Identification and Localization of Hydroxycinnamoyl and Flavonol Derivatives from Endive (*Cichorium endivia* L. cv. Geante Maraichere) Leaves

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Eight major phenolic compounds from endive (*Cichorium endivia* L.) leaves were separated and identified by analytical and semipreparative HPLC. An original enzymatic hydrolysis using hydroxycinnamoylquinate esterase is described for the characterization of five cinnamoyl esters. Three flavonoids were identified after acid hydrolysis. The acid–phenol, alcohol–acid, and sugar moieties released after enzymatic and acid hydrolysis were determined by using different HPLC methods. The cinnamoyl derivatives were shikimic, tartaric, and glucose esters, the phenol moiety of which was always caffeic acid. The flavonoids consisted of two kaempferol glycosides and one quercetin glycoside. After quantification in four fractions, e.g., foliar parenchyma and veins from either green or etiolated leaves, it was shown that caffeoyl esters represented more than 75% of the total phenolics. Among them, dicaffeoyltartaric esters were the major compounds (more than 50% of total phenolics). Foliar parenchyma fractions were richer in phenols than the vein fractions. Etiolation resulted in a large decrease in phenol content which was proportionately higher for the flavonol derivatives, mainly in the parenchyma fractions.

INTRODUCTION

The browning reaction in fruits and vegetables, following physiological disorders and physical and chemical stresses, has always been of considerable interest in food technology. The processing of fruits and vegetables for ready-to-use production is responsible for a marked enhancement of brown discoloration.

Browning of endive (*Cichorium endivia* L.) after cutting is the consequence of the enzymatic oxidation of natural phenolics. The phenomenon mainly occurs in the foliar parenchyma and the veins. Among the phenolics involved, hydroxycinnamoyl esters are important parameters in the quality of many fruits and vegetables (Macheix and Fleuriet, 1984), and a key role is played in brown discoloration mainly by orthodiphenols.

Hydroxycinnamoyltartaric esters have been found to be the major phenolics in chicory leaves (Scarpati and Oriente, 1958; Scarpati and D'Amico, 1960). They have also been reported in lettuce (Feucht et al., 1971; Winter and Herrmann, 1986) and grapes (Ong and Nagel, 1978; Singleton et al., 1986). Quinic esters of caffeic, *p*-coumaric, and ferulic acids occurred in *Cichorium intybus* roots (Dem'Yanenko and Dranik, 1971; Mialoundama and Paulet, 1975; Clifford et al., 1987). Winter and Herrmann (1986) have reported a caffeoylmalic acid in some Chicoriaceae. They have also found monocaffeoyltartaric acid in agreement with the findings of Scarpati and D'Amico (1960). All these derivatives exhibit similar spectrophotometric properties (Jaworski and Lee, 1987) with a peak at 325–330 nm and a shoulder at 290–310 nm (Ribereau-Gayon, 1968). Free caffeic acid has also been reported by Dem'Yanenko and Dranik (1971).

Chicory hydroxycinnamoyl esters were first separated by thin-layer or column chromatography; new HPLC techniques have recently been developed to obtain better separations. The esters have been generally identified by direct comparison of retention times with those of authentic samples (Winter and Herrmann, 1986).

To get more qualitative and quantitative information about the occurrence of the hydroxycinnamic acid derivatives, mainly caffeic acid esters and flavonoids in endive leaves, we have separated and analyzed the phenolic compounds by HPLC, using acid hydrolysis and a new method of enzymatic hydrolysis. The distribution of hydroxycinnamoyl ester and flavonoid contents have been determined in both foliar parenchyma and veins from green and etiolated leaves. This work was carried out in a general study concerning the browning discoloration of ready-to-use salads.

MATERIALS AND METHODS

Plant Material. Endive plants *C. endivia* L. (cv. Géante Maraîchère) were cultivated during the 1986 and 1987 seasons in the experimental plots of INRA GEVES (Les Vignières). Some plants were held in the dark for a week before they were harvested at commercial maturity. Leaves of green and etiolated endives were washed, cut, and separated into four groups, namely foliar parenchyma (GP) and veins (GV) from green leaves and foliar parenchyma (EP) and veins (EV) from etiolated leaves. Samples were frozen in liquid nitrogen, freeze-dried, ground into fine powder, and then stored at −20 °C in airtight bags until further use.

Sample Preparation, Extraction, and Purification of the Phenolics. The plant powder (2.5 g) was homogenized in 200 mL of 80% ethanol with 0.5% sodium bisulfite for 30 min at 4 °C. After filtration, ethanol was evaporated in vacuo at 40 °C. The concentrated extract was made 2% in metaphosphoric acid and 20% in ammonium sulfate. After removal of pigments and most lipids by three successive petroleum ether extractions (2/1 v/v), phenolic compounds were extracted three times with ethyl acetate (1/1 v/v) (Fleuriet and Macheix, 1972; Amiot et al., 1986). The combined fractions were vacuum-dried, and the residue was dissolved in 5 mL of methanol and filtered (0.22 μm) prior to HPLC analysis.

Separation of Phenolic Compounds by Analytical and Semipreparative HPLC. A Varian 5000 liquid chromatograph equipped with a Shimadzu integrator was used. Separa-

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tion of phenolics was carried out on a 200 × 4 mm, 5-μm C18 reversed-phase (RP) column (Chrompack). Phenolic compounds were eluted with a gradient of acetonitrile in water at pH 2.6 (Figure 1) at a flow rate of 1 mL/min. The effluent was monitored at 280, 325, and 360 nm. The same conditions were used for semipreparative HPLC. The column was a 450 × 8 mm, Nucleosil 120, 7-μm C18 RP column. Each collected fraction was extracted with ethyl acetate and evaporated to dryness in vacuo at 40 °C.

Enzymatic Hydrolysis of Phenolic Esters. Hydroxycinnamoylquinate esterase (HCQE) was extracted from 10 g of commercial *Aspergillus niger* pectinase (C10 Rapidase) by ammonium sulfate precipitation (80% saturation). After centrifugation (10000*g*, 30 min), the resulting precipitate was dissolved in 2 mL of 0.1 M sodium phosphate buffer at pH 6. The enzyme preparation (0.5 mL) was added to 1 mL of the phenolic substrate buffered at pH 6. Mild (10 min) and total (30 min) HCQE hydrolyses were achieved at 37 °C, under continuous stirring, and the reaction was stopped by addition of 3 drops of 12 N HCl (Varoquaux et al., 1982). Phenolic compounds were immediately extracted as indicated above. A blank without HCQE has shown that no hydrolysis occurred due to the acidification.

Acid Hydrolysis of Flavonols. The flavonol glycosides collected by semipreparative HPLC were hydrolyzed by heating at 100 °C for 10 min in an equal volume of 4 N HCl.

HPLC Analysis of the Products of Hydrolysis. The compounds released from the hydrolysis (enzymatic and acid) were identified by HPLC by comparing retention times with those of authentic samples. Three different chromatographs were carried out on each filtered hydrolysate.

Analysis of the Phenol Moiety. The acid–phenol moieties were analyzed by the previously described HPLC method.

Analysis of the Acid Moiety. The alcohol–acid moieties were analyzed by HPLC on a 300 × 6.5 mm anion-exchange column (Chrompack) fitted with a 75 × 2.1 mm guard column. Both columns were thermostated at 65 °C. The solvent (0.01 N sulfuric acid) flow was maintained at 0.6 mL/min with the effluent monitored at 210 nm.

Analysis of the Sugar Moiety. The sugar moieties were identified by HPLC on a 100 × 7.8 mm fast carbohydrates analysis column (Bio-Rad) eluted with distilled water at 85 °C. The flow was maintained at 0.5 mL/min and the effluent monitored by refractometry.

Standards. Each compound was identified by its retention time and/or by co-injection with the standards under the same conditions. Caffeic and tartaric acids, kaempferol, and quercetin were purchased from Sigma and chlorogenic and shikimic acids from Fluka. Esters of caffeoyltartaric acid were kindly given by Dr. V. Cheynier, IPV Station INRA-Montpellier, France.

Mass Spectrometry. Fast atom bombardment mass spectrometry was performed to confirm the identification of one caffeoyl ester.

Quantification. For peaks 1–5, quantification was performed at 325 nm by using caffeic acid as standard, assuming a similar molar extinction coefficient for caffeoyl derivatives. For peaks 6–8, it was performed at 360 nm by using kaempferol as standard, assuming a similar molar extinction coefficient for kaempferol and quercetin derivatives. Results are expressed in milligrams per gram of dry matter for each identified phenol (peaks 1–5 and 7) and in milligrams of equivalent kaempferol glucoside and quercetin galactoside for peaks 6 and 8, respectively. The variability of the method was checked in the following way. Five extractions were carried out from the same powder. The extraction variability calculated from the absorbance at 325 nm of the different extracts was estimated at 5.2%. Then the same extract was analyzed 10 times by HPLC. The variability calculated on the total area detected at 325 nm was estimated at 4%. Therefore, for each sample, two HPLC analyses from two different extracts were carried out (four results).

RESULTS AND DISCUSSION

HPLC analysis monitored at 325 nm showed only eight major compounds in the GP fraction (Figure 1). Detection at 280 nm resulted in a very similar chromatogram without new peaks (not shown); then endive extracts did not contain any benzoic derivatives or flavan-3-ols (catechins). The latter finding was confirmed by the lack of spot on TLC plates after vanillin hydrochloride spraying (Ribereau-Gayon, 1968). When monitored at 360 nm, HPLC analysis showed always eight compounds, but the absorbances of the peaks 1–5 decreased, whereas those of peaks 6–8 increased. Due to these spectral properties, peaks 1–5 can be considered hydroxycinnamoyl derivatives and peaks 6–8 flavonol compounds.

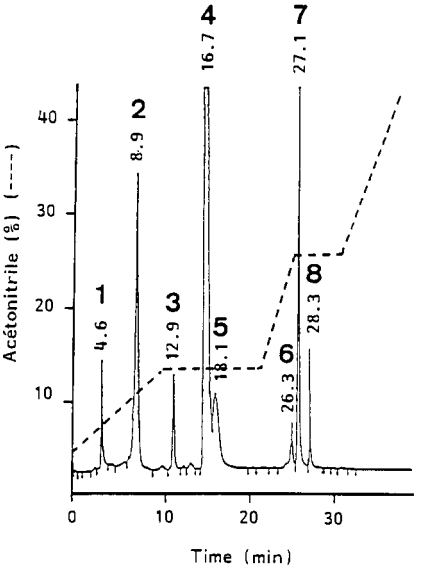

Figure 1. Analytical HPLC of phenolic compounds from GP fraction. 1, Caffeoylglucose; 2 and 3, caffeoylshikimic esters; 4 and 5, dicaffeoyltartaric esters; 6, kaempferol glycoside; 7, kaempferol 3-glucoside; 8, quercetin glycoside.

No free hydroxycinnamic acid was detected in endive leaves, in disagreement with the findings of Dem'Yanenko and Dranik (1971), who found hydroxycinnamic acids in roots of *C. intybus*. Since free phenolic acids are scarcely present in the plant kingdom and are considered phytotoxic (Macheix and Fleuriet, 1986), the above findings could be the result of hydrolysis of combined forms under severe extraction conditions.

Identification of Hydroxycinnamoyl Derivatives by Enzymatic Hydrolysis. Crude phenolic extract and each hydroxycinnamoyl derivative isolated by semipreparative HPLC were submitted to enzymatic hydrolysis by HCQE. Seto et al. (1988) developed a similar procedure using cellulase to establish the structure of sesquiterpene lactones from chicory roots. Mild hydrolysis by HCQE of the crude extract resulted in two new peaks linked to either a large decrease or a loss of peaks 1–5 without change on peaks 6–8 as shown by HPLC (Figure 2). The major new compound has a retention time (10 min) corresponding to that of caffeic acid, whereas the other one has a retention time (4.4 min) matching that of monocaffeoyltartaric esters (see Compounds **4** and **5** and Figure 6). After a total hydrolysis by HCQE, the latter compound completely disappeared just as did peaks 1–5, whereas the peak of caffeic acid largely increased (not shown). No phenol other than caffeic acid was released by HCQE hydrolysis, though the presence of *p*-coumaric and ferulic moieties was reported in the roots of closely related species, *C. intybus* (Dem'Yanenko and Dranik, 1971). Therefore, caffeic acid would be the only phenol–acid moiety of esters in endives leaves. This accounts for the UV–visible spectrum of crude phenolic extract, which is characteristic of caffeic derivatives. According to Macheix and Fleu-

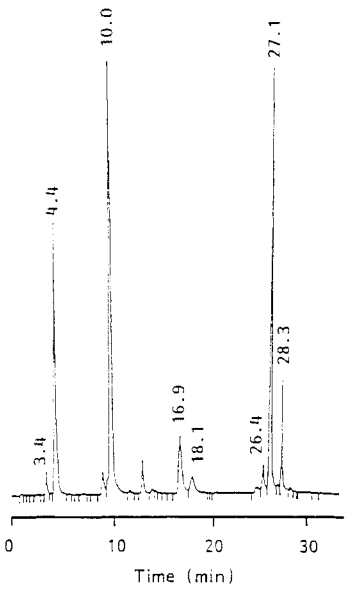

Figure 2. Analytical HPLC of phenolic compounds after mild hydrolysis by HCQE. Retention times: 3.4 and 4.4 min, monocaffeoyltartaric esters; 10.0 min, caffeic acid; 16.9 and 18.1 min, dicaffeoyltartaric esters; 26.4, 27.1, and 28.3 min, flavonol glycosides.

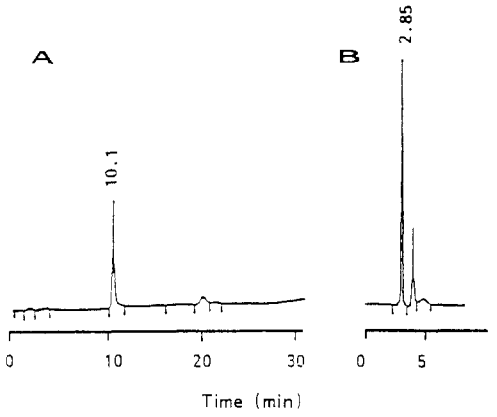

Figure 3. Mild hydrolysis products of compound 1. Analytical separation. (A) HPLC of phenolics. Retention time: 10.1 min, caffeic acid. (B) HPLC of sugars. Retention time: 2.85 min, glucose.

riet (1986), caffeic derivatives may represent 75–95% of the phenolic pool in some plant organs.

Compound 1. The enzymatic hydrolysis of compound 1 gave caffeic acid as phenol moiety (Figure 3A) and glucose as sugar moiety (Figure 3B). Since compound 1 was eluted in the void volume during HPLC analysis of the acid moiety, one can think that the carboxyl function of caffeic acid was involved in the ester bound with the sugar moiety. Therefore, compound 1 is a caffeoylglucose ester. This combination, frequently found in plants (Ribereau-Gayon, 1968) mainly in Solanaceae (Molgaard and Ravn, 1988), has not been previously reported in endive leaves.

Compound 2. Optical and chromatographic properties of compound 2 were close to those of chlorogenic acid. Thus, under certain chromatographic conditions by HPLC, the two compounds showed similar retention times. Caffeoyl-3-quinic and its isomers were reported in chicories (Dem'Yanenko and Dranik, 1971; Rees and Harborne, 1985; Winter and Herrmann, 1986). However, enzymatic hydrolysis by HCQE of compound 2 yielded caffeic acid (Figure 4A) and an acid (Figure 4B) whose retention time (8.2 min) differed from that of quinic acid (7.0 min) under our chromatographic conditions but corresponded to that

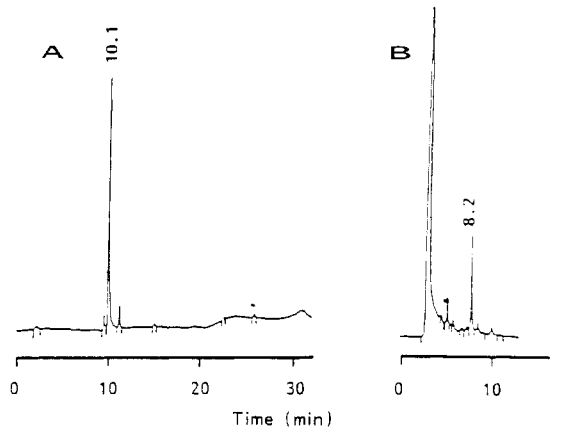

Figure 4. Mild hydrolysis products of compound 2. Analytical separation. (A) HPLC of phenolics. Retention time: 10.1 min, caffeic acid. (B) HPLC of acids. Retention time: 8.2 min, shikimic acid.

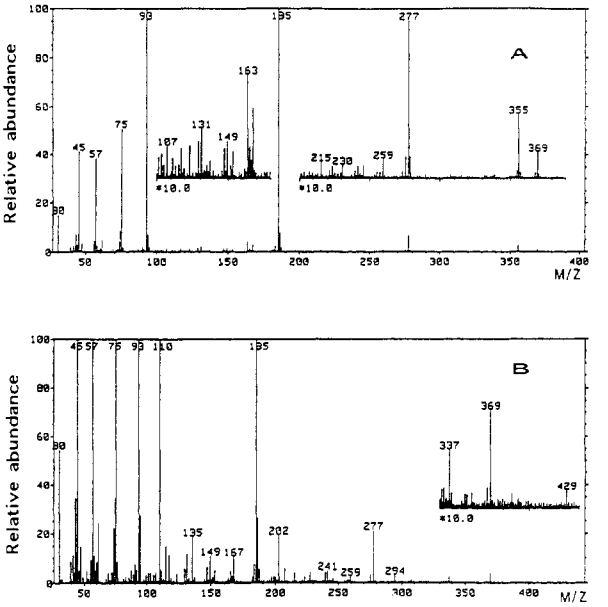

Figure 5. Fast atom bombardment mass spectrometry. (A) Chlorogenic acid. (B) Compound 2.

of shikimic acid. Moreover, fast atom bombardment mass spectrometry showed molecular ion peaks at m/z 355 ($C_{16}H_{18}O_9 + H^+$) for chlorogenic acid and 337 ($C_{16}H_{16}O_8 + H^+$) for compound 2 (Figure 5). The molecular weight of the acid, which esterified caffeic acid, was 174, in agreement with that of shikimic acid. Lastly, mild HCQE hydrolysis yielded only caffeic acid as phenol moiety, and no transitory intermediate was detected. Therefore, compound 2 can be considered a monocaffeoylshikimic ester.

Compound 3. The enzymatic hydrolysis gave the same results as compound 2, i.e., caffeic acid as the phenol moiety and shikimic acid as the acid moiety. Therefore, compounds 2 and 3 are probably isomeric forms of a monocaffeoylshikimic ester whose precise structures remain to be proved by proton nuclear magnetic resonance and infrared analysis. Hydroxycinnamoylshikimic esters would not generally accumulate in plants since shikimic acid is involved in the biosynthesis of numerous aromatic compounds (Maier and Metzler, 1965). Nevertheless, shikimic derivatives have been found in dates (Maier and Metzler, 1965), where 3-*O*-caffeoylshikimic ester (dactylifric acid) and its isomers were identified (Maier et al., 1964). Fukuoka (1982) found 5-*O*-caffeoylshikimic ester in bracken fern. Caffeoylshikimate has not been previously reported

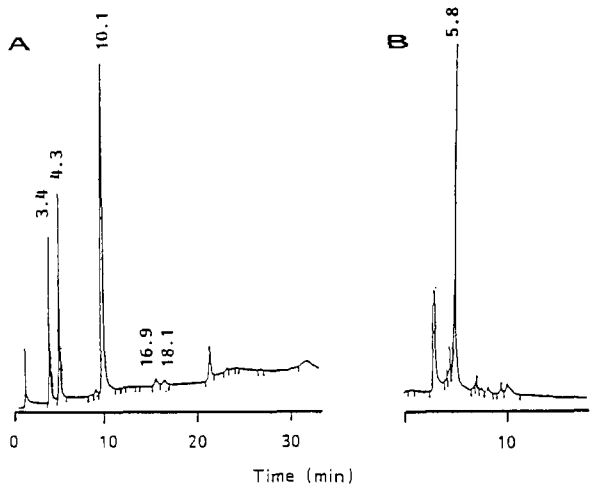

Figure 6. Mild hydrolysis products of compounds 4 and 5. Analytical separation. (A) HPLC of phenolics. Retention times: 3.4 and 4.3 min, monocaffeoyltartaric esters; 10.1 min, caffeic acid; 16.9 and 18.1 min, dicaffeoyltartaric esters. (B) HPLC. Retention time: 5.8 min, tartaric acid.

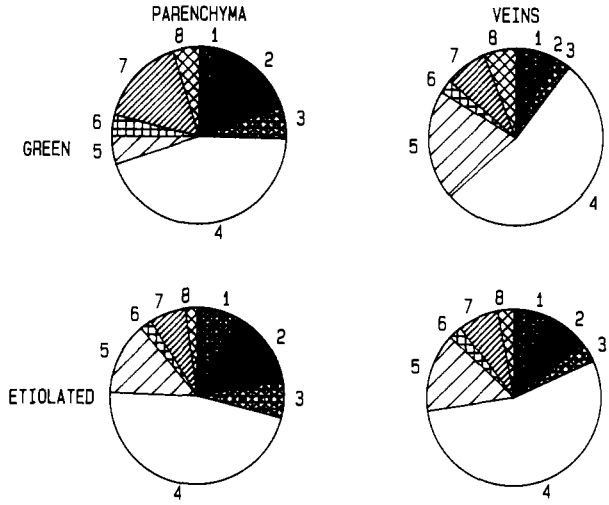

Figure 7. Relative distribution of individual phenol in foliar parenchyma and vein from green and etiolated endive leaves. 1, Caffeoylglucose; 2 and 3, caffeoylshikimic esters; 4 and 5, dicaffeoyltartaric esters; 6, kaempferol glycoside; 7, kaempferol 3-glucoside; 8, quercetin glycoside.

in chicories, though the presence of this ester could be expected since hydroxycinnamoyl-CoA shikimate hydroxycinnamoyl transferase (CST) is very active in cell suspensions and whole plants of *C. endivia* (Ulbrich and Zenk, 1980). This enzyme catalyzes the transfer of shikimic acid to coumaroyl-CoA to yield 5-*O*-coumaroylshikimic ester. However, the caffeic acid derivative cannot be a direct reaction product of CST since this enzyme is specific for *p*-coumaroyl-CoA and shows no activity toward caffeoyl-CoA (Ulbrich and Zenk, 1980). Then, caffeic esters of shikimic acid may come either from the orthohydroxylation of *p*-coumaric ester or from direct biosynthesis through specific transferases. In all cases, though shikimic and quinic acids have similar structures, two different enzymes specifically catalyze their esterification (Rhodes et al., 1979).

Compounds 4 *and* 5. Mild HCQE hydrolysis of compounds 4 and 5 generated caffeic acid (Figure 6A), and monocaffeoyltartaric esters identified on the basis of their retention times, which matched those of *cis*- and *trans*-monocaffeoyltartaric esters purified from grapes. These esters were not found in crude extract, in agreement with the findings of Winter and Herrmann (1986). Total hydrolysis resulted in the loss of compounds 4 and 5 and appearance of tartaric acid (Figure 6B). The caffeic acid to tartaric acid ratio averaged 1.8 ± 0.3. Thus, compounds 4 and 5 should be isomeric forms of dicaffeoyltartaric esters which have been previously reported in *C. intybus* (Scarpati and Oriente, 1958). Woeldecke and Herrmann (1974b) confirmed this structure by mass spectrometry and ^1H NMR, but the exact structure of each isomer was not determined. Dicaffeoyltartaric acid or chicoric acid is the major polyphenol in *C. endivia*. It has also been isolated from lettuce (Winter and Herrmann, 1986). Monocaffeoyl-, *p*-coumaroyl-, and feruloyltartaric acids found in grapes (Singleton et al., 1986) were not detected in endive leaves. No quinic esters were found, in agreement with the findings of Cheynier et al. (1986), who stated that in some plants quinic esters, which are the most common hydroxycinnamoyl derivatives, are replaced by tartaric esters.

Identification of Flavonol Derivatives by Acid Hydrolysis. *Compounds* 6 *and* 7. The acid hydrolysis of compounds 6 and 7 released kaempferol as flavonol. Glucose was combined to kaempferol in compound 7. Thus, the main flavonol derivative in endive leaves is kaempferol 3-glucoside, in agreement with the findings of Woeldecke and Herrmann (1974a) and Rees and Harborne (1985).

Table I. Phenolic Distribution in Foliar Parenchyma (P) and Veins (V) from Green (G) or Etiolated (E) Endive Leaves[a]

	GP	GV	EP	EV
dicaffeoyltartaric esters[b]	15.8 ± 0.8	2.6 ± 0.1	7.2 ± 0.3	2.3 ± 0.1
caffeoyl esters[c]	24.0 ± 1	3.0 ± 0.1	10.7 ± 0.5	2.9 ● 0.1
flavonols[d]	7.8 ± 0.4	0.57 ± 0.04	1.3 ± 0.1	0.43 ± 0.04
total phenols	31.8 ± 2	3.6 ± 0.2	12.0 ± 0.7	3.3 ± 0.2

[a] Results are given in milligrams per gram of dry matter for each individual phenol with the exception of peaks 6 and 8, which are expressed in milligrams of equivalent kaempferol glucoside and quercetin galactoside, respectively (the results are the mean of four chromatograms, i.e., two extractions followed by two HPLC analyses for each extraction). [b] Sum of peaks 4 and 5. [c] Sum of peaks 1–5. [d] Sum of peaks 6–8.

Compound 8. Quercetin was released by acid hydrolysis. The sugar moiety was not identified, but the presence of quercetin derivatives in endive leaves as quercetin 3-galactoside and quercetin 3-rhamnoside was previously noted by Rees and Harborne (1984).

Distribution of Phenolic Compounds in Foliar Parenchyma (P) and Veins (V) of Endive Leaves either Green (G) or Etiolated (E). The phenolic compositions of the four fractions, namely, P and V from both green and etiolated leaves, are illustrated in Figure 7 for each compound (peaks 1–8) and gathered by family in Table I. In all fractions, hydroxycinnamoyl derivatives accounted for at least 75% of the phenolic pool, and among them, the dicaffeoyltartaric esters were the major compounds since the two isomers represented more than 50% of the total phenolic content (Figure 7).

Foliar parenchyma fractions showed a much higher content in phenols compared to veins (Table I). This result could be related to their structural organization. Although the enzymes of phenolic metabolism are located in the endoplasmic reticulum (Hrazdina and Wagner, 1985), the main accumulation sites of phenolics are the cell walls and the vacuoles. In vein cells, hydroxycinnamic acids are involved in a rapid turnover to synthesize lignins which accumulate in the cell wall and are not extracted by our method, whereas in the foliar parenchyma, all phenolics

are located under soluble forms in the vacuoles and are therefore readily extractable. Furthermore, flavonol content was higher in foliar parenchyma than in veins, resulting probably from a preferential flavonol localization in foliar epidermic layers. In many examples, flavonoids accumulated in superficial tissues (Tissut, 1974), where they could either act as a screen against solar light (Weissenbock et al., 1986) or protect the tissues against several aggressions (Tissut and Ravanel, 1980). According to these authors, these flavonoids are mainly flavonols, in agreement with our findings.

Accumulation of phenolics preferentially took place in green leaves (Table I). Green foliar parenchyma (GP) showed the highest content in phenolics (32 mg/g DW) and mainly in caffeoyl derivatives (24 mg/g DW) compared to etiolated foliar parenchyma (EP). Etiolation modified both groups of compounds, but etiolated tissues exhibited higher relative contents in caffeoyl derivatives (89%) than green tissues (75%) (Figure 7). Therefore, the flavonol metabolism was more dependent on etiolation than the caffeoyl ester metabolism.

Changes in phenolic content during etiolation could be related to the decrease in activity of enzymes such as phenylalanine ammonia lyase, which are under the control of phytochrome (Tan, 1980). Moreover, the accumulation of flavonols in leaves depends on the quality and quantity of illuminant (Tissut and Ravanel, 1980) and on the activity of chalcone synthase which is also light dependent (Hahlbrock and Grisebach, 1979). Furthermore, according to the findings of McLure (1979), etiolation modifies the plant physiology since several phytohormones are also under the control of phytochrome and then may act on the accumulation or the synthesis of numerous phenolic compounds.

From a technological point of view, the preferential presence of caffeoyl derivatives in etiolated endive leaves would probably result in an increased sensitivity to enzymatic browning since, compared to flavonols, they are much better substrates for polyphenol oxidases (Goupy, 1989).

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