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ScienceDirect
Food Chemistry 105 (2007) 1695–1701
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical Nutritional and Clinical Methods

Simultaneous quantification of caffeoyl esters and flavonoids in wild and cultivated cardoon leaves

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Received 4 December 2006; received in revised form 3 April 2007; accepted 3 May 2007

Abstract

Cynara cardunculus is a diploid ($2n = 2x = 34$) species, native to the Mediterranean basin, which belongs to the family of *Asteraceae*. It includes globe artichoke, cultivated cardoon, as well as their progenitor wild cardoon. The species is a source of biophenols and its leaf extracts have been widely used in herbal medicine as hepatoprotectors and choleretics since ancient times. Globe artichoke leaves have been found to be rich in compounds originating from the metabolism of phenylpropanoids however, to our knowledge, the leaf polyphenolic composition of the two other forms within the species, cultivated and wild cardoon, have not yet been properly investigated. Two main classes of polyphenols have been detected by HPLC/DAD and HPLC/MS analyses: caffeoyl esters and flavonoids. The compounds which are the result of esterification of caffeoylquinic acid moiety with succinic acid, previously detected in other members of the *Asteraceae* family, were detected in cardoon leaves for the first time.

Keywords: *Cynara cardunculus* L.; Cardoon; Succinylcaffeoylquinic acid derivatives; Caffeoylquinic acids; Flavonoid; HPLC/DAD/MS

1. Introduction

Cynara is a small genus belonging to the *Asteraceae* (daisy) family; it comprises seven species native to the Mediterranean basin – the crop complex *cardunculus*, which includes the globe artichoke (*Cynara cardunculus* L. var. *scolymus* L.), the cultivated cardoon [*C. cardunculus*. L. var. *altilis* (DC)] and the wild cardoon [*C. cardunculus* L. var. *sylvestris* (Lamk) Fiori], *C. syriaca* Boiss, *C. cornigera* Lindely (syn. *sibthorpiana* Boiss. and Heldr.), *C. algarbiensis* Cosson, *C. baetica* (Spreng.) Pau (syn. *alba* Boiss.), *C. humilis* L. and *C. cyrenaica* Maire and Weiller. As previously suggested by Rottenberg and Zohary (1996), recent molecular studies (Acquadro, Portis, Lee, Donini, & Lanteri, 2005) confirmed that wild cardoon is the ancestor of both cultivated forms, which evolved separately as a result of different selection criteria. Globe artichoke was selected for the production of heads or capitula (immature inflorescences), which are used as a fresh, frozen or canned delicacy all over the world, while cultivated cardoon is used for the production of fleshy leaf-stalks.

In Europe, globe artichoke commercial production is, at present, mainly based on the cultivation of perennial, vegetatively propagated clones, which guarantee high yields of marketable product. In spite of that, molecular studies demonstrate that the varieties in cultivation are highly heterogeneous (Lanteri, Di Leo, Ledda, Mameli, & Portis, 2001; Portis, Mauromicale, Barchi, Mauro, & Lanteri, 2005). This reflects their multi-clonal composition, which is a direct consequence of the limited selection criteria applied by the farmers; a further source of variation might be due to spontaneous mutation, not necessarily detectable at the phenotypic level, and propagated over time in the absence of a meiotic sieve. On the other hand cultivated

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0308-8146/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2007.05.014

and wild cardoon are seed propagated but, being the species allogamous, a wide range of within population genetic variation has been also detected (Portis et al., 2005).

The cultivation of cardoon (*C. cardunculus* var. *altilis* DC) is much less widespread than that of the globe artichoke; it remains of regional importance in Spain, Italy and the south of France, where it is used in traditional dishes. The edible parts of the plant are the fleshy stems which are typically collected in late autumn–early winter (Portis, Barchi, Acquadro, Macua, & Lanteri, 2005); before the cooking are often subjected to a blanching process, i.e. tied together, wrapped with straw and buried underground in order to accentuate the flavour (blanching process), reduce bitterness problem and improve tenderness. Previous studies have shown that both cultivated and wild forms of *C. cardunculus* are a promising source of seed oil of importance with respect to quality and quantity, and the residue flour after extraction is usable as a component of animal feed (Foti, Mauromicale, & Racchia, 1999). Furthermore, wild and cultivated forms of *C. cardunculus* are a source of polyphenol antioxidants and other biopharmaceuticals (Adzet & Puigmacia, 1985; Sevcikova, Glatz, & Slanina, 2002; Slanina et al., 2001; Wang et al., 2003).

Cardoon leaves are traditionally employed as diuretics and hepatoprotectors, cholagogue, choleretic and antidiabetic effects are also reported in popular medicine (Paris & Moyse, 1971).

In a recent work the scavenging effect of cardoon leaf infusion against oxygen radicals, such as superoxide and hydroxyl, was investigated and the antioxidant properties were attributed to the presence of polyphenol compounds (Valentao et al., 2002).

Many studies report the polyphenolic composition of artichoke: in particular the major class of polyphenols are caffeic acid derivatives (Hausler, Ganzera, Abel, Popp, & Stuppner, 2002; Mulinacci et al., 2004) which, in heads, mainly occur as esters with quinic acid; leaves and heads of globe artichoke have been also found to be rich in mono and dicaffeoylquinic compounds and flavonoids (Schutz, Kammerer, Carle, & Schieber, 2004; Wang et al., 2003), these antioxidant molecules are also found in artichoke by-products, as raw artichoke, blanched artichoke and artichoke blanching waters; in particular, in thermally treated samples, a higher content of phenolics is reported and that could be due to the inactivation of polyphenol oxidase (Llorach, Espin, Tomas-Barberan, & Ferreres, 2002). In a recent paper, Koubaa and Damak (2003) have isolated from the seeds of *C. cardunculus* a new dilignan together with the known tracheloside, however, to our knowledge, the polyphenolic pattern of cardoon leaves has not yet been fully investigated. The aim of the present work was to analyse the polyphenolic composition and identify the new antioxidant molecules present in both wild and cultivated leaf samples of cardoon, in order to gain better understanding of their potential use in human health.

2. Materials and methods

2.1. Plant material

Leaves of globe artichoke cv. Romanesco (ART1) and Spinoso sardo (ART2) were obtained from the germplasm living collection of globe artichoke maintained at the C.R.A.S. (Centro Agrario Sperimentale, Sardinia, Italy). Leaves of wild cardoon were collected from three sites in Sardinia [Sassari (CW1), Oristano (CW2) and Nuoro (CW3)] and one in Sicily [Siracusa (CW4)]. Leaves of cultivated cardoon were sampled from plants subjected or not to blanching. The leaf samples CC1 and CC2, obtained from the variety 'Gobbo di Nizza' and 'Bianco avorio', respectively, derived from plants collected in the field in Scalenghe (Piedmont) and subjected to blanching; the samples CC3, obtained from a local selection grown in Ragusa (Sicily), and CC4, obtained from the variety 'Bianco avorio' grown in Scalenghe (Piedmont), derived from plants not subjected to blanching.

The blanching process was carried out about 30 days before harvest and was accomplished by tying up the outer branches a foot or so from the top of the plant and piling soil up around the plant as it grows.

Tissues from three fully developed leaves of each genotype in study were lyophilised within 12 h from collection. The dried materials were comminuted to a powder and kept in sealed bags at room temperature until proceeding to further extractions.

2.2. Extraction of polyphenol compounds

The dried leaf powder of cardoon (2.0 g) was extracted in quadruplicate with 3×50 mL of 70% v/v ethanol adjusted to pH 3.2 with formic acid, at room temperature, under stirring. The extracts were completely defatted with *n*-hexane (4×20 mL), then concentrated under vacuum (Rotavapor 144 R, Büchi, Switzerland), and rinsed with the extraction solvent to a final volume of 25 mL. Hydroalcoholic extracts were stored at −20 °C until use and remained stable for at least 12 months. Chlorogenic acid and cynarin were from Roth (Germany); luteolin 7-*O*-glucoside (cynaroside) and luteolin 7-*O*-rutinoside (scolymoside) and apigenin 7-*O*-glucoside were purchased from Extrasynthèse S.A. (Lyon, Nord-Genay, France). All solvents were HPLC grade and were obtained from Merck (Darmstadt, Germany).

2.3. HPLC/DAD and HPLC/MS analyses of cardoon leaf extract

The analysis was conducted using a HP-1100 liquid chromatograph equipped with a DAD detector and a HP 1100 MSD API-electrospray (Agilent Technologies, Palo Alto, USA) operating in negative ionization mode under the following conditions: gas temperature 350 °C,

nitrogen flow rate 10.0 L min^{-1}, nebulizer pressure 40 psi, quadrupole temperature 40 °C, and capillary voltage 3500 V. Fragmentor operated in the range 80–180 eV. Each sample was filtered through a 0.45 μm filter before HPLC analysis. Polyphenol compounds were separated using a 150 × 4.6 mm (5 μm) Luna RP18 (Phenomenex) maintained at 27 °C. Eluent was constituted by: (A) H_2O (pH 3.2 by formic acid), (B) CH_3CN. A four-step linear solvent gradient system was used, starting from 0% up to 100% of solvent B during a 30-min period, at a flow rate of 0.6 mL min^{-1}. The percentage of B reached 20% from 0 to 5 min, then 30% from 7 to 13 min, and finally, 100% from 20 to 30 min. UV–VIS spectra were recorded in the range 190–600 nm, and chromatograms were acquired at 350, 330, 310, 280 and 254 nm. Identification of individual polyphenols was carried out using their retention times, and both UV and MS spectra. In particular, the identity of 1-*O*-caffeoylquinic acid, 1,5-*O*-dicaffeoylquinic and luteolin-7-*O*-malonylglucoside, was ascertained with isolated compounds from hydroalcoholic extract of artichoke leaves (Romani, Pinelli, Cantini, Cimato, & Heimler, 2006). Quantification of the single polyphenol was directly performed by HPLC–DAD using a four-point regression curve built with the available standards. Curves with a correlation factor $r^2 > 0.998$ were considered. In particular, mono and di-esters of caffeoylquinic acid amounts were calculated at 330 nm using chlorogenic acid and cynarin as reference, respectively. Monosuccinyldicaffeoylquinic and disuccinyldicaffeoylquinic acid derivatives were quantified with cynarin as reference. Luteolin 7-*O*-glucuronide and luteolin 7-*O*-malonylglucoside were calibrated at 350 nm using cynaroside as reference. Finally, apigenin 7-*O*-rutinoside and apigenin 7-*O*-glucuronide were calibrated at 350 nm using apigenin 7-*O*-glucoside as reference.

2.4. Statistical analyses

The determinations of caffeoyl ester and flavonoid contents were carried out in quadruplicate, and results are given as means ± standard deviation (SD).

3. Results and discussion

In the present research, we were mainly interested in detecting possible differences in polyphenolic composition among the three forms within the species *C. cardunculus*, thus we planned to sample a single genotype (plant) for each variety or provenance in study. On the other hand the varieties and provenances were selected on the basis of their molecular genetic differentiation and might be considered as representative of the genetic variation which is present in the species.

The chromatographic profile of a wild cardoon leaf hydroalcoholic extract (CW2), recorded at 330 and 350 nm, is illustrated in Fig. 1. The following compounds were identified: 1-*O*-caffeoylquinic acid; 5-*O*-caffeoylquinic acid (chlorogenic acid); 1,5-*O*-dicaffeoylquinic acid and another dicaffeoylquinic acid derivative; two monosuccinyl dicaffeoylquinic acid derivatives, one disuccinyldicaffeoylquinic acid and the flavonoids, luteolin 7-*O*-rutinoside, luteolin 7-*O*-glucoside, luteolin-7-*O*-malonylglucoside, luteolin aglycone, and apigenin 7-*O*-rutinoside.

The compounds which are the result of esterification of caffeoylquinic acid moiety with succinic acid were previously identified by Chuda, Ono, Ohnishi-Kameyama, Nagata, and Tsushida (1996) from garland (*Chrysanthemum coronarium* L.), and by Maruta, Kawabata, and Niki (1995) from *Arctium lappa* L. roots, both edible plants belonging to the *Asteraceae* family; in particular the following molecules were reported: 1-*O*-,5-*O*-dicaffeoyl-3-*O*-

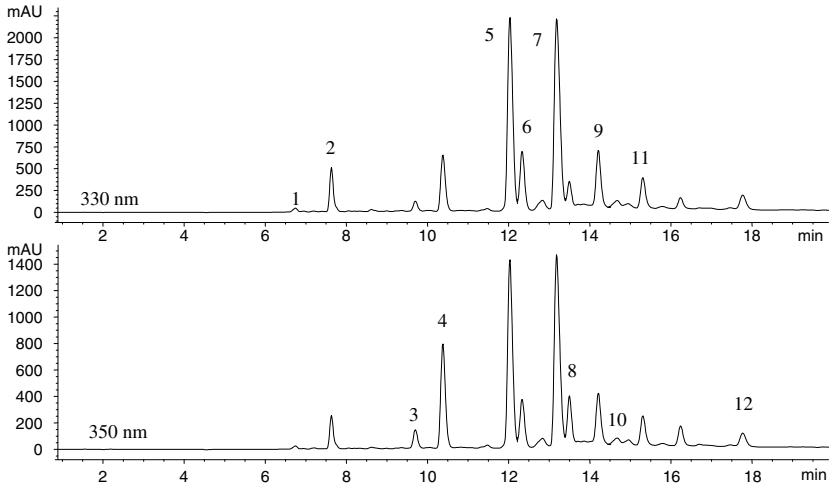

Fig. 1. Chromatographic profile of a wild cardoon hydroalcoholic leaf extract (sample CW2) acquired at 330 and 350 nm. Peak identification numbers: 1, 1-*O*-caffeoylquinic acid; 2, chlorogenic acid; 3, luteolin 7-*O*-rutinoside; 4, luteolin 7-*O*-glucoside; 5, 1,5-*O*-dicaffeoylquinic acid; 6, dicaffeoylquinic acid; 7, succinyldicaffeoylquinic acid; 8, luteolin 7-*O*-malonylglucoside; 9, succinyldicaffeoylquinic acid; 10, apigenin 7-*O*-rutinoside; 11, disuccinyldicaffeoylquinic acid; 12, luteolin.

succinylquinic acid, 1-*O*-,5-*O*-dicaffeoyl-4-*O*-succinylquinic acid, and 1-*O*-,5-*O*-dicaffeoyl-3-*O*-,4-*O*-disuccinylquinic acid. The presence of these esters in cardoon leaves strengthens the belief that succinylcaffeoylquinic acid might be a common compound in the *Asteraceae* family, although this molecule was not previously detected in *C. cardunculus* var. *scolymus* (Romani et al., 2006; Sanchez-Rabaneda et al., 2003). We have hypothesized the presence of these caffeoyl derivatives due to their UV spectra profile and the mass spectrometric fragmentation. As an example, Fig. 2 reports the mass spectrum of the monosuccinyldicaffeoylquinic acid derivative with six major mass signals at 615, 515, 453, 353, 291 and 191 *m*/*z*, corresponding to the quasi-molecular ion $[M-H]^-$ (615 *m*/*z*) and the fragments after two successive losses of caffeoyl moiety (453, 291 *m*/*z*), the fragment after the loss of succinyl moiety (515 *m*/*z*), and two mass signals of caffeoylquinic acid (353 *m*/*z*) and quinic acid (191 *m*/*z*), respectively.

Analogously, the mass signals at 715, 553, 453, 391, 353, 291 and 191 *m*/*z* are consistent with a disuccinyldicaffeoyl substitution of the quinic acid core (Fig. 3).

Table 1 reports the quantitative data of each polyphenol compound for all the analysed samples of cardoon leaves. In the case of dicaffeoylquinic acid derivatives, the sum of amounts of two compounds is reported (5 and 6). Data show that cultivated cardoon leaves, subjected to blanching (CC1 and CC2), have a lower content in polyphenols with respect to both the leaves of wild cardoon and the sample from Sicily (CC3). In particular, the main compounds are caffeoyl quinic derivatives, with chlorogenic acid (5-*O*-caffeoyl quinic acid) being the highest in content. The succinyldicaffeoylquinic derivatives, not previously reported in *C. cardunculus*, were mainly detected in the samples of wild cardoon collected in Sardinia, and the mono derivatives are also present in lower amounts in cultivated cardoon samples.

A wide range of variation in flavonoid content was found among samples (Table 1). High total flavonoid contents were detected in wild (CW4 = 107.99 μmol/g d wt) and cultivated (CC4 = 91.93 μmol/g d wt) cardoon, while in both globe artichoke samples the contents were rather low (see Table 2). Flavonoids are strongly UV-absorbing compounds, and accumulate mainly in the epidermal cells of plant tissues after UV-induction. UV-B (280–320 nm) can penetrate the ozone layer and damage plants, thus, the localization of flavonoids in the epidermal tissues suggests that they can serve as shields against potentially harmful radiation. The activation of flavonoid biosynthetic genes by UV-radiation has been pointed out in a number of studies and the role of flavonoids in UV protection has also been proven using mutants of *Arabidopsis* (Harvaux & Kloppstech, 2001) and petunia (Ryan, Swinny, Markham, & Winefield, 2002).

Interestingly, the two cultivated cardoon samples subjected to blanching and, therefore, not exposed to the light for a month before harvest, showed extremely low amounts of flavonoids (CC1 and CC2), whereas in the sample not submitted to the blanching process large amounts of these compounds were present, in particular three luteolin glycosides. These results confirm the key role of flavonoids in plant stress defence, such as from excess UV-light exposition (Romani et al., 2000). In recent years, many researchers have focused their attention on the antioxidant properties of flavonoids (Gordon,

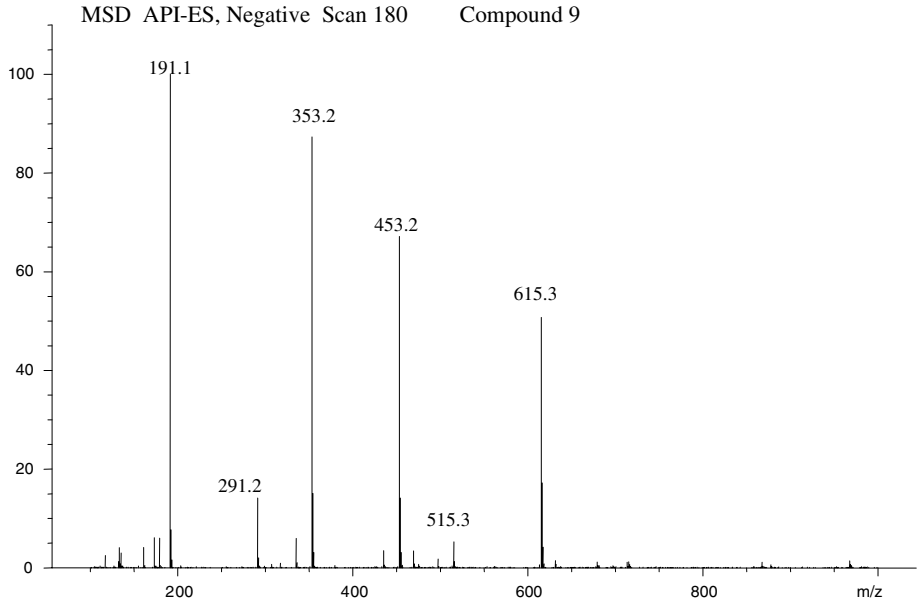

Fig. 2. Negative ion mass spectrum of succinyldicaffeoylquinic acid (compound 9) acquired during the API-electrospray HPLC–MS analysis at the following operating conditions: gas *T* 350 °C, nitrogen flow rate 10.0 L min^{-1}, nebulizer pressure 40 psi, quadrupole temperature 40 °C, and capillary voltage 3500 eV. Mass spectra were recorded in the range 0–1000 AMU.

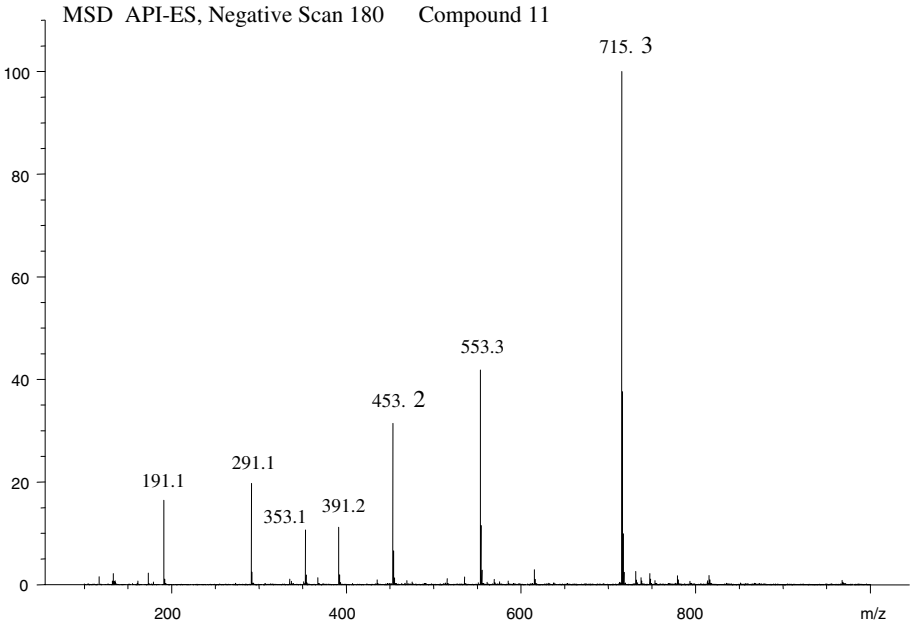

Fig. 3. Negative ion mass spectrum of disuccinyldicaffeoylquinic acid (compound 11) acquired during the API-electrospray HPLC–MS analysis at the following operating conditions: gas T 350 °C, nitrogen flow rate 10.0 L min^{-1}, nebulizer pressure 40 psi, quadrupole temperature 40 °C, and capillary voltage 3500 eV. Mass spectra were recorded in the range 0–1000 AMU.

1990; Rice-Evans, Miller, Bowell, Gramley, & Pridham, 1995) and it has been observed that an increase in their dietary intake may reduce the risk of some chronic diseases (Knekt et al., 2002).

Table 2 reports the relative amounts of each polyphenol sub-class: i.e. monocaffeoylquinic acids, dicaffeoylquinic acids, succinylquinic acids and flavonoids detected in leaf samples of wild and cultivated cardoon, as well as in two artichoke cultivars, Romanesco (ART 1) and Spinoso sardo (ART 2), chosen as reference samples, as artichoke is a very investigated species. From these data it appears that in wild cardoon samples the monocaffeoylquinic acids represent a low percentage (7.9–9.4%) of the total polyphenol content, with the exception of the sample from Sicily (CW4, 23.9%); the cultivated cardoons have higher values (CC1–CC4, 32.7–52.8%), and the artichoke leaf extracts are the richest with percentages ranging from 63.9% and 66.3%, due to their very high level of chlorogenic acid. Moreover, the dicaffeoylquinic acids show little variation and are in higher quantity with respect to the monoesters within wild cardoon samples (CW1–CW4, 31.8–39.2%); they are more variable among cultivated ones (CC1–CC4, 13.5–57.4%) and the artichokes have lower percentage values (12.9–19.3%).

Table 1
HPLC/DAD quali-quantitative analysis of polyphenolic compounds in different samples of wild and cultivated cardoon leaves

Compounds	CW1	CW2	CW3	CW4	CC1	CC2	CC3	CC4
1-*O*-Caffeoylquinic acid	2.38 ± 0.47	1.87 ± 0.51	1.58 ± 0.08	8.23 ± 0.68	2.95 ± 2.03	2.58 ± 0.02	5.07 ± 2.50	9.53 ± 2.63
Chlorogenic acid	11.69 ± 0.21	18.27 ± 3.65	11.32 ± 0.29	61.84 ± 2.09	28.70 ± 9.66	18.82 ± 0.72	48.97 ± 2.26	73.68 ± 4.83
Luteolin 7-*O*-rutinoside	5.19 ± 0.10	6.05 ± 0.47	3.55 ± 0.15	1.10 ± 0.21	nd	0.28 ± 0.24	0.48 ± 0.09	nd
Luteolin 7-*O*-glucoside	14.42 ± 0.40	34.24 ± 1.80	21.15 ± 0.29	27.29 ± 1.87	0.66 ± 0.94	0.35 ± 0.16	28.70 ± 0.19	33.55 ± 8.21
Dicaffeoylquinic acids	55.60 ± 9.76	80.57 ± 15.65	48.90 ± 1.61	114.57 ± 4.25	50.62 ± 9.54	9.44 ± 0.13	51.15 ± 2.37	29.17 ± 9.26
Succinyldicaffeoylquinic acid	29.16 ± 5.89	66.25 ± 8.79	24.86 ± 0.80	nd	2.84 ± 0.12	6.19 ± 0.38	nd	8.67 ± 0.41
Luteolin 7-*O*-malonylglucoside	12.85 ± 2.04	15.07 ± 2.34	7.35 ± 0.08	14.62 ± 0.41	1.11 ± 0.50	1.56 ± 0.28	18.59 ± 0.62	43.00 ± 0.50
Succinyldicaffeoylquinic acid	7.94 ± 0.44	16.39 ± 0.36	8.59 ± 0.47	nd	0.65 ± 0.92	0.94 ± 0.23	nd	2.20 ± 0.16
Apigenin 7-*O*-rutinoside	1.23 ± 0.02	2.65 ± 0.41	1.12 ± 0.07	1.11 ± 0.11	0.59 ± 0.11	0.08 ± 0.07	1.39 ± 0.214	nd
Disuccinyl dicaffeoylquinic acid	2.57 ± 1.83	6.51 ± 3.28	4.58 ± 0.02	nd	nd	nd	nd	nd
Luteolin	6.36 ± 0.16	5.41 ± 1.97	12.61 ± 0.27	0.80 ± 0.01	0.02 ± 0.01	0.28 ± 0.08	2.02 ± 0.28	1.68 ± 0.08
Luteolin 7-*O*-glucuronide	nd	nd	nd	34.61 ± 1.48	nd	nd	nd	13.70 ± 2.40
Apigenin 7-*O*-glucuronide	nd	nd	nd	23.72 ± 0.06	nd	nd	2.31 ± 0.01	nd
Apigenin	nd	nd	nd	4.74 ± 0.14	nd	nd	6.79 ± 0.54	nd

Mean values ± SD ($n = 4$) expressed as μmol/g d wt.
nd = not detected; CW = wild cardoon; CC = cultivated cardoon.

Table 2
HPLC/DAD quantitaive analysis of total polyphenol content (data are expressed as μmol/g d wt) and percentages of each polyphenol sub-class in the different leaf extracts of cardoon (wild and cultivated samples)

	Total polyphenols (μmol/g d wt)	Monocaffeoyl quinic acids (%)	Dicaffeoyl quinic acids (%)	Succinyl dicaffeoyl quinic acid (%)	Flavonoids (%)
CW1	149.39	9.4	37.2	26.6	26.8
CW2	253.28	7.9	31.8	35.2	25.0
CW3	145.61	8.9	33.6	26.1	31.4
CW4	292.63	23.9	39.2	0	36.9
CC1	88.14	35.9	57.4	3.9	2.7
CC2	40.52	52.8	23.3	17.6	6.3
CC3	165.47	32.7	31.0	0	36.4
CC4	215.18	38.7	13.5	5.1	42.7
ART 1	49.83	63.9	19.3	0	16.7
ART 2	100.19	66.3	12.9	0	20.7

CW = wild cardoon; CC = cultivated cardoon; ART = artichoke.

It is important to point out that the succinylcaffeoylquinic acid compounds have the highest variation among the analysed accessions: the percentages are higher in the wild cardoons (26.1–35.2%), with the exception of the sample coming from Sicily, with large variation and intermediate values in the cultivated ones (3.9–17.6%), while totally absent in the artichoke leaf extracts and in both Sicilian samples.

Finally, flavonoids are very different among the samples: in wild cardoons and unblanched cultivated cardoon their percentage is high (25.0–42.7%) with respect to artichokes (16.7–20.7%) and, as reported above, very low in cultivated cardoon subjected to the blanching process (2.7–6.3%).

The composition of phenolic compounds in plant tissues is a consequence of an interaction between genotype and environment and may greatly vary also at intra-specific level. In a previous work (Lanteri et al., 2004) aimed at evaluating molecular genetic divergence among wild and cultivated forms of *C. cardunculus* by means of AFLP (amplified fragment length polymorphism) markers, it was found that the highest genetic distance occurs between wild cardoon and globe artichoke, while cultivated cardoon occupies an intermediate position. Data on the polyphenolic composition of the samples in analysis do not appear directly related to molecular genetic distances, thus confirming that environmental conditions play a crucial role which is even more important than genotype in determining plant tissue polyphenol composition.

An interesting and challenging aspect for future research is to clarify the effect of genotype *versus* environmental interactions on the different classes of polyphenols as, by combining the knowledge gained from the studies concerning the effects of different polyphenol compounds on human health, it might be possible to produce plants with improved health properties.

Acknowledgements

The authors thank *Provincia di Grosseto* for providing the Tuscany accessions and for supporting by funds this research and *Cassa di Risparmio di Firenze* for the contribution to the acquisition of a part of the instrumentation used for this work. The technical support of Sandra Gallori is greatly acknowledged.

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