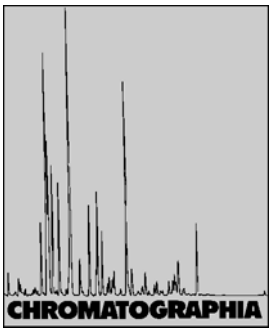
Optimization of the Chromatographic Determination of Polyphenols in the Aerial Parts of *Cichorium intybus* L.



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Key Words

Column liquid chromatography-DAD and column liquid chromatography-MS
Flavonoids
Polyphenols
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Cichorium intybus L.

Summary

The aim of this work was to contribute to the phytochemical characterization of *Cichorium intybus* L. var. *silvestre*, chicory. Semi-preparative HPLC analysis was applied to an extract of fresh wild chicory leaves to separate and collect the main polyphenolic compounds.

HPLC-diode-array detection (DAD), HPLC-MS, and NMR were used for the complete chemical characterization of all the compounds isolated. The molecules characterized were monocaffeoyl tartaric acid, chicoric acid, monocaffeoyl *p*-hydroxycinnamoyl tartaric acid, caffeoyl feruloyl tartaric acid, chlorogenic acid, quercetin-3-*O*-glucuronide, luteolin-7-*O*-glucuronide, and quercetin-3-*O*-glucoside.

The chromatographic behaviour of the main components of the extract of the leaves has been compared on three different stationary phases – LiChrosorb RP18, Luna C_{18}, and Luna Phenyl-Hexyl.

Introduction

Chicory (*Cichorium intybus* L.) is a Mediterranean herbaceous plant belonging to the Asteraceae family. This plant, indigenous to Europe, western Asia, Egypt, North America, and Italy [1], is mainly used as a food product, diffused as a pot herb or as a salad plant. In recent years cultivation of this plant has increased substantially, because it can be produced in the autumnal months when some types of vegetable are not available. Many Italian regions are involved in this cultivation. According to Bischoff's classification there are three varieties of *Cichorium intybus* L. – var. *silvestre*, or wild chicory; var. *sativus*, or chicory from roots; and var. *foliosum*, or chicory from leaves. Nowadays chicory roots are used as an additive to coffee (in Belgium, France, and, especially, in the USA); in the past the peeled, sliced, and roasted roots were mainly employed for the preparation of coffee-like drinks.

Since antiquity chicory has also been used as a medicinal plant. At the end of the first century A.D. Dioscoride advised the use of decoctions of the roots for liver diseases, and Galeno described the use of infusions of the leaves for eye diseases. Most studies on the pharmacological activity of chicory have focused on the roots as source of inulin, although other studies have provided evidence of diuretic, laxative, hypoglycaemic, and bradycardic effects [2]. In the past the leaves were used in laxative preparations, diuretic tea, and as bitter infusions or decoctions [3]; nowadays the use of chicory as a medicinal plant is reported in German monographs only [4].

As far as we are aware the literature contains few papers focusing on phytochemical investigation of *Chicorium intybus* L. A brief review by Dinelli and Morelli [5] describes the presence of bitter sesquiterpenes, e. g. lactucin and lactucopicrin, and coumarins, e. g. ciicorin and aesculin, in the radix juice; derivatives of hydroxycinnamic acid, including chicoric acid, and flavonoids such as apigenin, quercetin, and luteolin glycosides, are distributed mainly in the aerial parts of the plant.

The aim of this work was to contribute to the phytochemical characterization of *Cichorium intybus* L., var. *silvestre*, by investigating the polyphenol content in the fresh leaves. HPLC-DAD, HPLC-MS, and NMR, mainly, were used for the complete chemical characterization of all the isolated compounds. The chromatographic behaviour of the main components of the leaves on three different stationary phases has been compared.

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Table I. Linear solvent gradient used for analytical HPLC-DAD analysis.

H_2O (%)	CH_3CN (%)	Time (min)
100.0	0.0	0.10
85.0	15.0	20.00
85.0	15.0	25.00
75.0	25.0	35.00
75.0	25.0	43.00
0.0	100.0	53.00
0.0	100.0	57.00

Table II. Linear solvent gradient used for semi-preparative HPLC analysis.

H_2O (%)	CH_3CN (%)	Time (min)
90.0	10.0	0.1
86.0	14.0	10.0
86.0	14.0	15.0
78.0	22.0	7.0
55.0	45.0	5.0
55.0	45.0	7.0
0.0	100.0	5.0
0.0	100.0	3.0

Experimental

Materials

All solvents used were HPLC-grade; CH_3CN and MeOH for HPLC were from E. Merck (Darmstadt, Germany) and water was from J. T. Baker (Italy). CD_3OD (purity 99.8%; Euriso-top, France) was used as solvent for NMR analysis. The wild chicory sample was picked in the countryside near Florence in May 1999.

Sample Preparation

Wild chicory leaves (250 g) were extracted by stirring for two days with 8:2 (*v/v*) EtOH-H_2O (2 L; pH adjusted to 2 by addition of HCOOH). The ethanol was then evaporated under vacuum and the solution obtained was extracted with *n*-hexane and $CHCl_3$, to remove lipophilic compounds, and finally with EtOAc. The EtOAc extract was used for semi-preparative HPLC analysis.

Analytical Techniques and Equipment

HPLC-DAD Analysis

HPLC analysis was performed with a Hewlett-Packard (HP; Palo Alto, CA, USA) 1090L liquid chromatograph equipped with a DAD detector. The columns used were:

(i) 4.6 mm i. d. × 250 mm, 5 µm particle LiChrosorb RP18 (Merck) equipped with a 4 mm i. d. × 10 mm precolumn packed with the same stationary phase;
(ii) 150 mm × 3.00 mm i. d., 5 µm particle Luna C_{18} (Phenomenex, Germany) equipped with a 4 mm × 3.0 mm i. d. C_{18} precolumn; and
(iii) 250 mm × 4.6 mm i. d., 5 µm particle Luna Phenyl-Hexyl (Phenomenex) equipped with a 4 mm × 3.0 mm i. d. precolumn packed with the same stationary phase.

The column oven was maintained at 26 °C. The mobile phase was a linear gradient prepared from H_2O (pH adjusted to 3.2 by addition of H_3PO_4) and CH_3CN; the composition of the gradient is reported in Table I.

UV-Vis spectra were recorded in the range 190–450 nm and chromatograms were acquired at 230, 254, 280, 330, and 360 nm.

Semi-Preparative HPLC

Separation and collection of individual compounds was performed by use of a Perkin-Elmer (PE; Norwalk, CT, USA) Series 250 LC equipped with a PE LC 95 UV-Vis detector. Compounds were separated on a 250 mm × 10 mm i. d. LiChrocart RP18-7µ column (Merck) equipped with a 10 mm × 10 mm pre-column packed with the same stationary phase. The mobile phase was a linear gradient prepared from H_2O (pH adjusted to 3.2 by addition of HCOOH) and CH_3CN; the composition of the gradient is reported in Table II. The flow rate was 5.0 mL min^{-1}.

HPLC-MS Analysis

Mass spectra were acquired by means of an HP 1100 MSD API-electrospray coupled with an HP 1090L liquid chromatograph also equipped with DAD.

Negative ionization mode was used at a gas temperature of 350 °C, a nitrogen flow rate of 10.0 L min^{-1}, a nebulizer pressure of 30 psig, a quadrupole temperature of 30 °C, and a capillary voltage of 3500 V. The fragmentor potentials used were in the range 80–150 V.

The orthogonal positioning of the nebulizer relative to the capillary inlet enabled the use of the same conditions for HPLC-DAD analysis with H_2O adjusted to pH 3.2 by addition of HCOOH.

HPTLC Analysis

Screening by mono-dimensional HPTLC was performed on 5 cm × 5 cm silica gel $60F_{254}$ plates (Merck) in a Desaga separating chamber (Carlo Erba, Milano, Italy) for horizontal development comprising a solvent-proof body (Teflon) with a tray for the mobile phase and a tight-fitting glass lid; the mobile phase was transferred from the tray to the layer via an exchangeable sintered-glass plate. The mobile phases used were:

1. EtOAc-MeOH-H_2O-HCOOH, 76:13:10:1 (*v/v*);
2. EtOAc-MeOH-H_2O-HCOOH, 77:13:10:4 (*v/v*);
3. EtOAc-CH_3COOH-HCOOH-H_2O, 100:11:11:21 (*v/v*);
4. EtOAc-MeOH-H_2O, 77:13:10 (*v/v*); and
5. $CHCl_3$-MeOH-H_2O, 7:3:0.5 (*v/v*).

Mobile phases 1–3 were used for the more polar compounds and 4 and 5 for the more lipophilic derivatives. The spots were detected by spraying with a 1% methanolic solution of the complex of diphenylboric acid with ethanolamine, then with 5% ethanolic poly(ethylene glycol) (PEG 4000), and observing the fluorescence at 365 nm.

NMR Analysis

^1H and ^{13}C NMR spectra were acquired by use of Bruker AC and Varian Gemini 200-MHz spectrometers operating in FT mode with CD_3OD as solvent.

Enzymatic Hydrolysis

A β-glucuronidase enzyme (type B10; Sigma-Aldrich) with an activity of 10^7 units per solid gram was used. Samples (approximately 1–2 mg) were dissolved in acetic buffer solution (3 mL) at pH 5 prepared from 0.5 M CH_3COOH and 0.7 M CH_3COONa. The solution was maintained at 37 °C for 30 min. The samples were extracted with EtOAc, the organic solvent was removed under vacuum, and the dry residue was dissolved in 6:2:2 (*v/v*) MeOH-CH_3CN-H_2O, pH adjusted to 2 by addition of HCOOH, (1 mL). The extract and the aqueous solution were both analysed by HPLC-DAD.

Polarimetric Analysis

Analysis of a 1% methanolic solution of chicoric acid was performed with a Perkin-Elmer model 241 instrument, with a 10-cm cell, volume 2 mL, and a Na lamp with the D line at 589 nm.

Results and Discussion

The chemical structures of the compounds identified are reported in Figure 1. The first part of this section is devoted to the chromatographic conditions used to isolate and purify the individual compounds and to the analytical data collected for their chemical characterization. The HPLC behaviour of chicoric acid and its analogues on three different stationary phases are compared and discussed at the end of this section.

Semipreparative HPLC Analysis

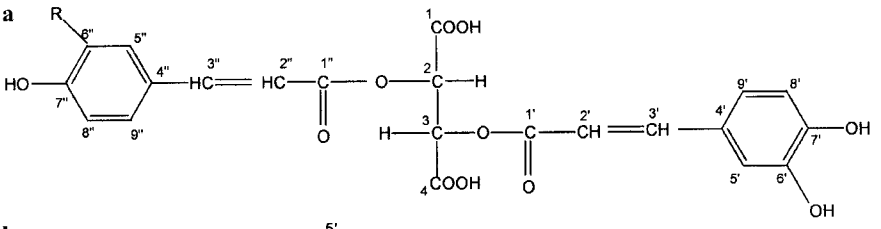
To obtain a representative extract of the leaves of wild chicory, classical extraction with EtOH-H_2O was performed at room temperature. To isolate and characterize the main polyphenols, an enriched extract was prepared from the total raw aqueous ethanol extract by liquid-liquid extraction with EtOAc. This final extract was submitted to semi-preparative HPLC on a Li-Chrosorb column – the best stationary phase for optimization of the separation of the peaks in this sample. Figure 2 shows the HPLC-DAD profiles of the EtOAc extract at 254 nm and 360 nm; the semi-preparative chromatographic profile is shown in Figure 3. Eight fractions, denoted A-H, were well separated and their purity was checked by HPLC-DAD and HP-TLC.

Chemical Characterization

The polyphenolic compounds were identified by the use of HPLC-DAD and HPLC-MS analysis and, when necessary, the chemical structures were confirmed by use of ^1H and/or ^{13}C NMR. All the molecules characterized are listed in Table III; they belong to three classes of polyphenolic compound – mono- and dicaffeoyl tartaric esters, caffeoyl quinic esters, and flavonoid glycosides.

HPLC-MS in negative-ion mode, with different fragmentation energies, was a very diagnostic tool for characterization of all the isolated compounds. Both for flavonoids and for caffeoyl derivatives this technique is highly sensitive and furnishes a complete fragmentation pattern, with peaks for the $[M-H]^-$ and $[2M-H]^-$ species always over 20% intensity.

Fraction C corresponded to a pure compound, the most abundant component of the EtOAc extract; analysis confirmed it to be chicoric acid, or dicaffeoyl tartaric acid. Figure 4 shows the chromatograms obtained at 254 nm and 330 nm and the UV-Vis and negative-ion mass spectra of chicoric acid. The UV-Vis spectrum is typical of the caffeoyl group and the negative-ion mass spectrum provided information on the molecular weight, and other characteristic fragments. Five ion species were recorded. Those at *m/z* 473 and 947 corresponded to the molecular ion and dimer ion, respectively; that at *m/z* 311 corresponded to the fragment after loss of the caffeic acid molecule; the peaks at *m/z* 179 and 149 corresponded to caf-



a		b		
R	Compound	R	R_1	Compound
OH	Chicoric acid (dicaffeoyl tartaric acid)	O-gluc	OH	Quercetin 3-*O*-glucoside
OCH_3	Caffeoyl feruloyl tartaric acid	O-glucu	OH	Quercetin-3-*O*-glucuronide
H	Caffeoyl *p*-hydroxycinnamoyl tartaric acid	H	O-glucu	Luteolin-7-*O*-glucuronide

Figure 1. The chemical structures of the identified compounds.

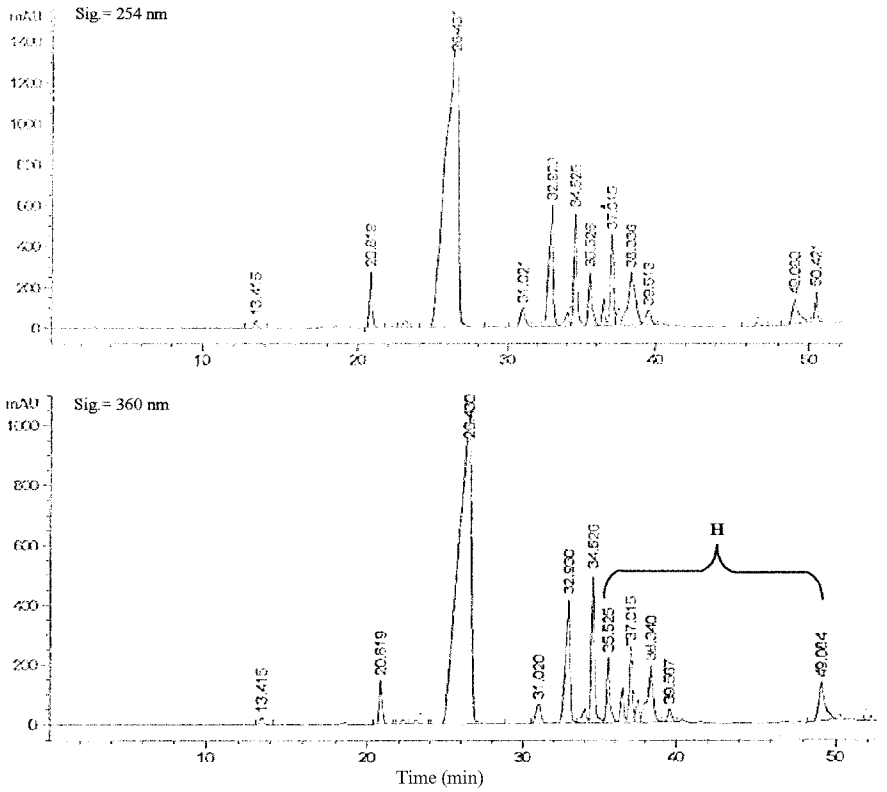


Figure 2. Chromatographic profiles at 254 and 360 nm of the EtOAc extract of wild chicory. The compounds with t_M between 35.52 and 49.0 min constitute fraction H.

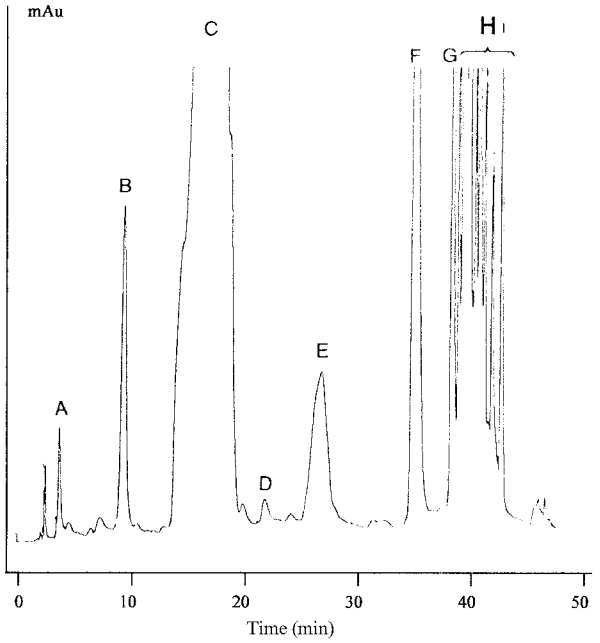


Figure 3. Semipreparative HPLC chromatographic profile at 254 nm of the EtOAc extract on the LiChrosorb column.

feic and tartaric acids, respectively. This compound has previously been found in this plant [6]. In the ^{1}H and ^{13}C NMR spectra the signals from caffeic and tartaric acids were easily identified and the chemical shifts were in accordance with previous studies [7–9]. By polarimetric analysis and by reference to previous work it was possible to determine the absolute configuration of the two stereogenic centres. Zhao and Burke [10] described a synthetic pathway used to prepare the two diastereoisomers of chicoric acid – 2*R*,3*R*-(–) and 2*S*,3*S*-(+). The (+) isomer was identical with natural chicoric acid previously extracted and characterized by Scarpati and Oriente [11]. Compound C was 2*S*,3*S*-(+) dicaffeoyl tartaric acid ester.

Table III. The compounds isolated by semipreparative HPLC-DAD.

A	Monocaffeoyl tartaric acid or caftaric acid
B	Chlorogenic acid
C	Chicoric acid
D	Monocaffeoyl *p*-hydroxycinnamoyl tartaric acid
E	Caffeoyl feruloyl tartaric acid
F	Quercetin-3-*O*-glucuronide + luteolin-7-*O*-glucuronide
G	Quercetin-3-*O*-glucoside

Three other caffeoyl tartaric esters, A, D, and E, were also found in the EtOAc extract. The mass spectra of compounds D and E (Figures 5a and 5b, respectively), show the presence of dimers at 915 *m/z* (D) and 975 *m/z* (E), as was observed for chicoric acid, together with the $[M - H]^-$ ions. Other characteristic fragments were related to ferulic (*m/z* 193), caffeic (*m/z* 179), *p*-hydroxycinnamic (*m/z* 163), and tartaric (*m/z* 149) acids. The fragments

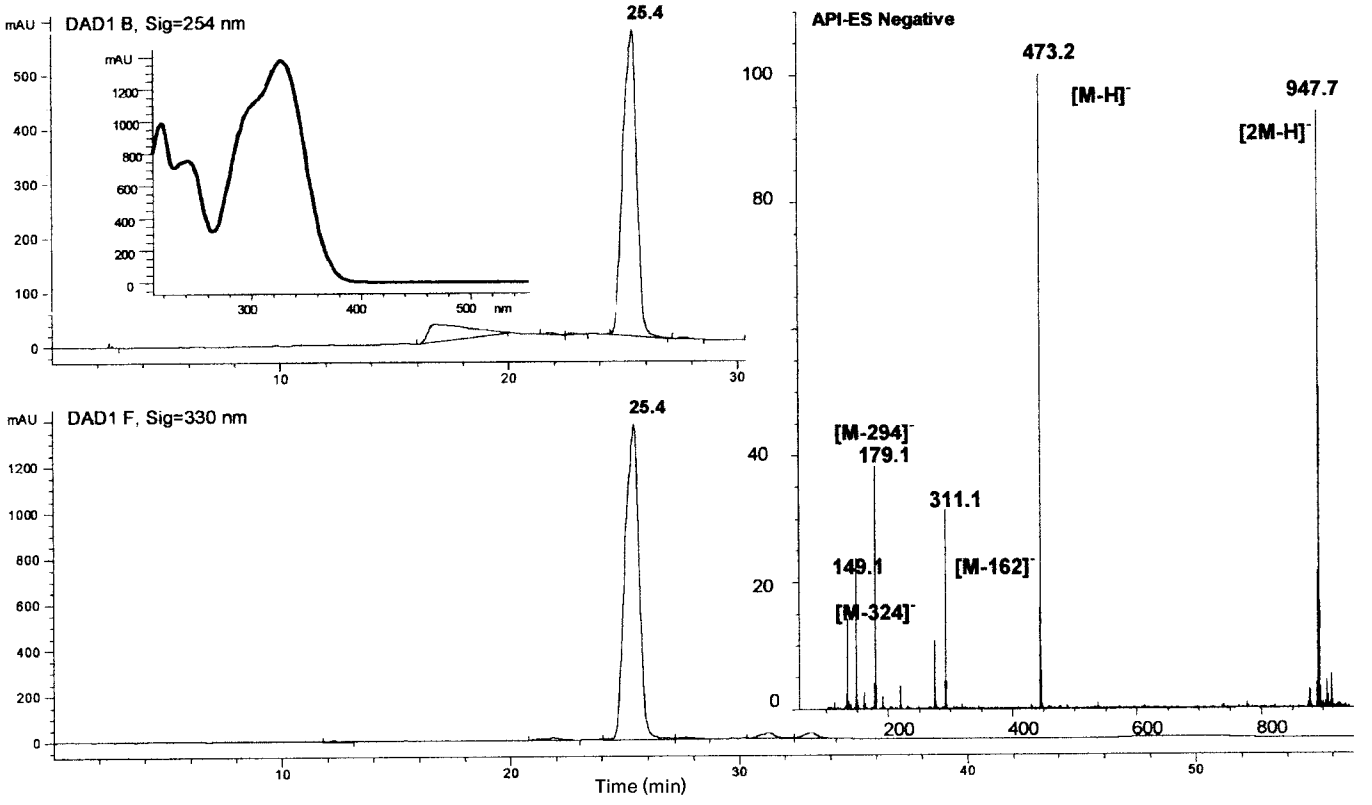


Figure 4. HPLC chromatograms obtained at 254 and 330 nm, UV-Vis spectrum of pure cichoric acid obtained by HPLC-DAD, and the negative-ion mass spectrum obtained by HPLC-MS.

obtained after loss of one cinnamic acid fragment are also displayed. To determine unequivocally the structure of these compounds, ^1H and ^{13}C NMR spectra were also recorded; the most diagnostic chemical shifts were:

Compound ***D***. ^1H NMR: δ 5.73 (H_2 and H_3), 6.45 ($H_{2'}$), 7.71 ($H_{3'}$), 7.09 ($H_{5'}$), 6.81 ($H_{8'}$), 6.49 ($H_{2''}$), 7.64 ($H_{3''}$), 7.50 ($H_{5''}$ and $H_{9''}$), 6.77 ($H_{6''}$ and $H_{8''}$);
Compound ***E***. ^1H NMR: δ 5.77 (H_2 and H_3), 6.32 ($H_{2'}$), 7.66 ($H_{3'}$), 7.09 ($H_{5'}$), 6.79 ($H_{8'}$), 6.97 ($H_{2''}$), 7.64 ($H_{3''}$), 7.50 ($H_{5''}$ and $H_{9''}$), 6.77 ($H_{6''}$ and $H_{8''}$);
^{13}C NMR: δ 181.0 (C_1 and C_4), 111.0 (C_2 and C_3), 166.1 ($C_{1'}$), 121.58 ($C_{2'}$), 145.14 ($C_{3'}$), 126.02 ($C_{4'}$), 112.62 ($C_{5'}$), 146.27 ($C_{6'}$), 148.09 ($C_{7'}$), 113.42 ($C_{8'}$), 166.10 ($C_{1''}$), 113.04 ($C_{2''}$), 146.14 ($C_{3''}$), 126.02 ($C_{4''}$), 110.05 ($C_{5''}$), 149.05 ($C_{6''}$), 147.69 ($C_{7''}$), 114.82 ($C_{8''}$), 122.65 ($C_{9''}$), 55.00 (OCH_3).

Compounds D and E were identified as *p*-hydroxycinnamoyl-, tartaric-, and feruloyl-caffeoyl tartaric acids respectively; as far as we are aware this is the first report of the isolation and identification of these molecules.

Among the flavonoid compounds, HPLC-DAD analysis of fraction F indicated the presence of a single peak (t_M = 34.7 in Figure 6), but investigation of the peak purity by UV-Vis spectroscopy revealed the presence of two flavonoid structures with very different structures. Mass spectrometry confirmed these data, showing presence of two molecular ions corresponding to quercetin glucuronide (*m/z* 477) and luteolin glucuronide (*m/z* 461), and their respective aglycones, at *m/z* 301 and 285. Enzymatic hydrolysis confirmed the presence of a glucuronide group bound to the aglycones. The chromatographic profiles of fraction F before and after hydrolysis are compared in Figure 6. The decrease in the intensity of the peak with t_M 34.7, related to the glucuronides, and the appearance of a peak with t_M 47.1, corresponding to the luteolin and quercetin aglycones, again overlapped, are evident.

The two components of fraction F were unequivocally identified, by ^1H NMR and ^{13}C NMR analysis, as quercetin-3-*O*-glucuronide and luteolin-7-*O*-glucuronide, by comparison of results with previous data [9]. The pattern of the aromatic protons in the ^1H NMR spectrum, in particular, is very useful for defining the site of binding. As far as we are aware

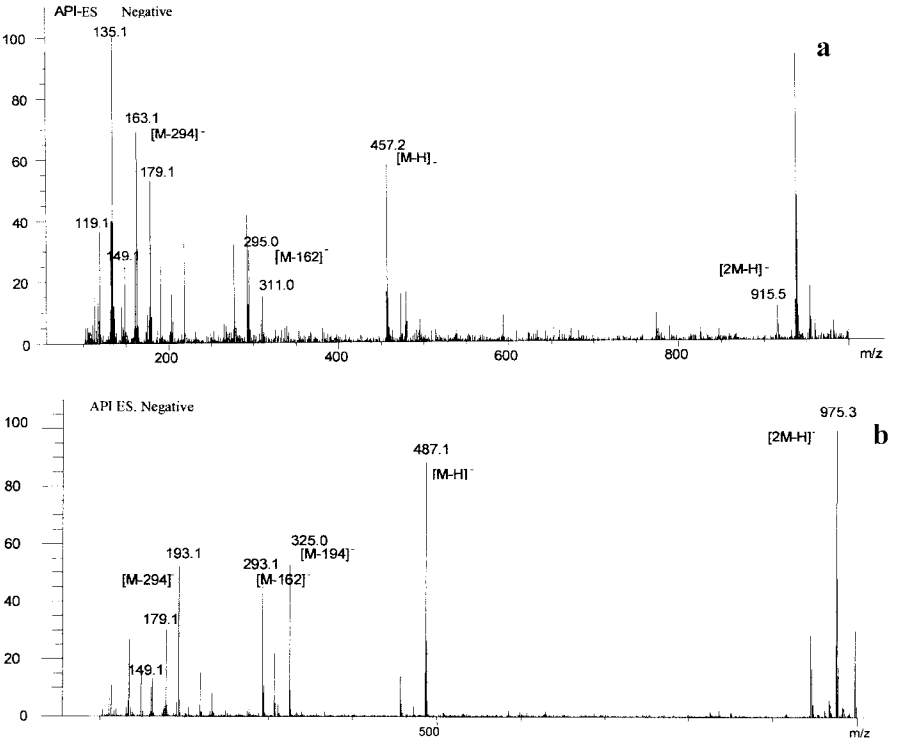


Figure 5. Negative-ion mass spectra of compounds D (**a**) and E (**b**) at a fragmentor potential of 120 V.

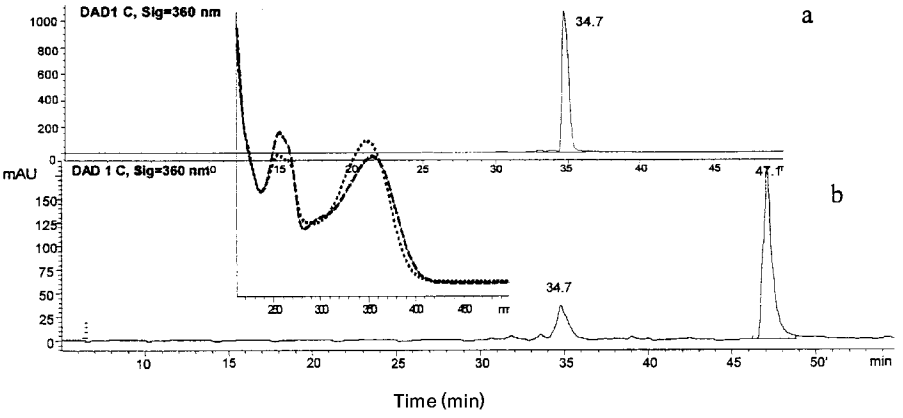


Figure 6. Chromatographic profiles at 360 nm of fraction F before (**a**) and after (**b**) enzymatic hydrolysis.

this is the first report of the presence of these two flavonoids in chicory. Fraction G was identified as a quercetin-3-*O*-glucoside by comparison with pure standard.

Although fraction H was a very complex mixture of different products (Figure 7), it represented a small amount of the total EtOAc extract, as is apparent from Figure 3. Preliminary characterization of the main compounds of this fraction by HPLC-DAD and -MS only indicated the presence of luteolin derivatives and caffeoyl derivatives such as quinic esters. These latter compounds belong to a chemical group already found in chicory leaves; fraction B of Figure 3 corresponds to the pure chlorogenic acid. Fraction H will be the object of further studies for the complete identification of all these minor compounds.

Chromatographic Behaviour

To find the optimum chromatographic conditions for qualitative and quantitative determination of the polyphenols of chicory leaves, direct HPLC-DAD analysis of the aqueous alcoholic extracts of wild chicory leaves was performed on

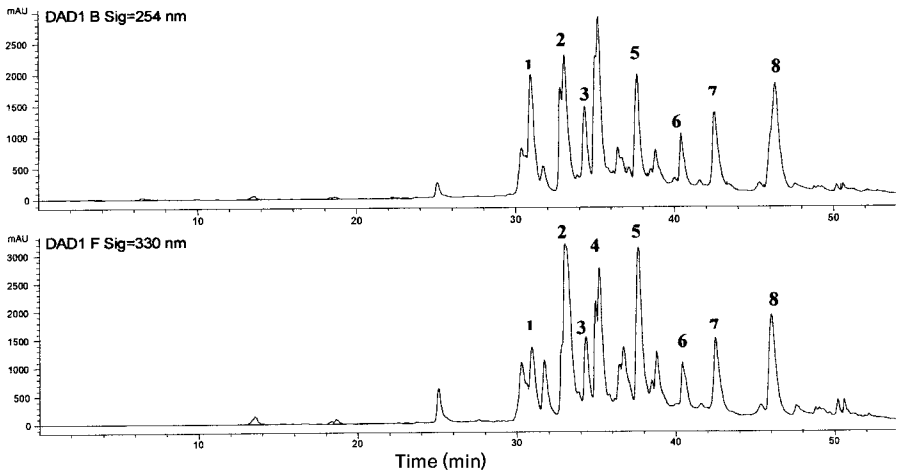


Figure 7. Chromatographic profiles of fraction H at 254 and 330 nm: **1**. quercetin-3-*O*-glucoside; **2**. dicaffeoylquinic acid; **3**, **4**. flavonoid derivatives; **5**. dicaffeoyl derivative; **6**, **7**. luteolin derivatives; **8**. flavonoid derivative.

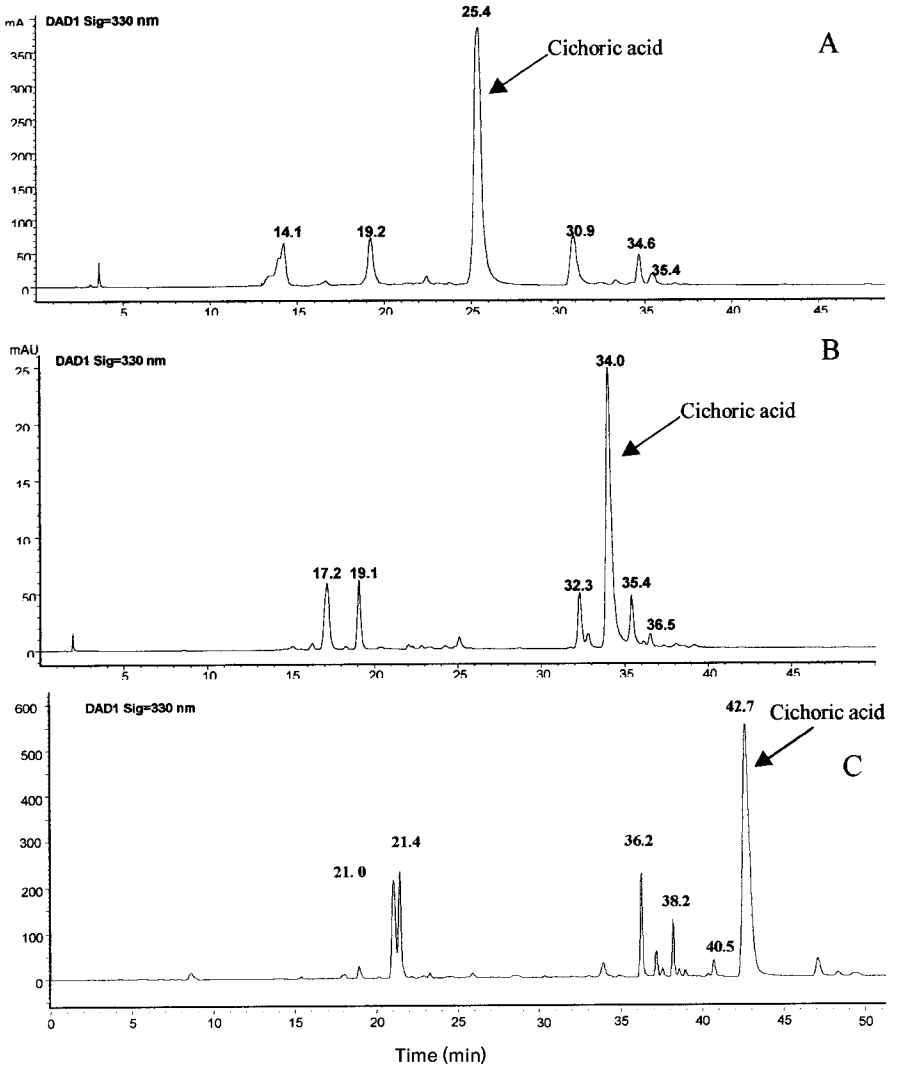


Figure 8. Chromatographic profiles obtained from the extract of chicory leaves by use of the LiChrosorb (**A**), Luna C_{18} (**B**), and Luna Phenyl-Hexyl (**C**) columns.

three different types of column, LiChrosorb (RP18), Luna (C_{18}), and Luna (Phenyl-Hexyl) under the same mobile-phase conditions; the resulting profiles at 330 nm are compared in Figure 8.

The use of the Luna (C_{18}) column resulted in unexpected substantial modifications of some t_M values compared with those on LiChrosorb. Particularly apparent was a ten-minute shift of cichoric acid toward higher t_M. For the other compounds, e. g. chlorogenic acid and flavonoids, t_M values were approximately the same as those obtained with the LiChrosorb column. For monocaffeoyl tartaric acid there was a three-minute difference between t_M values on the LiChrosorb and Luna columns. The behaviour of chicoric acid was probably attributed to two main factors – different packing and characteristics of the stationary phase in the LiChrosorb and Luna (C_{18}) columns, and the peculiar conformations of this molecule. To clarify the latter aspect the spatial conformations of di-caffeoyl tartaric esters were evaluated by application of a method of molecular mechanics able to determine the minimum conformational energy. Force field calculations were performed by use of the AMBER method, in dynamic simulation at 25 °C [6]. This revealed that the more stable conformations of cichoric acid were always those with the two aromatic groups facing each other on the same part of the molecule. This particular structure could increase the strength of interaction with more lipophilic substrates, e. g. the Luna (C_{18}) phase compared with the LiChrosorb column, i. e. this greater affinity of cichoric acid for more lipophilic phases can be correlated with the presence of the aromatic rings of the caffeoyl moieties.

When the same analytical separation was performed on the Luna Phenyl-Hexyl column the t_M of cichoric acid was increased further, as shown in Figure 8C. It can be postulated that on this column a charge-transfer interaction occurs between the aromatic rings of the molecule and the phenyl groups of the stationary phase. To confirm the spatial distribution of the ester groups as mainly responsible for this chromatographic behaviour, the same HPLC-DAD investigations were also conducted with other molecules containing two caffeoyl groups bound with a ester linkage, to a different central legant, quinic acid. For this purpose a previously analysed artichoke extract [12] rich in dicaffeoyl-quinic esters, e. g. cinarin and its

isomers, was analysed on the three columns under the same elution conditions. For this sample only small t_M differences were observed for the LiChrosorb C_{18} and Luna C_{18} columns.

These results suggest that chicoric acid, a compound recently reported to have interesting biological properties as an HIV1 integrase inhibitor [13], seems, because of its peculiar conformation, to be particularly sensitive to differences between reversed stationary phases.

It is apparent from the chromatographic profiles in Figure 8 that peak shape and resolution are better for the Phenyl-Hexyl column (C) than for the others column, except for the peaks eluting after 21.0 and 21.4 min. With this method of elution, therefore, the use of an irregular reversed C_{18} phase seems to be more suitable for the HPLC analysis of chicory extracts, which usually contain large amounts of chicoric acid. In Figure 8 A, in fact, the peak corresponding to this molecule lies in a part of the chromatogram free from the presence of other compounds, and its elution time is also shorter. The range of t_M values of the other main phenols in the extract is, moreover, also wider than that observed in Figures 8B and 8C.

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