dicaffeoylquinic acid **7**) as well as three hydroxycinnamic acids (caffeic acid **1**, *p*-coumaric acid **2** and ferulic acid **3**) were further identified (Figure 3). *p*-Coumaric acid was previously identified by Pereira et al. (17) while as far as we know this is the first time that dicaffeoylquinic acids have been detected and quantified in this species and could contribute in the characterization of the phenolic profile of this plant and seasonal variation in the leaf tissue.

Regarding the seasonal analyses, diCQAs were present in all collection times but in significant smaller amount with respect to the simple CGAs. Caffeic acid was present in its highest concentration in May (0.45 mg/g) while *p*CoA and FA had their higher concentrations in May and July respectively (0.35 mg/g and 0.46 mg/g respectively). On the other hand, the lower concentration of diCQAs was observed in May with a total of 0.56 mg/g. Apparently, it seems the concentrations of these hydroxycinnamic acids is less affected by the seasonal changes.

In summary, a total of fifteen hydroxycinnamic acid derivatives were quantified and their total quantification was ranging between 68.59 mg/g and 23.49 mg/g (Figure 4). Results from total concentrations of each collection time showed that there are not significant changes during the vegetative growth (between April and May) but after this period it was detected an important decrease in the total concentration, confirming results already reported in the literature where, for most of the chlorogenic acids, variations were studied from April to August. It was also found a decrease of their content during this period (15, 16).

Caffeoylquinic acids represent the main compounds, with the highest concentration in April (54.43 mg/g) (Figure 5). Specifically, we have found that *trans* 3-CQA was the major compound while *p*-coumaric acid was the minor one for each collection time. It may be noted that, when a consistent

comparison can be performed, the total amount of quantified phenolic acid derivatives is higher than the one previously reported in the literature due to differences in both extraction and quantification methods. (16, 17, 21, 22) In particular, by comparing the present data (May month) expressed as the sum of 3- and 5-caffeoylquinic acids with those reported by Pereira et al. 2007 (17) (sampling 31st May; average of 6 different cultivars), an increase of about 47% can be calculated. However, by comparing the sum of 3- and 4-*p*-coumaroylquinic acids with data reported in the same work, the increase is still evident but remarkably lower (about 4.5%). The variation in phenolic acid derivatives content in walnut leaves could be partially due also to the natural climatic differences that occur over the years (22) and to a defence response to stressful environment (32). In Figure 6 the relative percentage of the different hydroxycinnamic acid derivatives with respect to the sum of the all hydroxycinnamic acid derivatives quantified is reported; it can be noticed that CQAs represent around 77% with higher concentration in April and July and *p*CoQAs around 18% with a higher peak in May, while diCQAs and cinnamic acids showed the highest percentages in September (3.74% and 2.87% respectively).

Conclusions

A rapid and reliable method for the quantification of chlorogenic acids by means of UHPLC analysis has been developed and fifteen different chlorogenic acids derivatives have been identified and quantified. Dicaffeoylquinic acids were for the first time identified in *Juglans regia* L. The extraction method, in comparison with other proposed in the literature (e.g. methanol extraction) revealed to be successful since comparable amounts of chlorogenic acids derivatives could be obtained and results confirm that walnut leaves infusion could be considered as an interesting source of polyphenolic compounds and could contribute to antioxidants intake to the human diet. Furthermore, seasonal variations could be used to choose spring or early summer as the best period for walnut leaves harvest in order to maximize antioxidants content of the infusion preparation. When quantitative data are discussed geographical location, cultivar and crop season could

influence remarkably the concentration of this class of polyphenols, furthermore environmental pollution is something to take into consideration and needs to be deeply investigated to elucidate the role of stress induction of urban plants with subsequent possible production of higher amount of CGAs, as data collected in this study seems to suggest.

Since we have successfully characterized different *cis* and *trans* isomers, this method could be applied in the analyses of other chlorogenic acids rich matrices exposed to UV radiations, such as coffee leaves, in order to determine the exact amount of minor compounds such as *p*CoQAs in *cis* and *trans* forms, which till now were only identified by HPLC/MS analysis. Determination of complete isomers profile could give important information to elucidate seasonal variation on the biosynthetic pathway of formation of CGAs and genetic variations that can be involved in the defense mechanism of the plant.

Funding

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie [grant agreement No 642014].

References

1. Bjørklund, G., Chirumbolo, S.; Role of oxidative stress and antioxidants in daily nutrition and human health; *Nutrition*, (2017); 33: 311-321.
2. Umeno, A., Horie, M., Murotomi, K., Nakajima, Y., Yoshida, Y.; Antioxidative and antidiabetic effects of natural polyphenols and isoflavones; *Molecules*, (2016); 21: 708/1-708/15.
3. Li, W., Guo, Y., Zhang, C., Wu, R., Yuding Yang, A., Gaspar, J., Kong, A.-N. T.; Dietary Phytochemicals and Cancer Chemoprevention: A Perspective on Oxidative Stress, Inflammation, and Epigenetics; *Chemical Research in Toxicology*, (2016); 29: 2071-2095.
4. Du, G., Sun, L., Zhao, R., Du, L., Song, J., Zhang, L., He, G., Zhang, Y., Zhang, J.; Polyphenols: Potential source of drugs for the treatment of ischaemic heart disease; *Pharmacology & Therapeutics*, (2016); 162: 23-34.
5. Carito, V., Ceccanti, M., Tarani, L., Ferraguti, G., Chaldakov, G. N., Fiore, M.; Neurotrophins' Modulation by Olive Polyphenols; *Current Medicinal Chemistry*, (2016); 23: 3189-3197.
6. Krga, I., Milenkovic, D., Morand, C., Monfoulet, L.-E.; An update on the role of nutrigenomic modulations in mediating the cardiovascular protective effect of fruit polyphenols; *Food & Function*, (2016); 7: 3656-3676.
7. a) Sinisi, V., Stevaert, A., Berti, F., Forzato, C., Benedetti, F., Navarini, L., Camps, A., Persoons, L., Vermeire, K.; Chlorogenic compounds from coffee beans exert activity against respiratory viruses; *Planta Medica*, (2017); in press. DOI: 10.1055/s-0042-119449 b) Sinisi, V., Forzato, C., Cefarin, N., Navarini, L., Berti, F.; Interaction of chlorogenic acids and quinides from coffee with human serum albumin; *Food Chemistry*, (2015); 168: 332-340.
8. Scalbert, A., Williamson, G.; Dietary intake and bioavailability of polyphenols; *Journal of Nutrition*, (2000); 130: 2073S-2085S.
9. a) Piston, M., Machado, I., Branco, C. S., Cesio, V., Heinzen, H., Ribeiro, D., Fernandes, E., Chiste, R. C., Freitas, M.; Infusion, decoction and hydroalcoholic extracts of leaves from artichoke (*Cynara cardunculus* L. subsp. *Cardunculus*) are effective scavengers of physiologically relevant ROS and RSN; *Food Research*

- International*, (2014); 64: 150-156. b) Brown, P. N., Mudge, E. M., Paley, L.; Determination of phenolic constituents in Echinacea raw materials and dietary supplements by HPLC-UV: collaborative study. *J. AOAC Int.*, (2016); 99: 1197-1203.
10. Clifford, N., Kirkpatrick, Jo., Kuhnert, N., Roozendaal, H.; LC-MSⁿ analysis of the *cis* isomers of chlorogenic acids. *Food Chemistry*, (2008); 106: 379-385.
11. Gostin, I.; Air pollution stress and plant response. In *Plant Responses to Air Pollution*. Kulshrestha U., Saxena P. (Eds) Springer, Singapore, (2016), pp.99-117.
12. Craig, A. P.; Fields, C., Liang, N., Kitts, D., Erickson, A.; Performance review of a fast HPLC-UV method for the quantification of chlorogenic acids in green coffee bean extracts; *Talanta*, (2016); 154: 481-485.
13. Abrankó, L., Clifford, M. N.; An unambiguous nomenclature for the acyl-quinic acids commonly known as chlorogenic acids; *J. Agric. Food Chem.* (2017); 65: 3602-3608
14. Regueiro, J., Sánchez-González, C., Vallverdú-Queralt, A., Simal-Gándara, J., Lamuela-Raventós, R., Izquierdo-Pulido, M.; Comprehensive identification of walnut polyphenols by liquid chromatography coupled to linear ion trap-Orbitrap mass spectrometry; *Food Chemistry*, (2014), 152: 340-348
15. Solar, A., Colarič, M., Usenik, V., Stampar, F.; Seasonal variations of selected flavonoids, phenolic acids and quinones in annual shoots of common walnut (*Juglans regia* L.); *Plant Science*, (2006); 170: 453-461.
16. Amaral, J. S., Seabra, R. M., Andrade, P. B., Valentão, P., Pereira, J. A., Ferreres, F.; Phenolic profile in the quality control of walnut (*Juglans regia* L.) leaves; *Food Chemistry*, (2004); 88: 373-379.
17. Pereira, J. A.; Oliveira, I.; Sousa, A.; Valentão, P., Andrade, P. B., Ferreira, I. C. F. R., Ferreres, F., Bento, A., Seabra, R., Estevinho, L.; Walnut (*Juglans regia* L.) leaves: phenolic compounds, antibacterial activity and antioxidant potential of different cultivars; *Food and Chemical Toxicology*, (2007); 45: 2287-2295.
18. Kuhnert, N.; Karaköse, H.; Jaiswal, R.; Analysis of chlorogenic acids and other hydroxycinnamates in food, plants and pharmacokinetic studies; In *Handbook of analysis of active compounds in functional foods*, Nollet, L. M. L.; Toldra, F., Eds. CRC Press: 2012; pp 461-512.
19. Kusilic-Bilusic, T.; Katalinic, V.; Dragovic-Uzelac, V.; Ljubenkovic, I.; Krisko, A.; Dejanovic, B.; Jukic, M.; Politeo, O.; Pifat, G.; Milos, M.; Antioxidant and acetylcholinesterase inhibiting activity of several aqueous tea infusions in vitro; *Food Technology and Biotechnology*, (2008); 46: 368-375.
20. Gawlik-Dziki, U.; Durak, A.; Pecio, L.; Kowalska, I.; Nutraceutical potential of tinctures from fruits, Green husks, and leaves of *Juglans regia* L.; *The Scientific World Journal* (2014); 2014: Article ID 501392, 10 pages, <http://dx.doi.org/10.1155/2014/501392>
21. Nour, V., Trandafir, I., Cosmulescu, S.; HPLC determination of phenolic acids, flavonoids and juglone in walnut leaves; *J. Chromat. Science*, (2013); 51: 883-890.
22. Amaral, J. S., Valentão, P., Andrade, P. B., Martins, R. C., Seabra, R. M.; Do cultivar, geographical location and crop season influence phenolic profile of walnut leaves?; *Molecules*, (2008); 13: 1321-1332.
23. Gutiérrez Ortiz, A. L., Colombari, S., Berti, F., Forzato, C., Navarini, L., LC-MS identification of *cis*-isomers of caffeoylquinic acids and *p*-coumaroylquinic acids; poster presented at 5th MS Food Day, October 11-13, 2017, Bologna; ISBN: 9788890738838.
24. Dokli, I., Navarini, L., Hamersak, Z.; Synthesis of 3,4-, and 5-*O*-feruloylquinic acids; *Tetrahedron:Asymmetry*, (2013); 24: 785-790.
25. Gutiérrez Ortiz, A. L.; Berti, F.; Navarini, L., Monteiro, A., Resmini, M., Forzato, C.; Synthesis of *p*-coumaroylquinic acids and analysis of their interconversion; *Tetrahedron: Asymmetry*, (2017); 28: 419-427.
26. Clifford, M. N.; Madala, N. E.; Surrogate Standards: A cost-effective strategy for identification of phytochemicals; *J. Agric. Food Chemistry*, (2017); 65: 3589-3590.
27. Santos, A., Barros, L., Calheta, R. C., Dueñas, M., Carvalho, M., Buelga, S. C., Ferreira, I.; Leaves and decoction of *Juglans regia* L.: different performances regarding bioactive compounds in vitro antioxidant and antitumor effects; *Industrial Crops and Product*, (2013); 51: 430-436.
28. Clifford, M.N.; Johnston, K. L.; Knight, S.; Kuhnert, N.; Hierarchical scheme for LC-MSⁿ identification of chlorogenic acids; *J. Agric. Food Chem.* (2003); 51 (10): 2900-11.
29. Ncube, N.; Mhlongo, M.; Piater, L.A.; Steenkamp, P.A.; Dubery, I. A.; Madala, N.E.; Analyses of chlorogenic acids and related cinnamic acid derivatives from *Nicotiana tabacum* tissues with the aid of UPLC-QTOF-MS/MS based on the in-source collision-induced dissociation method, *Chemistry Central Journal*, (2014); 8:66.
30. Karaköse, H.; Jaiswal, R.; Deshpande, S.; Kuhnert, N.; Investigation of the Photochemical Changes of Chlorogenic Acids Induced by Ultraviolet Light in Model Systems and in Agricultural Practice with *Stevia rebaudiana* Cultivation as an Example, *J. Agric. Food Chem.* (2015), 63, 3338-3347.
31. Kahnt, G.; Trans-*cis* equilibrium of hydroxycinnamic acids during irradiation of aqueous solutions at different pH, *Phytochemistry* (1967); 6: 755-758.
32. Cosmulescu, S.; Trandafir, I.; Nour, V.; Seasonal variation of the main individual phenolics and juglone in walnut (*Juglans regia*) leaves, *Pharmaceutical Biology* (2014); 52 (5): 575-80.

Table I. Dimension of Fresh Leaves (cm) at the same growth stage per each month

		April	May	July	September
Length (cm)	mean	5.27	11.12	15.14	12.61
	St. dev.	1.85	1.20	1.48	3.59
Width (cm)	mean	2.36	5.20	7.35	6.42
	St. dev.	0.79	0.46	0.94	1.73

Table II. Phenolic compound concentrations of walnut leaves^a (mg/g dry weight)

Nr.	Comp.	April	May	July	September
1	CA	0.28 (0.00)	0.45 (0.00)	0.38 (0.02)	0.22 (0.00)
<i>c-1a</i>	<i>cis</i> 3-CQA	1.27 (0.02)	1.40 (0.13)	1.29 (0.02)	0.63 (0.00)
<i>t-1a</i>	<i>trans</i> 3-CQA	35.85 (0.87)	34.02 (1.29)	25.13 (0.46)	12.57 (0.54)
<i>c-1b</i>	<i>cis</i> 4-CQA	<LOD	<LOD	<LOD	<LOD
<i>t-1b</i>	<i>trans</i> 4-CQA	9.34 (0.01)	5.65 (0.02)	4.40 (0.14)	2.55 (0.01)
<i>c-1c</i>	<i>cis</i> 5-CQA	<LOD	<LOD	<LOD	<LOD
<i>t-1c</i>	<i>trans</i> 5-CQA	7.98 (0.04)	4.29 (0.10)	2.46 (0.07)	1.56 (0.00)
2	<i>p</i> CoA	0.22 (0.00)	0.35 (0.08)	0.19 (0.00)	0.18 (0.00)
<i>c-2a</i>	<i>cis</i> 3- <i>p</i> CoQA	1.42 (0.00)	2.18 (0.40)	1.22 (0.03)	0.96 (0.00)
<i>t-2a</i>	<i>trans</i> 3- <i>p</i> CoQA	7.15 (0.01)	8.30 (0.00)	4.24 (0.10)	2.18 (0.01)
<i>c-2b</i>	<i>cis</i> 4- <i>p</i> CoQA	<LOD	<LOD	<LOD	<LOD
<i>t-2b</i>	<i>trans</i> 4- <i>p</i> CoQA	1.58 (0.01)	1.27 (0.06)	0.99 (0.02)	0.84 (0.00)
<i>c-2c</i>	<i>cis</i> 5- <i>p</i> CoQA	<LOD	<LOD	<LOD	<LOD
<i>t-2c</i>	<i>trans</i> -5 <i>p</i> CoQA	1.74 (0.00)	1.19 (0.00)	0.56 (0.01)	0.34 (0.00)
3	FA	0.29 (0.01)	0.33 (0.01)	0.46 (0.02)	0.27
<i>t-3a</i>	<i>trans</i> 3-FQA	0.46 (0.00)	0.45 (0.01)	0.26 (0.00)	0.22 (0.00)
<i>t-3b</i>	<i>trans</i> 4-FQA	<LOD	<LOD	<LOD	<LOD
<i>t-3c</i>	<i>trans</i> 5-FQA	<LOD	<LOD	<LOD	<LOD
4	SA	<LOD	<LOD	<LOD	<LOD
5	3,4-diCQA	0.31 (0.01)	0.20 (0.01)	0.27 (0.01)	0.33 (0.00)
6	3,5-diCQA	0.44 (0.02)	0.18 (0.00)	0.30 (0.01)	0.33 (0.00)
7	4,5-diCQA	0.27 (0.03)	0.18 (0.01)	0.31 (0.03)	0.22 (0.01)
Total^b		68.59 (1.02)	60.44 (2.13)	42.47 (0.93)	23.40 (0.59)

^aValues are expressed as mean (standard deviation) of duplicate analyses. ^bTotal: sum of all identified compound.

Table III. *Trans/cis* ratio of compounds **1a** and **2a**

<i>Trans/cis</i> ratio	April	May	July	September
<i>t-1a/c-1a</i>	28.2	24.3	19.5	20.0
<i>t-2a/c-2a</i>	5.0	3.8	3.5	2.3

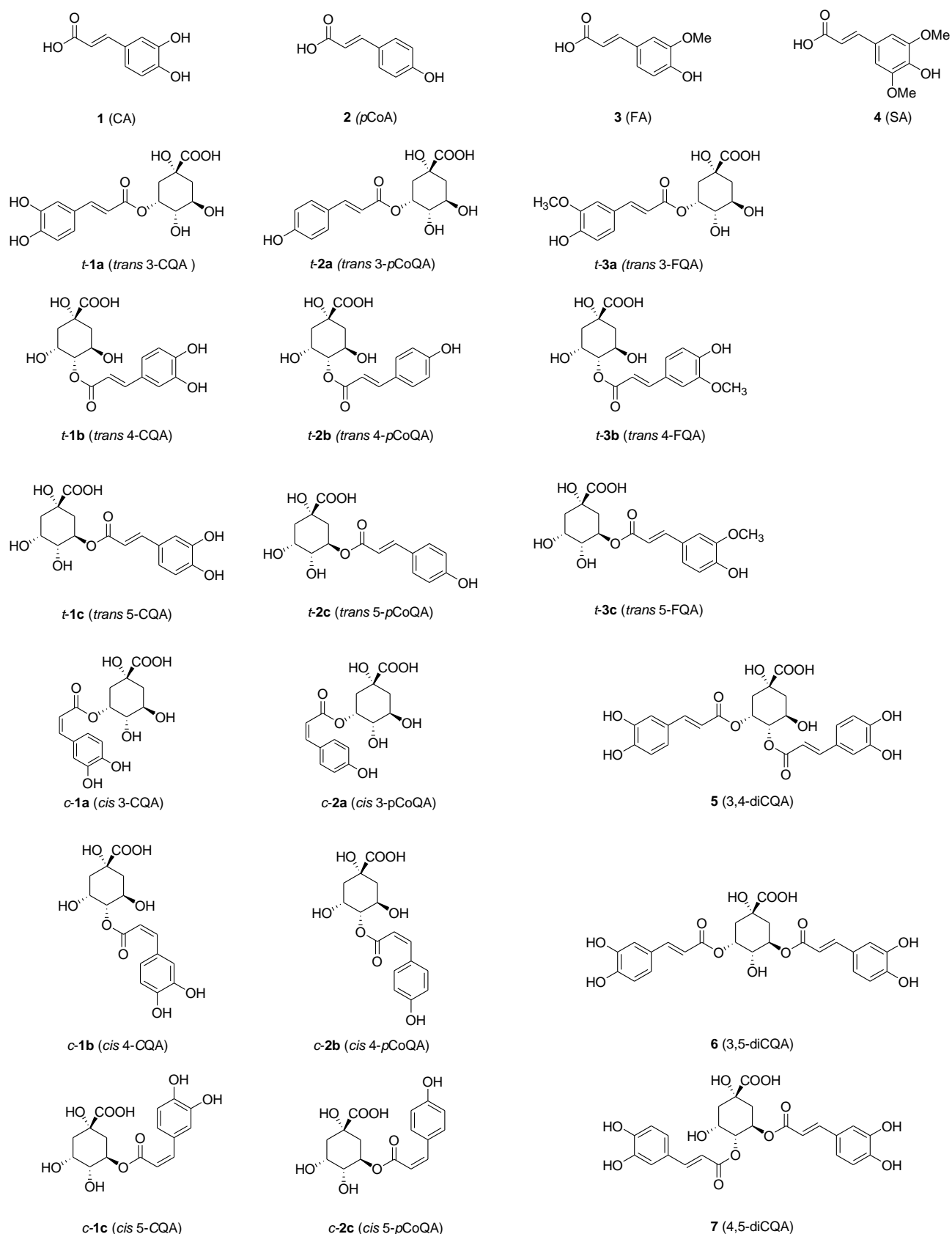


Figure 1. Chemical structures of the hydroxycinnamic acid derivatives: caffeic acid **1**; *p*-coumaric acid **2**; ferulic acid **3**, sinapic acid **4**; *trans* 3-caffeoylquinic acid **t-1a**; *trans* 3-*p*-coumaroylquinic **t-2a**; *trans* 3-feruloylquinic acid **t-3a**; *trans* 4-caffeoylquinic acid **t-1b**; *trans* 4-*p*-coumaroylquinic **t-**

2b; *trans* 4-feruloylquinic acid **t-3b**; *trans* 5-caffeoylquinic acid **t-1c**; *trans* 5-*p*-coumaroylquinic **t-2c**; *trans* 5-feruloylquinic acid **t-3c**; *cis* 3-caffeoylquinic acid **c-1a**; *cis* 3-*p*-coumaroylquinic **c-2a**; *cis* 4-caffeoylquinic acid **c-1b**; *cis* 4-*p*-coumaroylquinic **c-2b**; *cis* 5-caffeoylquinic acid **c-1c**; *cis* 5-*p*-coumaroylquinic **c-2c**; 3,4-dicaffeoylquinic acid **5**; 3,5-dicaffeoylquinic acid **6**, 4,5-dicaffeoylquinic acid **7**.

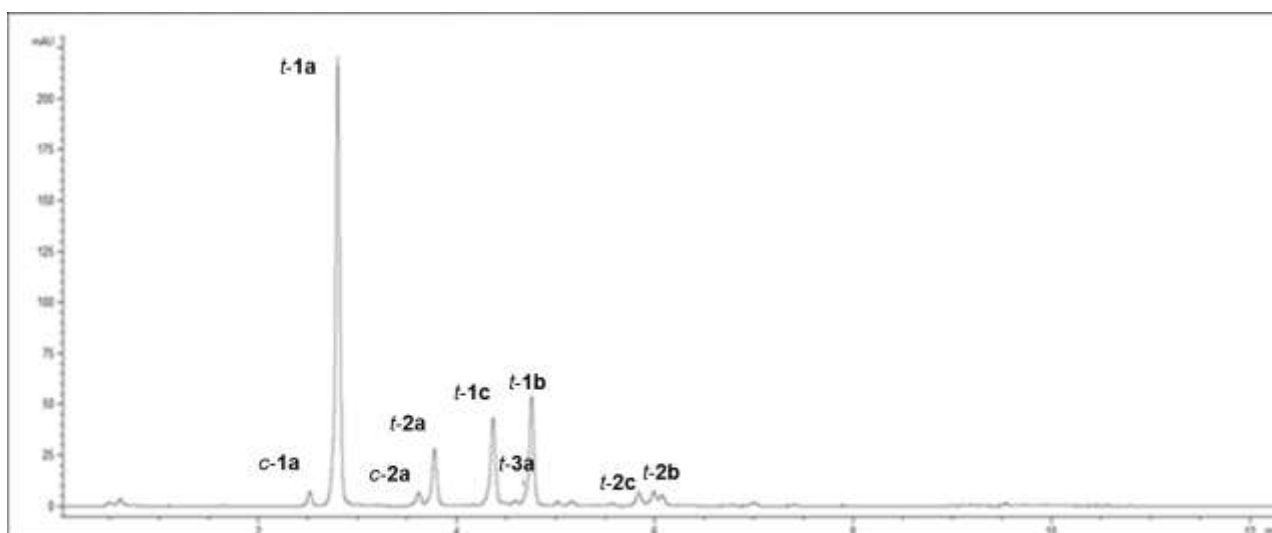


Figure 2. UHPLC of 1:10 diluted samples. hydroxycinnamic acid derivatives identified in walnut leaves. Detection at $\lambda=324\text{nm}$: *cis* 3-CQA **c-1a**; *trans* 3-CQA **t-1a**; *cis* 3-*p*CoQA **c-2a**; *trans* 3-*p*CoQA **t-2a**, *trans* 5-CQA **t-1c**; *trans* 3-FQA **t-3a**; *trans* 4-CQA **t-1b**; *trans* 5-*p*CoQA **t-2c**; *trans* 4-*p*CoQA **t-2b**.

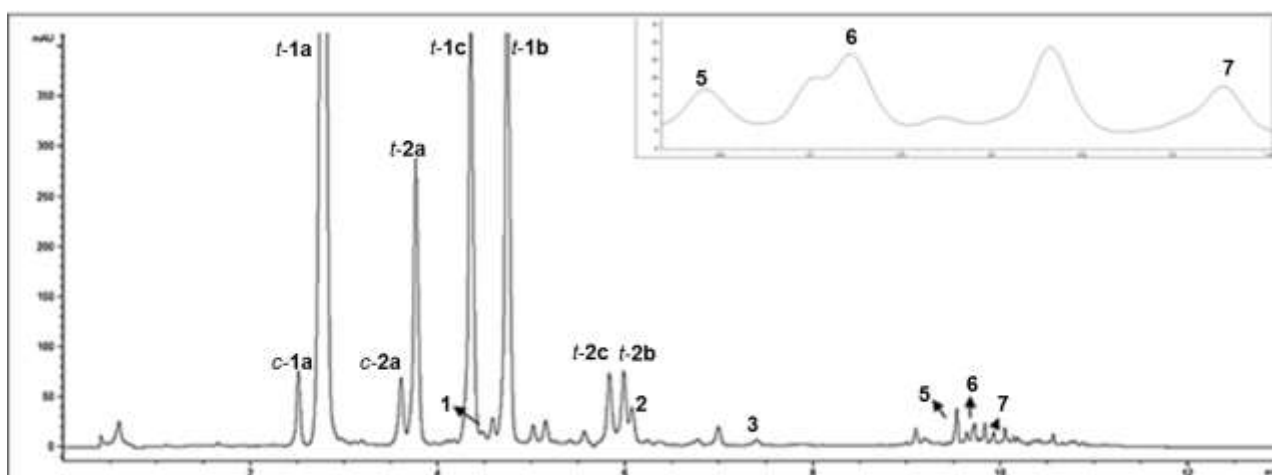


Figure 3. UHPLC of hydroxycinnamic acid derivatives identified in walnut leaves. Detection at $\lambda=324\text{nm}$. *cis* 3-CQA **c-1a**; *trans* 3-CQA **t-1a**; *cis* 3-*p*CoQA **c-2a**; *trans* 3-*p*CoQA **t-2a**, *trans* 5-CQA

t-**1c**; *trans* 3-FQA *t*-**3a**; *trans* 4-CQA *t*-**1b**; *trans* 5-*p*CoQA *t*-**2c**; *trans* 4-*p*CoQA *t*-**2b**; *p*CoA **2**; FA **3**; 3,4-diCQA **5**; 3,5-diCQA **6**; 4,5-diCQA **7**.

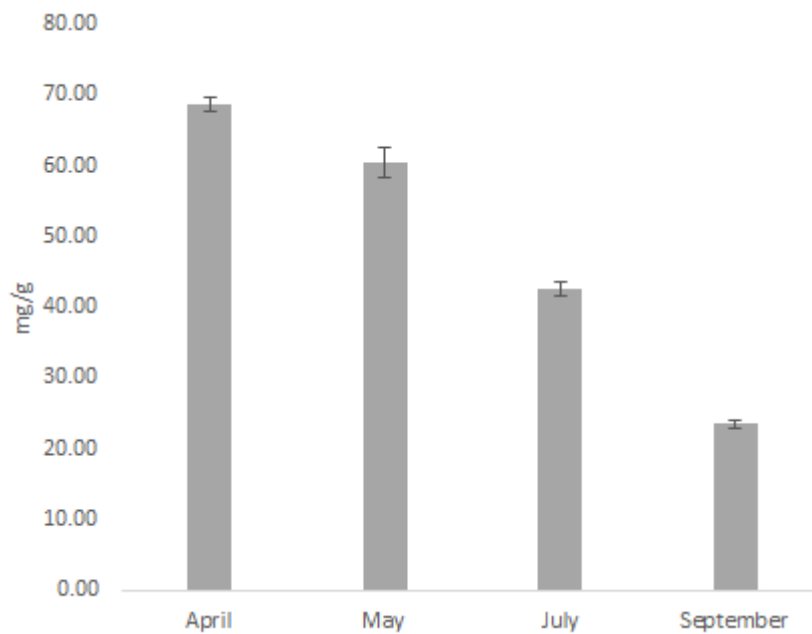


Figure 4. Total concentration (mg/g, dry weight) of hydroxycinnamic acid derivatives identified in walnut leaves, between April and September. Error bars are on the top of each column.

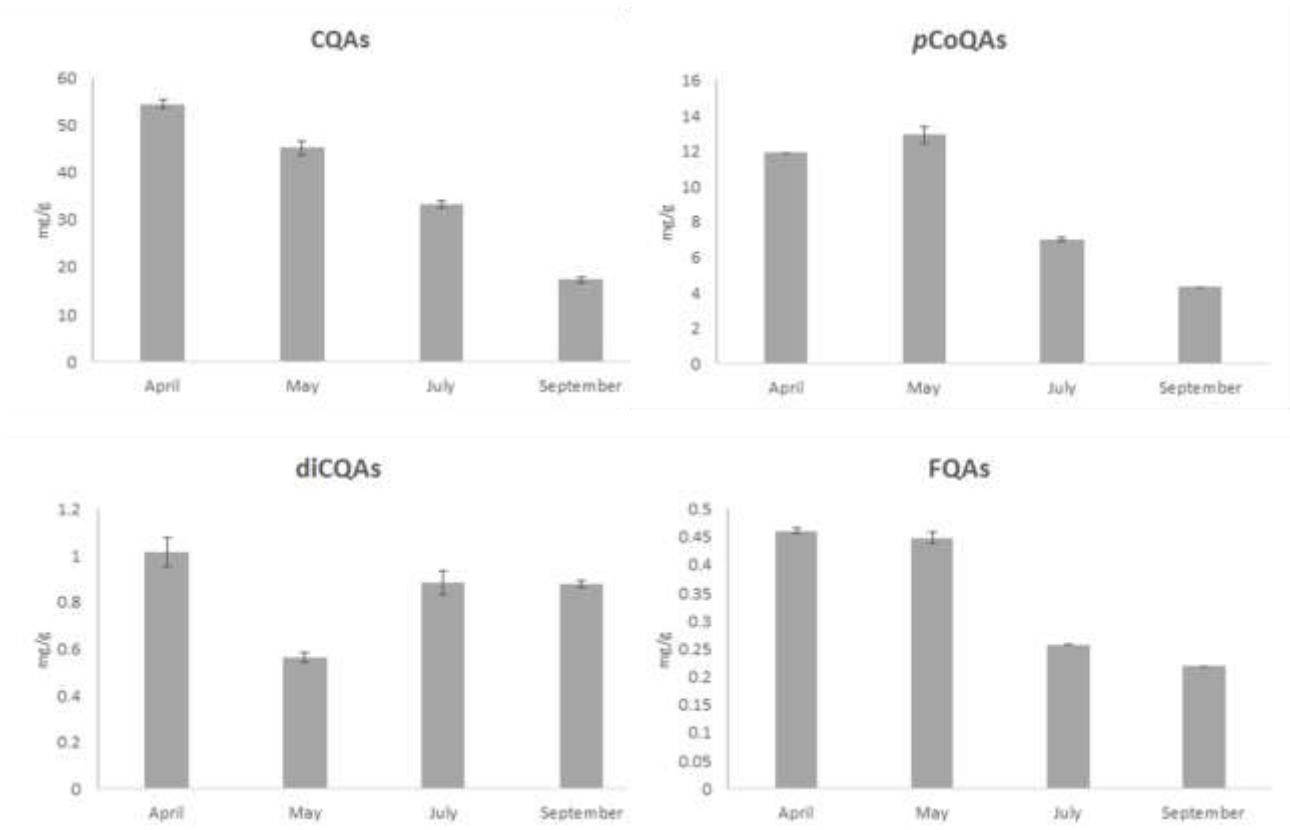


Figure 5. Concentrations of the different chlorogenic acids (mg/g, dry weight) identified in walnut leaves between April and September i.e. caffeoylquinic acids (CQAs), *p*-coumaroylquinic acids (*p*CoQAs), dicaffeoylquinic acids (diCQAs) and feruloylquinic acid (FQAs). Error bars are on the top of each column.

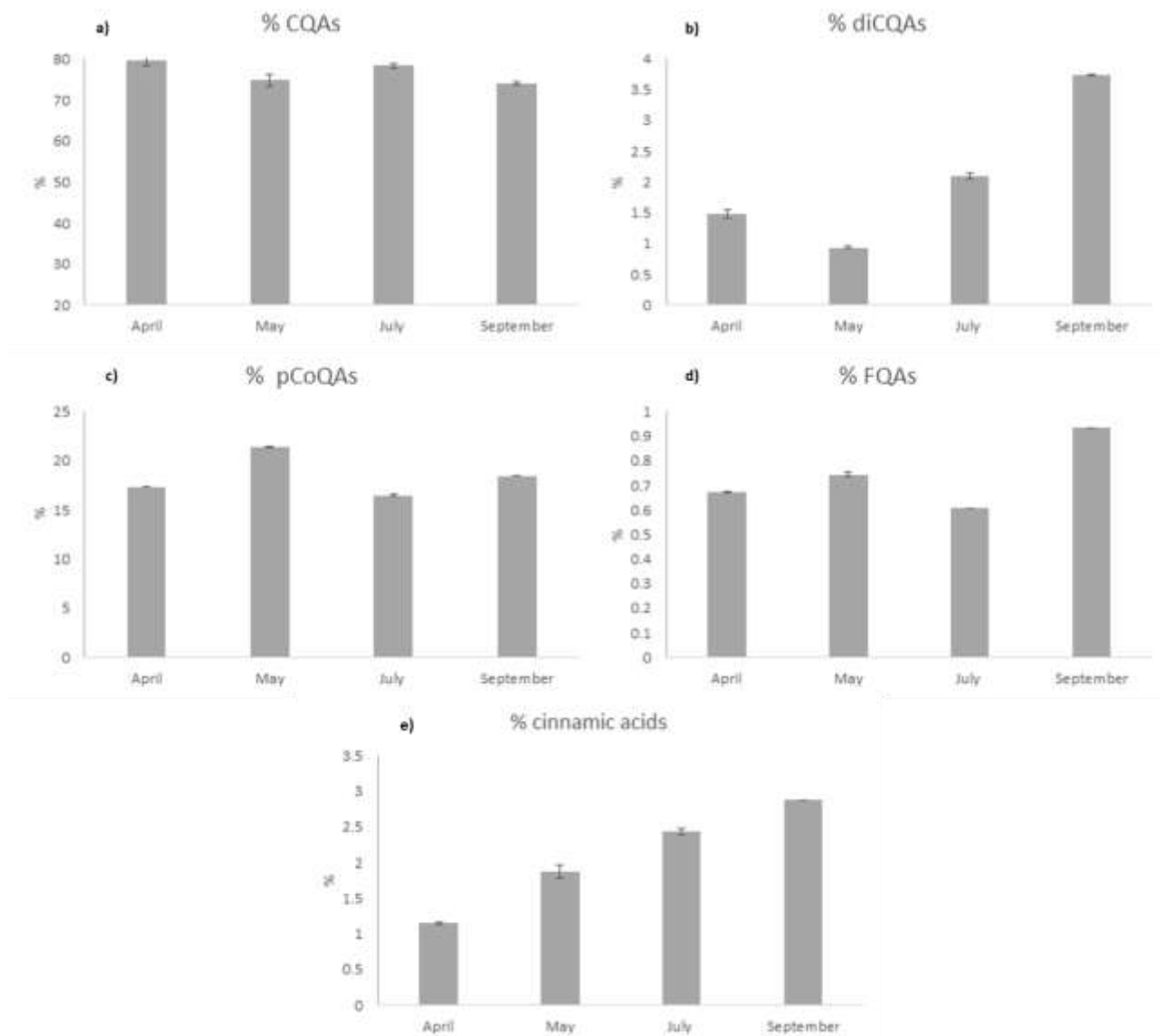


Figure 6. Percentages of the different hydroxycinnamic acid derivatives with respect to the quantified phenolic acid derivatives in walnut leaves, between April and September. a) % of caffeoylquinic acids (CQAs), b) % of *p*-coumaroylquinic acids (*p*CoQAs), c) % of dicaffeoylquinic acids (diCQAs), d) % of feruloylquinic acid (FQAs), e) % of cinnamic acids.