Journal of Chromatographic Science, 2018, Vol. 56, No. 8, 753–760
doi: 10.1093/chromsci/bmy041
Advance Access Publication Date: 14 May 2018
Article

Article

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

Anggy Lusanna Gutiérrez Ortiz[1], Federico Berti[1], Luciano Navarini[2], Paola Crisafulli[3], Silvia Colomban[3], and Cristina Forzato[1,*]

[1]Dipartimento di Scienze Chimiche e Farmaceutiche, Università di Trieste, via L. Giorgieri 1, 34127 Trieste, Italy, [2]illycaffè S.p.A., via Flavia 143, 34100, Trieste, Italy, and [3]Aromalab, illycaffè S.p.A., AREA Science Park, Padriciano 99, 34012 Trieste, Italy

[*]Author to whom correspondence should be addressed. Email address: cforzato@units.it

Received 12 April 2017; Revised 2 March 2018; Editorial Decision 22 March 2018

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, dicaffeoylquinic, mono-feruloylquinic and *cis* and *trans* mono-*p*-coumaroylquinic acid isomers were detected and quantified by Ultra High Pressure Liquid Chromatography and the seasonal variations of these secondary metabolites were investigated.

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 higher amount with respect to other antioxidants present in our common diet (8). Among them, the chlorogenic acids (CGAs) family is widely distributed in the plant kingdom. 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 (1,3-diCQA) (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 ultraviolet (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 CGAs in green coffee extracts and seven main CGAs isomers were quantify 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 International Union of Pure and Applied Chemistry (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 CGAs 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.

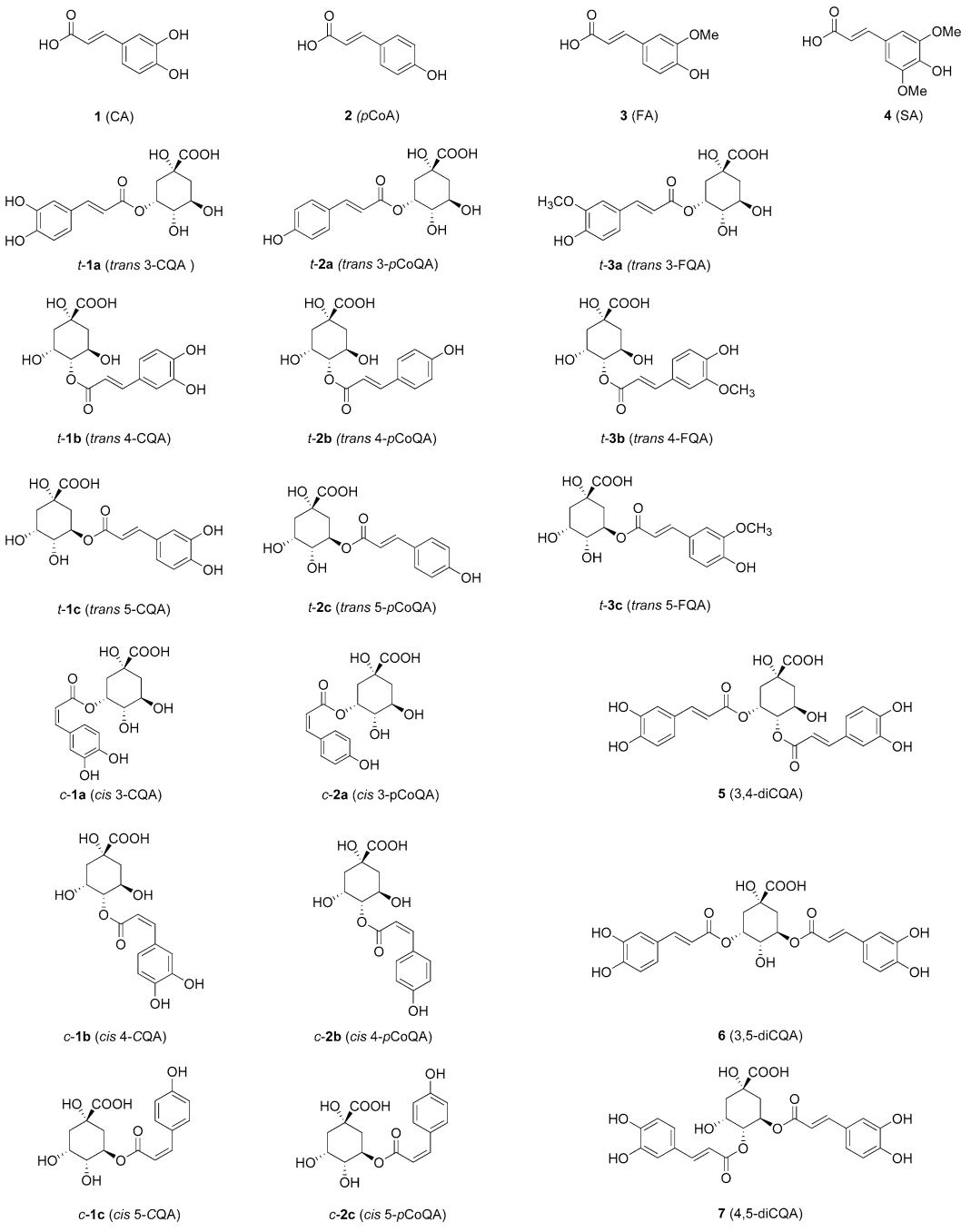

Figure 1. Chemical structures of the hydroxycinnamic acid derivatives: caffeic acid **1**; *p*-coumaric acid **2**; ferulic acid **3**, sinapic acid **4**; *trans* 3-caffeoylquinic acid *t*-**1a**; *trans* 3-*p*-coumaroylquinic acid *t*-**2a**; *trans* 3-feruloylquinic acid *t*-**3a**; *trans* 4-caffeoylquinic acid *t*-**1b**; *trans* 4-*p*-coumaroylquinic acid *t*-**2b**; *trans* 4-feruloylquinic acid *t*-**3b**; *trans* 5-caffeoylquinic acid *t*-**1c**; *trans* 5-*p*-coumaroylquinic acid *t*-**2c**; *trans* 5-feruloylquinic acid *t*-**3c**; *cis* 3-caffeoylquinic acid *c*-**1a**; *cis* 3-*p*-coumaroylquinic acid *c*-**2a**; *cis* 4-caffeoylquinic acid *c*-**1b**; *cis* 4-*p*-coumaroylquinic acid *c*-**2b**; *cis* 5-caffeoylquinic acid *c*-**1c**; *cis* 5-*p*-coumaroylquinic acid *c*-**2c**; 3,4-dicaffeoylquinic acid **5**; 3,5-dicaffeoylquinic acid **6**, 4,5-dicaffeoylquinic acid **7**.

The establishment of a reliable, rapid and cost-effective method to extract, identify and quantify these compounds in plants via Ultra High Pressure Liquid Chromatography (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 CGAs. 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 CGAs 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 synthetized standards of FQAs and *p*CoQAs, not commercially available (24, 25). Quantitative analyses are expressed as 5-CQAs equivalents in order to assure reliable results (26).

Experimental

Chemicals

3-CQA, 4-CQA, 5-CQA, 3,4-diCQA, 3,5-diCQA and 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 pCoQA 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 ^{1}H and ^{13}C 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 (21 April, 3 May, 21 July and 9 September). 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, 1 g 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 UHPLC.

Instrumentation

Analysis of CQAs, diCQAs, FQAs and *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 × 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 (1,000:1 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 11 min, 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-pCoQA) *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-CQA *c*-**1a**, *cis* 4-CQA *c*-**1b**, *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:H_2O (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 *J. 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:

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
	SD	1.85	1.20	1.48	3.59
Width (cm)	Mean	2.36	5.20	7.35	6.42
	SD	0.79	0.46	0.94	1.73

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

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) and September (67%WL).

UHPLC analyses were performed at different dilutions in order to have a better identification of all CGAs. 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 CGAs 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-CQA (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 three 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 µg/mL for LOQ and 0.26 µg/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 CGA is the *trans* 3-CQA 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-CQA 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 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

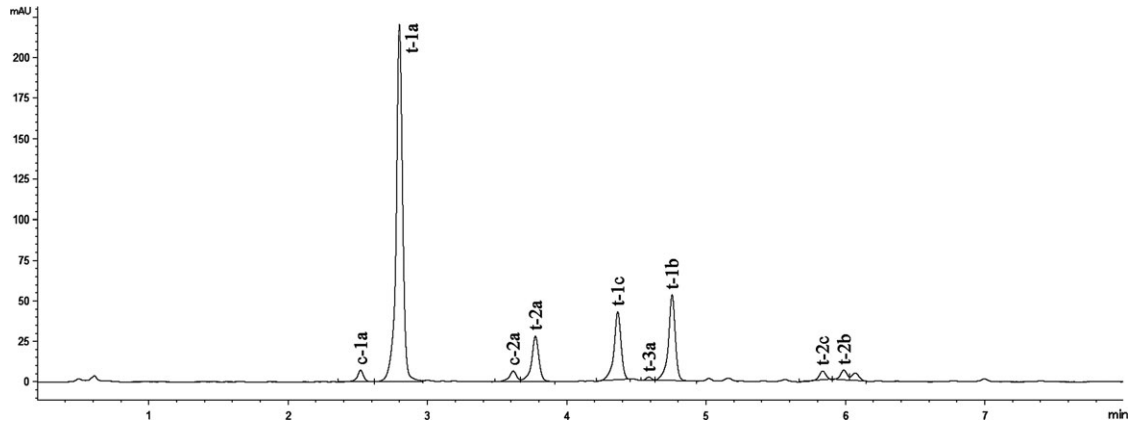

Figure 2. UHPLC of 1:10 diluted samples. hydroxycinnamic acid derivatives identified in walnut leaves. Detection at λ = 324 nm: *cis* 3-CQA *c*-**1a**; *trans* 3-CQA *t*-**1a**; *cis* 3-*p*CoQA *c*-**2a**; *trans* 3-*p*CoQA *t*-**2a**, *trans* 5-CQA *t*-**1c**; *trans* 3-FQA *t*-**3a**; *trans* 4-CQA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

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)
c-1a	*cis* 3-CQA	1.27 (0.02)	1.40 (0.13)	1.29 (0.02)	0.63 (0.00)
t-1a	*trans* 3-CQA	35.85 (0.87)	34.02 (1.29)	25.13 (0.46)	12.57 (0.54)
c-1b	*cis* 4-CQA	<LOD	<LOD	<LOD	<LOD
t-1b	*trans* 4-CQA	9.34 (0.01)	5.65 (0.02)	4.40 (0.14)	2.55 (0.01)
c-1c	*cis* 5-CQA	<LOD	<LOD	<LOD	<LOD
t-1c	*trans* 5-CQA	7.98 (0.04)	4.29 (0.10)	2.46 (0.07)	1.56 (0.00)
2	*p*CoA	0.22 (0.00)	0.35 (0.08)	0.19 (0.00)	0.18 (0.00)
c-2a	*cis* 3-*p*CoQA	1.42 (0.00)	2.18 (0.40)	1.22 (0.03)	0.96 (0.00)
t-2a	*trans* 3-*p*CoQA	7.15 (0.01)	8.30 (0.00)	4.24 (0.10)	2.18 (0.01)
c-2b	*cis* 4-*p*CoQA	<LOD	<LOD	<LOD	<LOD
t-2b	*trans* 4-*p*CoQA	1.58 (0.01)	1.27 (0.06)	0.99 (0.02)	0.84 (0.00)
c-2c	*cis* 5-*p*CoQA	<LOD	<LOD	<LOD	<LOD
t-2c	*trans*-5*p*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
t-3a	*trans* 3-FQA	0.46 (0.00)	0.45 (0.01)	0.26 (0.00)	0.22 (0.00)
t-3b	*trans* 4-FQA	<LOD	<LOD	<LOD	<LOD
t-3c	*trans* 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)

[a]Values are expressed as mean (standard deviation) of duplicate analyses.
[b]Total: sum of all identified compound.

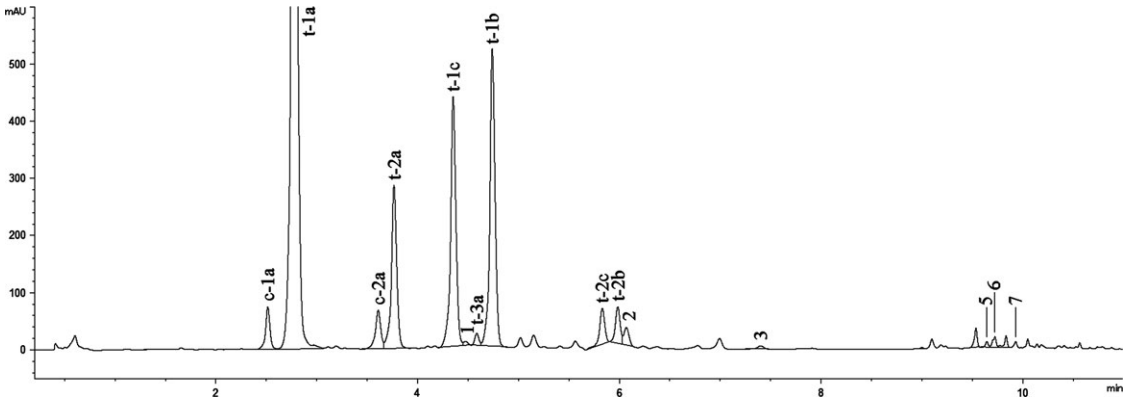

Figure 3. UHPLC of hydroxycinnamic acid derivatives identified in walnut leaves. Detection at λ = 324 nm. *cis* 3-CQA *c*-**1a**; *trans* 3-CQA *t*-**1a**; *cis* 3-*p*CoQA *c*-**2a**; *trans* 3-*p*CoQA *t*-**2a**, *trans* 5-CQA *t*-**1c**; *trans* 3-FQA *t*-**3a**; *trans* 4-CQA *t*-**1b**; *trans* 5-*p*CoQA *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**.

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

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

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 diCQA 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 CGAs, variations were studied from April to August. It was also found a decrease of their content during this period (15, 16).

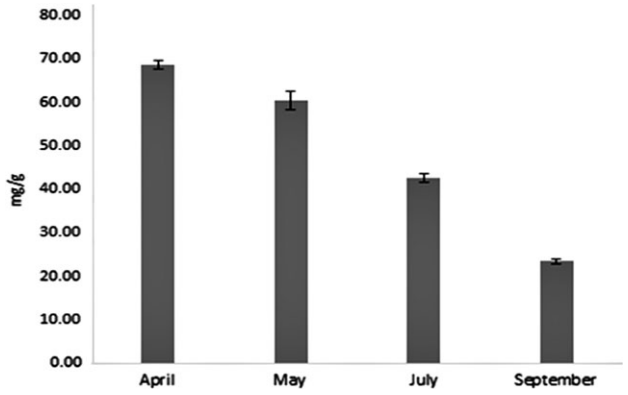

Figure 4. Total concentration (mg/g, dry weight) of hydroxycinnamic acid derivatives identified in walnut leaves, between April and September. Error bars are on the top of each column.

CQAs 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-CQAs with those reported by Pereira *et al.* 2007 (17) (sampling 31 May; average of six different cultivars), an increase of ~47% can be calculated. However, by comparing the sum of 3- and 4-*p*CoQA with data reported in the same work, the increase is still evident but remarkably lower (~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 CGAs by means of UHPLC analysis has been developed and fifteen different CGAs derivatives have been identified and quantified. diCQA were for the first time identified in *J. regia* L. The extraction method, in comparison with other proposed in the literature (e.g. methanol

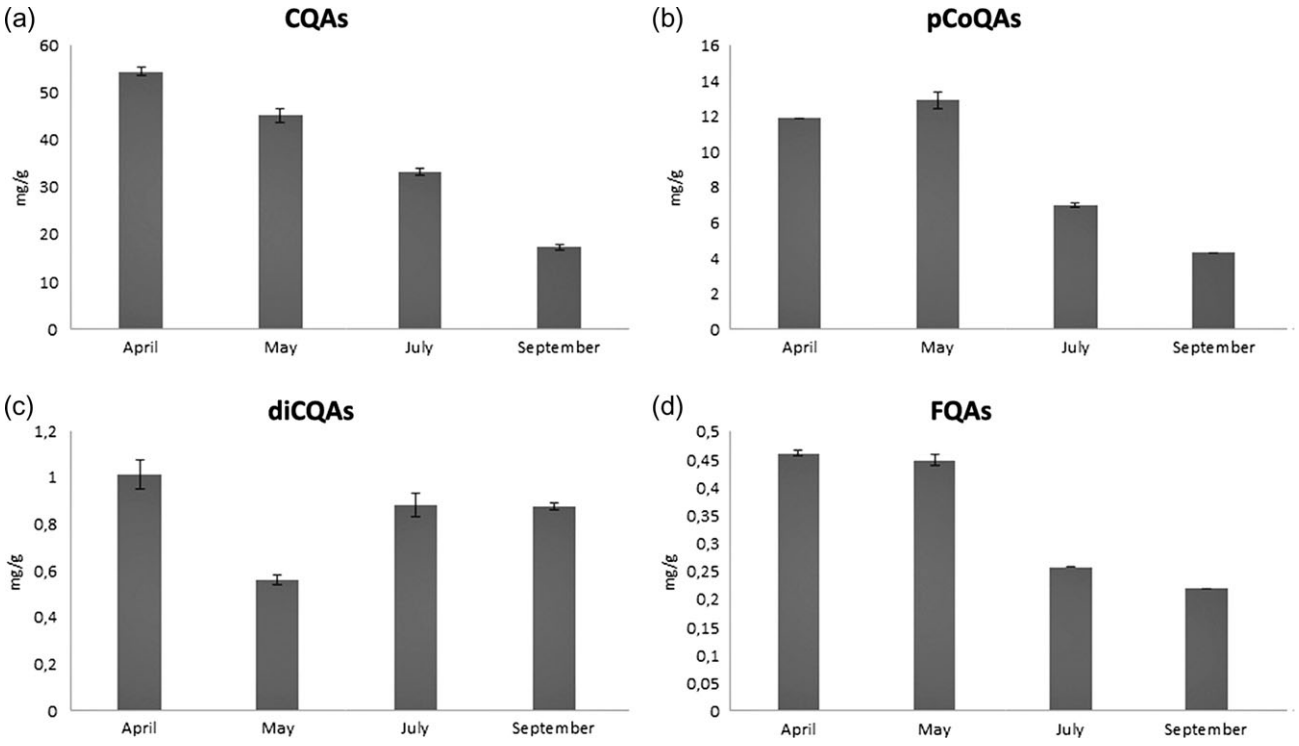

Figure 5. Concentrations of the different chlorogenic acids (mg/g, dry weight) identified in walnut leaves between April and September, i.e. caffeoylquinic acids (CQAs), *p*-coumaroylquinic acids (*p*CoQAs), dicaffeoylquinic acids (diCQAs) and feruloylquinic acid (FQAs). Error bars are on the top of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

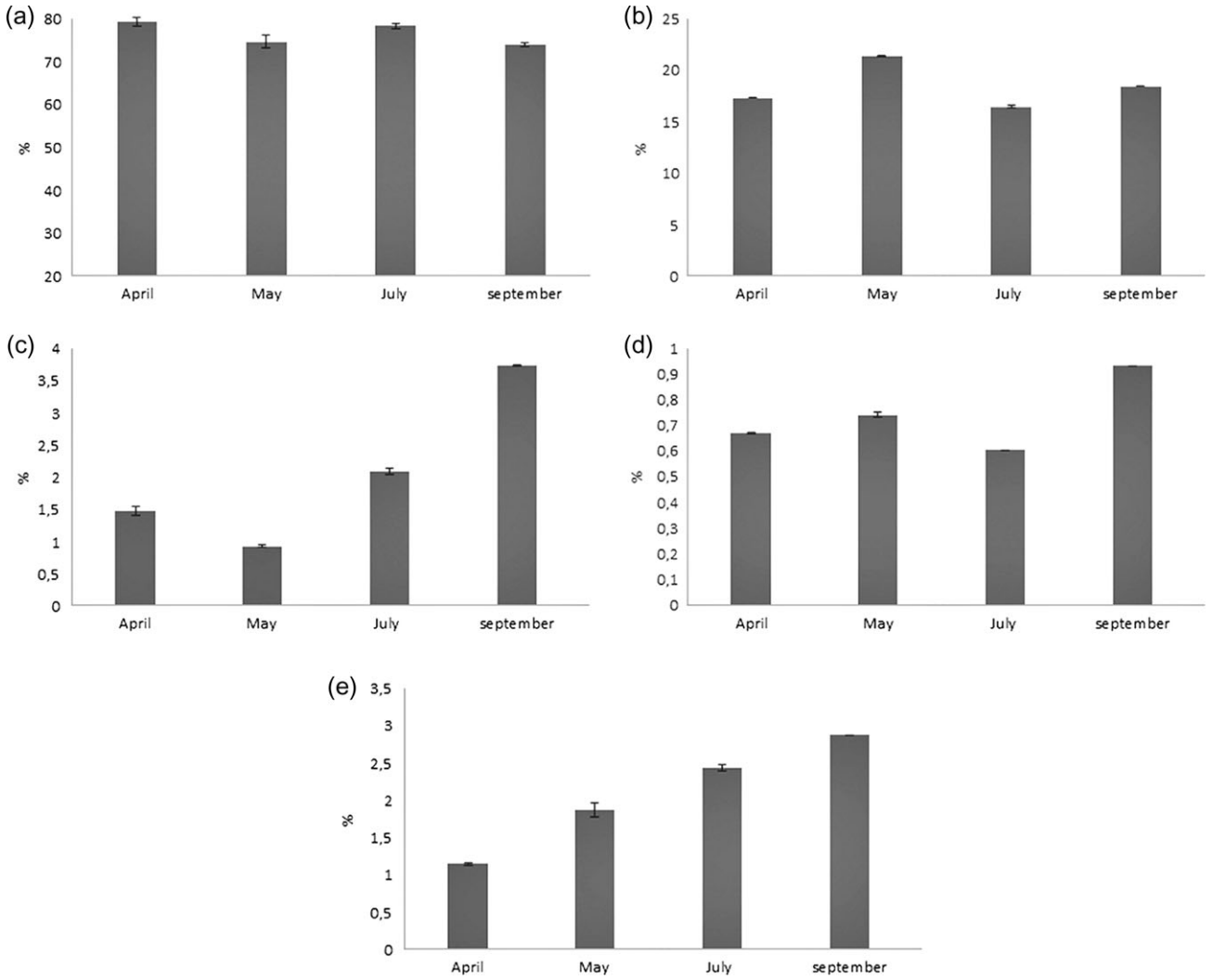

Figure 6. Percentages of the different hydroxycinnamic acid derivatives with respect to the quantified phenolic acid derivatives in walnut leaves, between April and September. (a) % of caffeoylquinic acids (CQAs), (b) % of *p*-coumaroylquinic acids (*p*CoQAs), (c) % of dicaffeoylquinic acids (diCQAs),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

extraction) revealed to be successful since comparable amounts of CGAs 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 CGAs 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 Sklodowska-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., Yuging Yang, A., Gaspar, J., *et al.*; 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., *et al*.; 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., *et al*.; Chlorogenic compounds from coffee beans exert activity against respiratory viruses; *Planta Medica*, (2017); 83: 615–623. 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., *et al*.; Infusion, decotion 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. Journal of AOAC International, (2016); 99: 1197–1203.
10. Clifford, N., Kirkpatrick, J.O., Kuhnert, N., Roozendaal, H.; LC-MS^n analysis of the *cis* isomers of chlorogenic acids; *Food Chemistry*, (2008); 106: 379–385.
11. Gostin, I.; Air pollution stress and plant response. In Kulshrestha, U., Saxena, P. (eds.); *Plant Responses to Air Pollution*. 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 acylquinic acids commonly known as chlorogenic acids; *Journal of Agricultural and Food Chemistry*, (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., *et al*.; Walnut (*Juglans regia* L.) leaves: phenolic compounds, antibacterial activity and antioxidant potential of different cultivars; *Food and Chemical Toxicology*, (2007); 45: 2287–2295.
18. Kunhert, N., Karaköse, H., Jaiswal, R.; Analysis of chlorogenic acids and other hydroxycinnamates in food, plants and pharmacokinetic studies. In Nollet, L.M.L., Toldra, F. (eds.); *Handbook of analysis of active compounds in functional foods*. CRC Press, NY, (2012); pp. 461–512.
19. Kusilic-Bilusic, T., Katalinic, V., Dragovic-Uzelac, V., Ljubenkov, I., Krisko, A., Dejanovic, B., *et al*.; 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; *Journal of chromatographic 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., Colomban, 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; *Journal of Agricultural and Food Chemistry*, (2017); 65: 3589–3590.
27. Santos, A., Barros, L., Calhelha, R.C., Dueñas, M., Carvalho, M., Buelga, S.C., *et al*.; Leaves and decoction of *Junglans 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; *Journal of Agricultural and Food Chemistry*, (2003); 51(10): 2900–2911.
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 derivates 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; *Journal of Agricultural and Food Chemistry*, (2015); 63: 3338–3347.
31. Kahnt, G.; *Trans-cis* equilibrium of hydroxycinnamic acids during irradiation of acqueous 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): 575–580.