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Short communication

Hydroxycinnamic acid derivatives occurring in *Cichorium endivia* vegetables

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ABSTRACT

The hydroxycinnamic acid derivatives found in *Chicorium endivia* var. *crispum* and var. *latifolium* polyphenolic extracts were detected and characterized by high-performance liquid chromatography (HPLC) combined with photodiode array detector (DAD) and electrospray ionization-tandem mass spectrometry (ESI-MS/MS). The method provides data (molecular weight and diagnostic fragment ions) on the molecular structure of compounds. The combined approach enabled identification of four hydroxycinnamic derivatives in each chicory extract; three derivatives (5-*O*-caffeoylquinic acid, 3,4-di-*O*-caffeoylquinic acid, and 5-*O*-feruloylquinic acid) were found in both chicories, while 3,5-di-*O*-caffeoylquinic acid was typical of var. *crispum* and *cis*-caftaric acid of var. *latifolium*.

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1. Introduction

Over the last 20 years consumption of fresh vegetables and fruit has been encouraged due to their protective action against several dangerous and diffuse chronic diseases. Such protective effect is thought to be related to their content in fibers, antioxidant vitamins, carotenoids as well as polyphenols [1]. The protective action of polyphenols has been attributed to their antioxidant properties and to their ability to transfer electron-free radicals and to chelate metal catalysts [2]. More recently, an influence on the expression of a number of genes involved in inflammatory processes [3], on the production of radical-generating enzymes [4] and the synthesis and activity of antioxidant enzymes has been documented [5,6].

Polyphenolic compounds are found in many plants, but concentrations and chemical forms vary markedly depending on the plant source. The most abundant polyphenolic groups are phenolic acids, flavanoids including flavonols, flavan-3-ols, flavanones, flavones, anthocyanidins, isoflavons, and stilben derivatives.

Phenolic acids, which occur in many types of fruit and vegetables, including green and red salad [7–10], comprise hydroxycinnamic acids (e.g. *p*-coumaric, ferulic, or caffeic acid); these bind to other compounds (e.g. (−)-quinic acid and *cis*-tartaric acid), generating different derivative groups of which chlorogenic acids (CGA) are the most extensively studied [7]. Specimen structures are shown in Fig. 1.

Liquid chromatography–mass spectrometry (LC–MS) is held to be the most powerful approach for qualitative identification of polyphenolic compounds [11]. LC–MS has been widely used to identify all kinds of phenolic acids in different food matrices, including coffee beans [9,12], strawberry, dried plums, apple pomace, turnip top, fruit juices, and fruit drinks [13–17].

In a previous study we found strong antiradical properties in the polyphenolic fraction of two *Cichorium* salads, *Cichorium endivia* var. *crispum* and var. *latifolium* [18]. *C. endivia* L., a member of the sunflower family (asteraceae, compositae), is a typical Mediterranean plant indigenous to Europe, Western Asia and North America [19]. Both varieties contain a number of chlorogenic acid derivatives [20,21], which could be responsible for the antioxidant properties documented in their phenolic extract; however their composition in hydroxycinnamic acid derivatives has not been exhaustively described. The aim of this work was to identify and define the chemical structure of the hydroxycinnamic acid derivatives found in *C. endivia* var. *crispum* and var. *latifolium* using high-performance liquid chromatography (HPLC) coupled with photodiode array detector (DAD) and electrospray ionization-tandem mass spectrometry (ESI-MS/MS).

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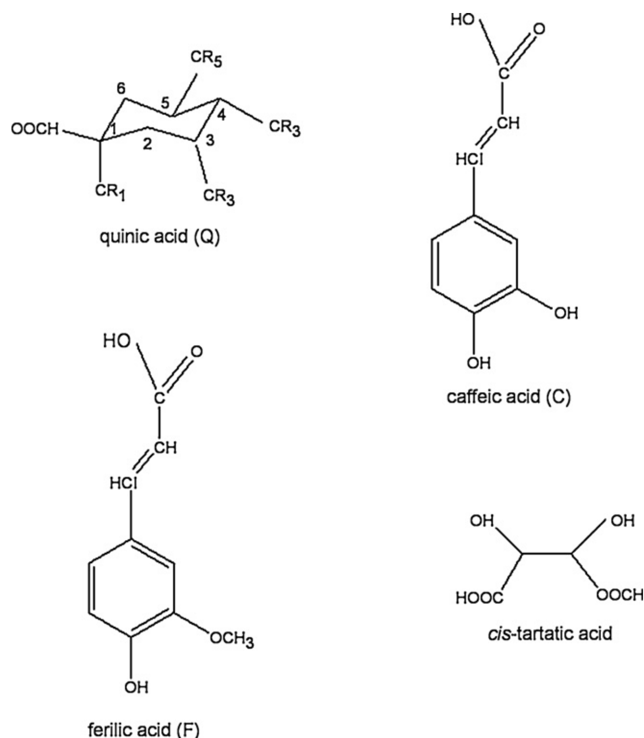

Fig. 1. Structures of selected chlorogenic acids (IUPAC numbering).

2. Experimental

2.1. Chemicals

HPLC-grade and analytical-grade organic solvents, HPLC–MS grade water and methanol, and the standard compound 5-*O*-caffeoylquinic acid (chlorogenic acid, 5-CQA) were purchased from Sigma–Aldrich (Milan, Italy). HPLC-grade water was prepared with a Milli-Q water purification system (Millipore Corp., Billerica, MA, USA).

2.2. Plant materials

Five clumps of *C. endivia* var. *crispum* and five of var. *latifolium* were purchased at a local market. Fresh leaves (20 g) were washed, cut into small pieces, suspended in 12.5 ml of MeOH–HCOOH (99:1, v/v) and shaken for 1 h in an ice bath in the dark. The mixture was then centrifuged for 5 min at 8750 g; the insoluble residue was re-extracted three times with the same solvent. The pale green extracts were pooled, filtered through a 0.45-μm Millipore membrane of cellulose acetate/cellulose nitrate mixed esters and then directly analyzed by HPLC–DAD and HPLC–ESI-MS/MS.

2.3. HPLC conditions

The LC equipment (Thermo Fisher Scientific, San Jose, CA, USA) comprised a Surveyor LC pump Plus, an autosampler (Finningan Surveyor) with a 25-μl loop and a DAD detector. Separation of hydroxycinnamic acid derivatives was performed on a Gemini® C18 analytical column (150 mm × 2.0 mm i.d., 5 μm, Phenomenex, Torrance, CA, USA) connected to a Hypersil Gold C18 guard column (10 mm × 2.1 mm i.d., 5 μm, Phenomenex, Torrance, CA, USA), using a gradient of increasing methanol concentrations in water acidified with 0.1% formic acid (v/v) (Table 1). Column and autosampler temperatures were held constant at 4 °C. The mobile phase flow rate was adjusted to 0.3 ml/min; the chromatogram was recorded at 280, 320, 350, and 370 nm. Spectral data were acquired in the range of 200–600 nm for all peaks.

Table 1
Solvent gradients

Time (min)	A (%)	B (%)
0	98	2
10	95	5
60	60	40
70	40	60
80	0	100
90	0	100

Data were acquired and processed using the Thermo Fisher Scientific Excalibur 2.0 software.

2.4. Mass spectrometry conditions

A Finnigan LCQ Advantage ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) was connected to the HPLC instrument via an ESI interface for HPLC–MS analysis. The ion trap was set to operate in data-dependent, zoom scan, full scan, and MS^n mode to obtain fragment ion m/z.

MS operating conditions (negative ion mode) had previously been optimized by flow injection analysis, using 5-CQA (5 ppm water acidified with 0.1% formic acid–methanol solution, 50:50, v/v) to 3.5 kV ionization voltage, a capillary temperature of 260 °C, a sheath gas flow rate of 50 arbitrary units, and an auxiliary gas flow rate of 20 arbitrary units; 5-CQA was fragmented with 35% collision energy. For full MS analysis, spectra were recorded in the range of 100–1000 m/z. The width used to isolate precursor ions was set at 3.0 units. MS^n data were acquired in the automatic data-dependent mode.

Thermo Fisher Scientific Excalibur 2.0 software was used for data acquisition and processing.

2.5. Preparation of the standard solution and method validation

2.5.1. Standard solution and calibration curve

An external standard method was used for quantitation. Briefly, 1 mg of the 5-CQA standard was accurately weighed and dissolved in a 10-ml volumetric flask with methanol to obtain a stock solution, and stored in a refrigerator at −20 °C until use. Working standard solutions were prepared daily by dilution with distilled water–methanol (50:50, v/v) in the concentration range 0.5–100 μg/ml [20]; the calibration curve was determined on five levels of concentration with three injections per level. LC chromatogram peak areas were plotted against the known concentrations of the standard solutions to establish calibration equations. A linear regression equation was calculated by the least squares method [22].

2.5.2. Limit of detection

The detection limit (LOD) was assessed by diluting the standard solution using a concentration sequence. The LOD was calculated as the lowest amount of analyte required to obtain a signal/noise ratio of 3:1 [22].

2.5.3. Limit of quantification

The limit of quantification (LOQ) was calculated as the lowest concentration of analyte required to yield a signal/noise ratio of 10:1 [22].

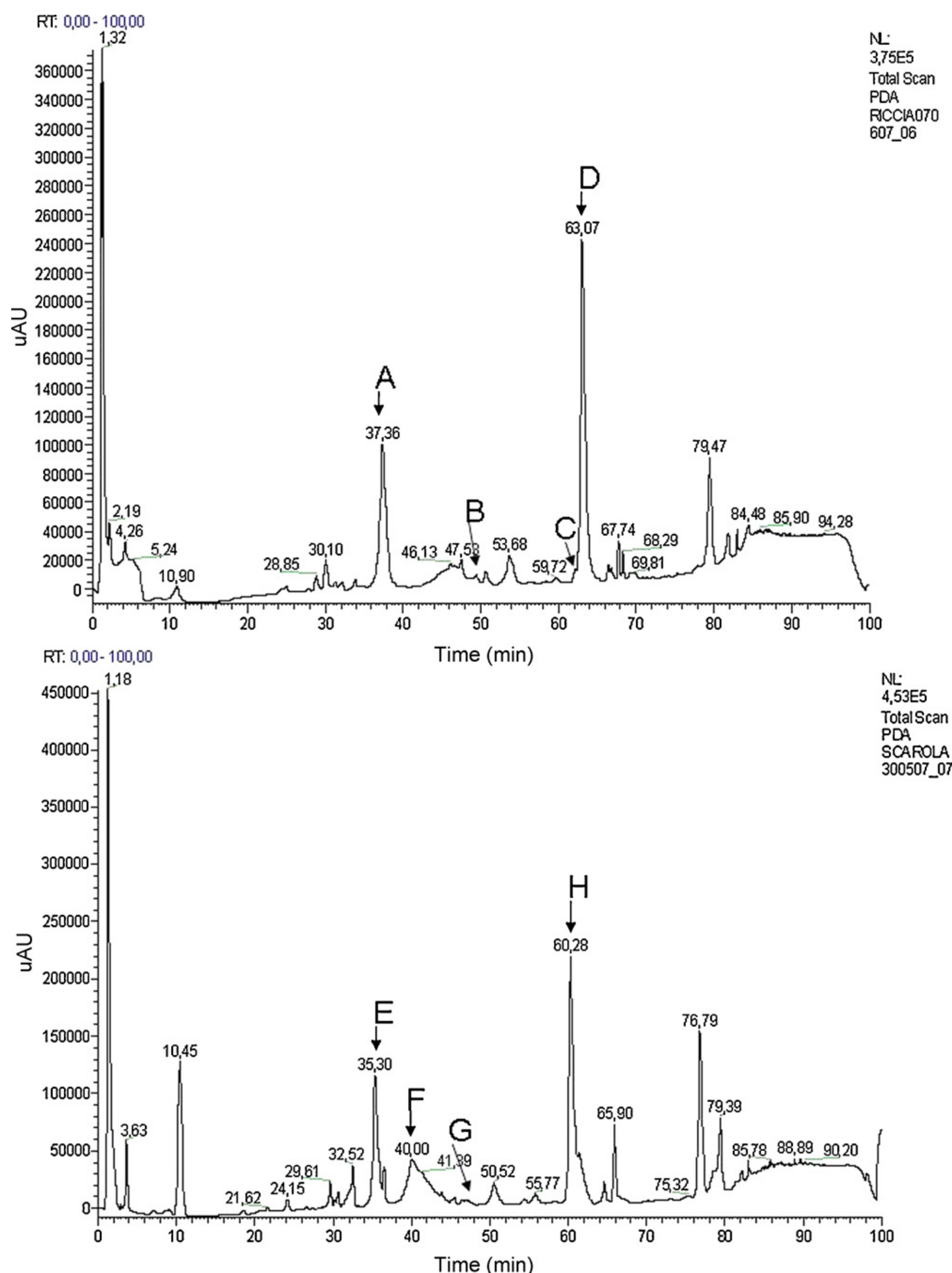

Fig. 2. LC–UV-DAD profiles of *Chicorium endivia* var. *crispum* (A) and var. *latifolium* (B) extracts (320 nm).

2.5.4. Repeatability

Three different concentrations of the 5-CQA standard solution (5, 25, and 50 μg/ml) were used for intra- and interday repeatability testing [22]. The areas of the three consecutive injections performed at each concentration on 3 different days were used to calculate %RSD interday repeatability (%RSD interday). Intraday repeatability data were the areas of six non-consecutive injections performed at each concentration on the same day (%RSD intraday).

2.5.5. Recovery

HPLC accuracy was determined by recovery tests analyzing three concentrations (5, 25, and 50 μg/ml) of the 5-CQA standard. Tests were conducted in triplicate. Analyte recovery was expressed as the ratio of mean measured concentration to nominal value [22].

2.6. Identification

The extract 5-CQA peak was assigned based on retention time and UV spectra of the standard compound using HPLC–DAD; the peak was further confirmed by HPLC–ESI-MS/MS. The peaks of the other hydroxycinnamic acid derivatives were identified by HPLC–ESI-MS/MS.

3. Results and discussion

The polyphenolic compounds occurring in *C. endivia* var. *crispum* and var. *latifolium* were extracted and analyzed using an HPLC–DAD method. UV spectra indicated the presence of some hydroxycinnamic acid derivatives in the free acid form that were then identified using HPLC–ESI-MS/MS.

Table 2
Negative ion ESI mass spectra of the hydroxycinnamic acid derivatives of *C. endivia* var. *crispum* and *latifolium* extracts

Compound	Vegetable *C. endivia*	$[M-H]^-$ *m/z*	HPLC–ESI(−)-MS^2 *m/z* (% base peak)	Structure
A	var. *crispum*	353	191 (100)	5-*O*-Caffeoylquinic acid
E	var. *latifolium*		179 (5)	
B	var. *crispum*	367	191 (100)	5-*O*-Feruloylquinic acid
G	var. *latifolium*		173 (5)	
C	var. *crispum*	515	353 (100) 191 (10) 179 (4)	3,5-Di-*O*-caffeoylquinic acid
D	var. *crispum*	515	353 (100)	3,4-Di-*O*-caffeoylquinic acid
H	var. *latifolium*		173 (24) 179 (17) 335 (11)	
F	var. *latifolium*	311	149 (100) 179 (41) 135 (3)	*cis*-Caftaric acid

3.1. Optimization of chromatographic conditions and HPLC–DAD method validation

In this work, during some tentative gradient elution procedures, a binary mobile phase (water–methanol) was chosen. Given the presence of polyphenolic compounds in the extracts, a small amount of formic acid was added to the mobile phase to reduce compound ionization and polarity. The optimum mobile phase was obtained with solvent A (water acidified with 0.1% formic acid, v/v) and solvent B (methanol) in the gradient mode, as shown in Table 1, with a flow rate of 0.3 ml/min. To gain additional information on the phenolic composition of the two extracts, the UV spectra of all peaks recorded in the chromatogram were investigated by DAD. Simultaneous monitoring was performed at 280 nm (flavanols), 320 nm (hydroxycinnamic acids), 350 nm (flavones), and 370 nm (flavonols); all spectra were recorded from 200 to 600 nm.

HPLC–DAD method validation was carried out using the 5-CQA standard. Five standard solutions from 0.5 to 100 μg/ml were assayed by sequentially injecting each of the five concentrations from lowest to highest three times. Peak area versus concentration plots were obtained and least squares regression analysis was used to fit lines to the data. The following regression line was obtained: $y = 31{,}595x + 12{,}427$ with a correlation coefficient of 0.9969 over the concentration range. The relative standard deviations showed acceptable reproducibility, with RSDs ranging from 0.27% to 1.09%. The LOD and LOQ were determined by testing dilutions of the lowest 5-CQA concentration. The LOD was 0.1 μg/ml and the LOQ 0.260 μg/ml. As regards method repeatability, intraday repeatability RSDs ($n = 6$) of 5-CQA were 2.25%, 1.25%, and 1.13% for 5, 25, and 50 μg/ml, respectively, while interday repeatability RSDs (3 days, $n = 9$) at the same concentrations were 2.09%, 1.44%, and 1.35%. The accuracy of the HPLC–DAD method was determined by recovery tests performed using three 5-CQA standard methanolic solutions at 5, 25, and 50 μg/ml concentrations. Recovery ranges were, respectively 96–106%, 97–103%, and 96–105% for the three concentrations, with RSDs less than 18%.

3.2. Identification of hydroxycinnamic acid derivatives

The HPLC–DAD profiles of *C. endivia* var. *crispum* and var. *latifolium* recorded at 320 nm are shown in Fig. 2. Both exhibited numerous peaks with distinct absorption maxima, due to different polyphenolic species. Four peaks in each extract (A, B, C, and D for var. *crispum* and E, F, G, and H for var. *latifolium*) had identical UV spectra, characterized by absorption bands at 320–325 nm and 242 nm and by a sharp diagnostic shoulder at 290–300 nm that is typical of compounds containing a hydroxycinnamic moiety.

5-*O*-Caffeoylquinic acid (peak A in var. *crispum* and peak E in var. *latifolium*) was identified by comparing its retention time and UV spectra with that of the standard compound. Its mean concentration, determined in five different clumps per variety analyzed in triplicate, was 92.02 ± 1.46 μg/ml of extract in var. *crispum* and 69.78 ± 1.97 μg/ml in var. *latifolium*.

The chemical structure of 5-*O*-caffeoylquinic acid was confirmed by ESI-MS/MS analysis. In fact, its full negative ion mass spectra exhibited intense $[M-H]^-$ ion at *m/z* 353 (MW 354), consistent with a caffeoylquinic acid derivative; the molecular ion fragmentation yield fragment ions corresponded to the quinic acid (base peak, *m/z* 191) and caffeic acid (*m/z* 179) moieties; 5-CGA gives the same base peak as 3-CGA but differed from it by a comparatively more intense caffeic acid-derived ion at *m/z* 179: in fact, a mean intensity of 5% of the caffeic acid-derived ion allowed assignment to 5-*O*-caffeoylquinic acid, as reported by Clifford [23,24] and Schütz [25].

Investigations of the chemical structure of the other hydoxycinnamic acid derivatives by HPLC–ESI-MS/MS and the comparison of obtained mass data with those ones reported in literature [23–25] allowed structure assignment (Table 2). In *C. endivia* var. *crispum* extract, the molecular ion $[M-H]^-$ of compound B was detected at *m/z* 367 (MW 368). MS^2 analysis showed the presence of two main fragment ions at *m/z* 191 and at *m/z* 173; the first fragment ion, corresponding to the base peak, was attributed to the quinic acid moiety; the second appears to be attributable to the ferulic acid moiety. The compound was therefore identified as a feruloylquinic acid; the fact that *m/z* 191 is the base peak led to its identification as 5-*O*-feruloylquinic acid isomer. Compound C partly co-eluted with compound D in the UV profile; both had $[M-H]^-$ at *m/z* 515 (MW 516) as well as fragment ions at *m/z* 353, due to chlorogenic acid after expulsion of one caffeic acid moiety in the MS^2 experiment, enabling its attribution to di-caffeoylquinic acid isomers. The two isomers can be differentiated by their secondary peaks; identification of *m/z* 191 (mean intensity, 10%), and 179 (mean intensity, 4%) in MS^2 spectra of compound C besides the base peak at *m/z* 353 allowed the compound to be identified as 3,5-di-*O*-caffeoylquinic acid [25]. The presence of *m/z* 335, besides *m/z* 353, *m/z* 173 and 179 in MS^2 spectra of compound D was characteristic of 3,4-di-*O*-caffeoylquinic acid [7,23].

The *latifolium* variety contained three other hydroxycinnamic derivatives besides 5-CQA; the MS and MS^2 spectra of compound G and H evidenced the molecular ions and corresponding fragmenta-

tion ions found in compounds B and D (var. *crispum* extract), i.e. 5-*O*-feruloylquinic acid and 3,4-di-*O*-caffeoylquinic acid, respectively. Analysis of compound F disclosed a molecular ion at *m*/*z* 311 (MW 312), fragments of both tartaric acid (base peak, *m*/*z* 149) and caffeic acid (*m*/*z* 179), and a low signal produced by the caffeic acid decarboxylation (*m*/*z* 135). The compound was thus identified as caftaric acid; in particular it was its *cis*-isomer, because of the lack of *m*/*z* 623 in MS spectra, corresponding to adduct formation of two individual molecules of caftaric acid, typically of the *trans*-isomer [25].

4. Conclusions

The method used in this study proved accurate and reproducible, and suitable for the exhaustive qualitative evaluation of *Chicorium* vegetable extracts. HPLC–DAD profiles identified several compounds belonging to different polyphenolic classes, including hydroxycinnamic acid derivatives; combined use of HPLC–DAD and ESI-MS/MS enabled molecule identification and discrimination between different isomers of the same class.

Our findings indicate that the hydroxycinnamic acid derivative composition of two varieties of a common Mediterranean plant (*C. endivia*) differed in one constituent. They shared three hydroxycinnamic acid derivatives, 5-*O*-caffeoylquinic acid, 3,4-di-*O*-caffeoylquinic acid and 5-*O*-feruloylquinic acid, while the *crispum* variety also contained 3,5-di-*O*-caffeoylquinic acid and var. *latifolium* contained *cis*-caftaric acid. In addition, the mean 5-*O*-caffeoylquinic acid concentration determined in var. *crispum* was significantly higher ($p < 0.01$) than that determined in var. *latifolium*.

Research is under way to identify and quantify the other characteristic polyphenol compounds of the two vegetables.

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