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Identification of Phenolic Constituents in *Cichorium endivia* Var. *crispum* and Var. *latifolium* Salads by High-Performance Liquid Chromatography with Diode Array Detection and Electrospray Ioniziation Tandem Mass Spectrometry

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ABSTRACT: Chicory is a widely consumed vegetable and a source of phenolic compounds. Phenolic acid and flavonoid derivatives were identified in *Cichorium endivia* var. *crispum* and var. *latifolium* and fully characterized using complementary information from two different high-performance liquid chromatography detectors, diode array and mass spectrometer, in positive and negative modes. We describe about 40 phenolic compounds, some of which have never previously been reported in these plants, such as hydroxycinnamic acid derivatives (i.e., different mono- and dicaffeoylquinic acid isomers) and mono- and diglycosides of quercetin, kaempferol, and myricetin (differing also by the glycosylation site). These data provide a contribution to a more exhaustive identification of phenolic compounds in *C. endivia* vegetables.

KEYWORDS: *Cichorium endivia var. crispum, Cichorium endivia var. latifolium, polyphenols, hydroxycinnamic acid derivatives, flavonols, HPLC-PDA-ESI/MS^n*

INTRODUCTION

Epidemiological and intervention studies have provided evidence of beneficial health effects of dietary fruits and vegetables; at least part of such effects have been attributed to secondary metabolites commonly referred to as "phenolics", including flavonoids and phenolic acids.[1]

The term phenolics encompasses more than 8000 naturally occurring compounds, all of which share a phenol moiety as a common structural feature. The current classification divides the broad category of phenolics into polyphenols and simple phenols, based solely on the number of phenol subunits present. Polyphenols possessing at least two phenol subunits include the flavonoids. On the other hand, naturally occurring phenolic acids possess one carboxylic acid functionality and contain two distinguishing constitutive carbon frameworks, the hydroxycinnamic and hydroxybenzoic structures. Despite an identical basic skeleton, the number and position of the hydroxyl groups on the aromatic ring create the variety.

Selected flavonoids have been seen to have beneficial effects in reducing the risk of various diseases, including cardiovascular conditions,[2] cancer,[3] atherosclerosis, and age-related conditions.[4] Therefore, it is widely believed that these phytochemicals may provide health benefits as antioxidants, by acting against free radical-mediated lipid peroxidation and chronic inflammation.[5,6]

Polyphenols in food and beverages have been reported to exert a variety of biological effects related to their antioxidant activity, such as radical scavenging, metal chelation, enzyme activity modulation, cell signaling pathway modulation, and effects on gene expression;[7] however, mounting evidence is showing that they contribute to preventing other conditions not directly related to antioxidant activity, such as oral disease, including tooth decay,[8] as well as their potential therapeutic application to inflammation not confined to the gastrointestinal tract but involving other sites and conditions, including arthritis, asthma, and encephalomyelitis.[9]

Their widespread occurrence and biological value make the phenolic compounds found in plants interesting to identify and characterize. High-performance liquid chromatography (HPLC) coupled with electrospray ionization mass spectrometry (ESI-MS) is the method of choice for such investigations. Indeed, its ability to provide information about the molecular mass and fragmentation pattern of analytes makes mass spectrometry (MS) the most effective tool to explore polyphenolic structures. HPLC-MS has successfully been applied to characterize the phytochemical profiles of several fruit and vegetable extracts.[10] Data-dependent acquisition, where user-specified criteria are applied to select the ion of interest for subsequent fragmentation, are among the most useful approaches employed to identify unknown compounds by MS. Using this approach, single-stage MS provides the putative molecular weight (MW) that can be used in combination with UV detection for a first tentative structure assignment; structure elucidation and confirmation can then be obtained by tandem MS analysis via the fragmentation pathway.[11−13]

Chicory is a typical, widely consumed Mediterranean plant indigenous to Europe, Western Asia, and North America; therefore, it could be a good source of dietary polyphenols. Chicory (*Cichorium intybus* L.) is a diploid plant species belonging to the Asteraceae family (Compositae); *Cichorieae* tribe includes approximately 100 genera and many hundreds species of which some genera are used as salad vegetables.

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Table 1. R_t, UV, MS, and MS/MS Data of Polyphenolic Compounds in *C. endivia* Var. *crispum* and Var. *latifolium*

no.	R_t (min)	UV λ_{max} (nm)	precursor ion m/z	LC-PDA-ESI-MS^n m/z (% of base peak)	identification
1[a]	2.2	210	133	MS^2 [133]: 115 (100), 89 (3)	malic acid
2[a]	3.9	258, 311	163	MS^2 [163]: 145 (100)	*p*-coumaric acid
3[a]	6.3	220, 272	191	MS^2 [191]: 173 (50), 127 (30), 111 (100)	quinic acid
4[a,b]	11.9	260	179	MS^2 [179]:135 (100)	caffeic acid
5	16.2	223, 279	299	MS^2 [299]:281 (10), 248 (5), 203 (100)	unknown
6	19.4	241, 327 310sh	311 623	MS^2 [311]: 179 (80), 149 (100), 135 (5)	*trans*-caftaric acid
7[b]	28.8	213, 260	313	MS^2 [313]: 253 (100), 223(14), 175 (18)	unknown
8[b]	30.7	219, 267	313	MS^2 [313]: 253 (100), 223(10), 175 (35)	unknown
9[b]	31.9	257, 329	339 679	MS^2 [339]: 177 (100), 149 (2), 133 (5)	caffeoyl tartaric acid dimethyl ester
10[b]	38.9	222, 264	313	MS^2 [313]: 151 (100)	vanillin hexoside or caffeoylvanillin
11	40.2		439	MS^2 [439]: 421 (100), 259 (20), 215 (15)	unknown
12	41.0	252, 306	307	MS^2 [307]: 191 (100), 133 (8)	malic ester of quinic acid
13[b]	46.0	215, 256	275	MS^2 [275]: 257 (100)	unknown
14[b]	48.0	250, 323 303sh	353	MS^2 [353]: 191 (100), 179 (50), 135 (5)	3-caffeoylquinic acid
15[a]	49.1	246, 324 310sh	353	MS^2 [353]: 191 (100), 179 (3)	5-CQA
16[b]	55.3	256, 352	727[c]	MS^2 [727]: 479 (100), 303 (28)	quercetin-monoglucuronil-*O*-(6″-malonyl)-glucoside
17[b]	56.5	264, 366	611[c]	MS^2 [611]: 449 (20), 287 (100)	kaempferol *O*-diglucoside
18[b]	57.8	242, 323 301sh	367	MS^2 [367]: 191 (100), 173 (10)	*trans*-5-feruloylquinic acid
19[b]	60.0	255, 351	463	MS^2 [463]: 301 (100), 300 (84)	quercetin-7-*O*-glucoside
20[b]	61.4	262, 365	697[c]	MS^2 [697]: 535 (65), 449 (100), 287 (30)	kaempferol-3-*O*-glucosyl-7-*O*-(6″-malonyl)-glucoside
21	64.5	242, 323 301sh	367	MS^2 [367]: 191 (100), 173 (10)	*cis*-5-feruloylquinic acid
22[a,b]	66.0	255, 355	481[c]	MS^2 [481]: 319 (100), 335 (30)	myricetin-3-*O*-glucoside
23	69.9	248, 324 301sh	515	MS^2 [515]: 353 (100), 335 (30)	1,3-dicaffeoylquinic acid
24[b]	70.9	248, 325 302sh	515	MS^2 [515]: 353 (100), 299 (35), 317 (28), 255 (10), 203 (5)	1,4-dicaffeoylquinic acid
25	71.0		483	MS^2 [483]: 285 (100)	unknown
26	71.9	248, 323 302sh	515	MS^2 [515]: 353 (100), 299 (25), 255 (18), 203 (8)	3,4-dicaffeoylquinic acid
27	72.0		411	MS^2 [411]: 259 (100)	galloyl derivative
28[b]	72.2	229, 325 302sh	515	MS^2 [515]: 353 (100), 203 (8), 191 (18)	3,5-dicaffeoylquinic acid
29[b]	72.9	257, 368	551[c]	MS^2 [549]: 389 (54), 303 (100)	quercetin-3-*O*-(6″-malonyl)-glucoside
30[b]	74.5	265, 346	463[c]	MS^2 [463]: 287 (100)	kaempferol-monoglucuronide
31	75.1	241, 327 305sh	473	MS^2 [473]: 311 (100), 293 (20), 179 (45), 149 (30)	chicoric acid
32	76.2	268, 366 318sh	447	MS^2 [447]: 285 (100), 284 (74), 257 (45), 255 (18)	kaempferol-7-*O*-glucoside
33	76.9	265, 346	479[c]	MS^2 [479]: 435 (100), 303 (56), 289 (25)	quercetin-monoglucuronide
34	77.1	240, 330	499	MS^2 [499]: 353 (100), 191 (30), 179 (25)	caffeoylquinic acid rhamnoside
35	78.0	265, 365	533[c]	MS^2 [535]: 449 (35), 287 (100), 286 (38)	kaempferol-7-*O*-(6″-malonyl)-glucoside
36	79.5	264, 366	533[c]	MS^2 [535]: 449 (28), 287 (42), 286 (100)	kaempferol-3-*O*-(6″-malonyl)-glucoside
37	79.9		529	MS^2 [529]: 379 (100)	vanillic acid derivative
38[a,b]	81.3	266, 318 300sh	447	MS^2 [447]: 327 (35), 285 (70), 284 (100), 257 (5), 255 (35)	kaempferol-3-*O*-glucoside
39[b]	84.6		579	MS^2 [579]: 392 (40), 301 (100)	kaempferide derivative
40[a]	85.1	255, 349 268sh	625	MS^2 [625]: 463 (100), 462 (70), 301 (46)	quercetin-3,7-di-*O*-glucoside
41[b]	86.2	287, 312	403[c]	MS^2 [403]: 257 (100)	pinocembrin-*O*-rhamnoside

[a]As compared with standard compound. [b]Not found in *C. endivia* var. *latifolium*. [c]Positive ionization mode.

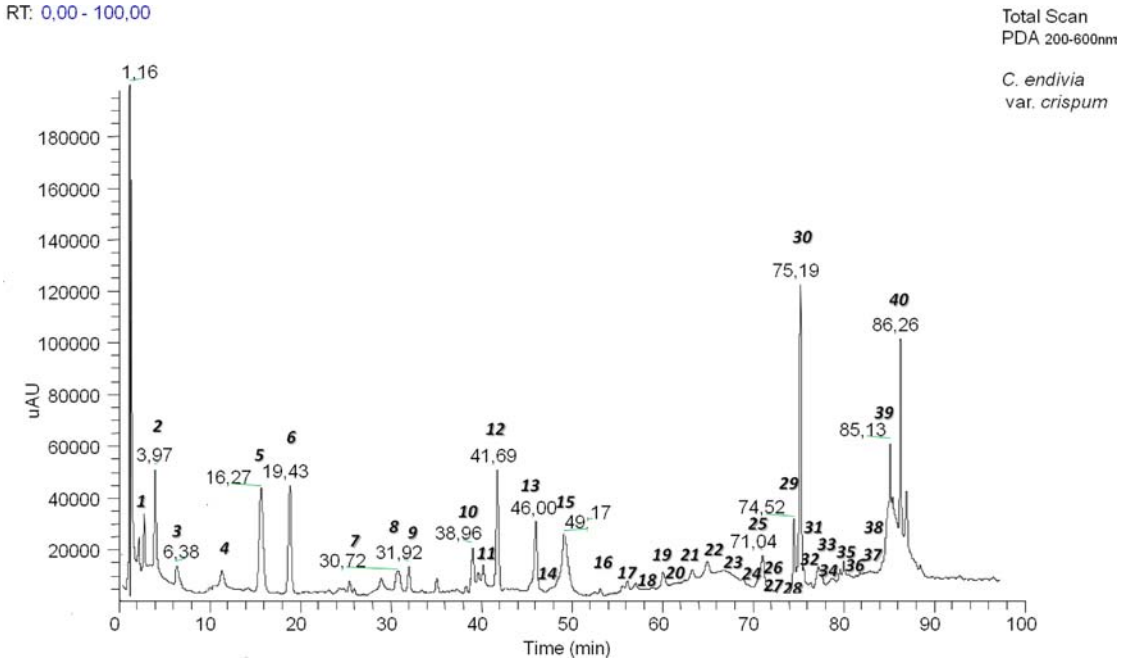

Figure 1. PDA chromatogram of *C. endivia* var. *crispum* extract.

Among these, the genus *Cichorium* consists of two cultivated species, *Cichorium endivia* and *C. intybus*, characterized by green and red leaf colors, respectively. Both of these species consist of different varieties, that is, *latifolium* and *crispum* for *C. endivia*.

In *C. endivia* var. *crispum* salads, chlorogenic and isochlorogenic acids, three caffeoyl acid derivatives, chicoric acid, and two kaemperol derivatives have already been identified,[14] but given the absence of a complete characterization and quantification of the different isomeric forms of phenolic acid and flavonoid derivatives in *C. endivia*, this work was devised to investigate the phenolic compound contents in *C. endivia* var. *latifolium* and var. *crispum*.

MATERIALS AND METHODS

Materials and Chemicals. HPLC-grade and HPLC-MS-grade water and methanol were purchased from Sigma-Aldrich (St. Louis, MO). Standard 5-*O*-caffeoylquinic acid (chlorogenic acid, 5-CQA), malic acid, *p*-coumaric acid, caffeic acid, keampferol, kaempferol-3-*O*-glucoside, quercetin-3,7-di-*O*-glucoside, and myricetin-3-*O*-glucoside were purchased from the same supplier. Quinic acid was purchased from Acros Organics (Geel, Belgium). HPLC-grade water, used for sample preparation, was obtained with a Milli-Q water purification system (Millipore, Billerica, MA). Filtration membranes (0.45 μm cellulose acetate/cellulose nitrate mixed esters) were also from Millipore.

Polyphenolic Extract Preparation from Vegetable Samples. Five clumps of *C. endivia* var. *crispum* and five of var. *latifolium* were purchased at a local market in autumn (September−December). 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 a fresh aliquot of the same mixture. The pale green extracts were pooled, filtered through a 0.45 μm membrane, and then directly injected in the HPLC-MS system.

Liquid Chromatography−Tandem Mass Spectrometry. HPLC-photodiode array detector (PDA)-ESI/MS^n analyses were performed using a Thermo Finnigan Surveyor Plus HPLC apparatus equipped with a quaternary pump, a Surveyor UV−vis PDA detector, a Surveyor Plus autosampler, and a vacuum degasser connected to an LCQ Advantage Max ion trap mass spectrometer (all from Thermo Fisher Scientific, Waltham, MA) through an ESI source.

Separation was achieved on a Gemini C18 analytical column (150 mm × 2.0 mm i.d., 5 μm) with a Hypersil Gold C18 guard column (10 mm × 2.1 mm i.d., 5 μm; both from Phenomenex, Torrance, CA). The mobile phase consisted of 0.1% formic acid in water (eluent A) and methanol (eluent B) at a flow rate of 0.3 mL/min.

The injection volume was 10 μL. Gradient elution was carried out using the following timetable: from 2 B to 5% B in 10 min, then to 40% B in 50 min, to 60% B in 10 min, and to 100% B in 10 min. An isocratic elution with 100% B was then carried out for 10 more minutes. The resulting total run time was 90 min, followed by column reconditioning. The sample tray and column temperatures were set at 4 °C.

The chromatogram was recorded at several wavelengths, characteristic of different polyphenol classes, for example, 280 nm for phenolic acids, 320 nm for hydroxycinnamic acids, and 370 nm for flavonols. Spectral data were acquired in the range of 200−600 nm for all peaks.

The ion trap operated in data-dependent, full scan (100−1000 m/z), zoom scan, and MS^n mode to obtain fragment ion m/z with a collision energy of 35% and an isolation width of 3 m/z. When greater discrimination was required, additional targeted MS^2 and MS^3 experiments were performed on selected pseudomolecular ions.

The negative- and positive-ion mode ESI source parameters had previously been optimized by flow injection analysis using 5-CQA and kaempferol (5 ppm in 0.1% formic acid−methanol solution, 50:50, v/v) to a ionization voltage of 3.5 kV, 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.

The Thermo Fisher Scientific Excalibur 2.0 software was used for data acquisition and processing. Three independent assays were performed to analyze each methanolic extract from endive leaves by HPLC-PDA-ESI/MS^n; no relevant variations attributable to the nature of the detected fragments or their relative intensities were observed.

RESULTS AND DISCUSSION

Compound attribution to each class based on chromatographic behavior, UV−visible (UV−vis) spectra and mass spectra, and comparisons with the literature are addressed below and summarized in Table 1.

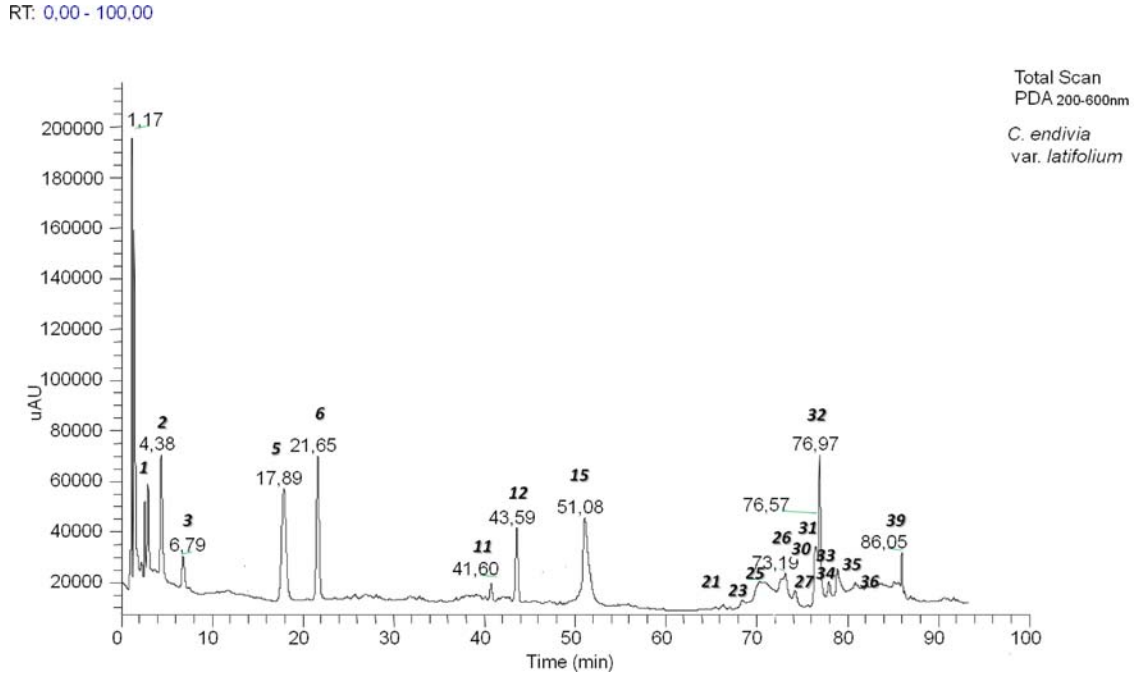

Figure 2. PDA chromatogram of *C. endivia* var. *latifolium* extract.

Chromatographic peaks were preliminarily classified into hydroxycinnamic acid, flavonol, and flavanone derivatives, mainly glycosides, according to PDA UV−vis spectra. Phenolic compounds exhibit absorbance maxima in the 275−285 nm wavelength region due to the aromatic ring in their molecular structure. Phenolic acids and flavonoids have characteristic UV−vis absorbances: hydroxybenzoic acids are detected at 280 nm, hydroxycinnamic acids at 320 nm, flavonols between 350 and 385 nm, and flavanones in the 277−295 nm range with a shoulder at 300−330 nm.

The peak assignment of the conjugated forms of phenolic compounds was based on the comparison of their spectral characteristics with those of the representative standards of the phenolic classes.[15] The chromatographic retention time of each phenolic compound compared to that of the external standard, when available, was used to support its identity. When commercial standards were not available, the analytes were identified by combining MS^n data with the respective literature data.

Polyphenols in nature generally occur as conjugates of sugars, usually *O*-glycosides.[16] In MS analysis, cleavage of the glycosidic linkage and concomitant H rearrangement leads to elimination of the sugar residue, namely, 162 (hexose; glucose or galactose), 146 (deoxyhexose; rhamnose), 132 (pentose; xylose or arabinose), and 176 atomic mass unit (amu) (glucuronic acid). The results of MS^2 and MS^3 product ion spectra of flavonol 3,7-di-*O*-glycosides using negative ion ESI-MS demonstrate that they can be differentiated from isomeric mono-*O*-diglycosides, and the glycosylation positions can be determined.[17]

Ionization was performed both in positive- and in negative-ion mode. Combined use of ionization in the two modes affords extra certainty of the determination of the molecular mass. In the negative ionization mode, hydroxybenzoic and hydroxycinnamic acids deprotonated easily, whereas in the positive mode, they possibly formed adducts with the cations in the sample or mobile phase.[18] Flavonol and flavanone glycosides also showed response in both ionization modes.[15,19]

Figures 1 and 2 show the PDA chromatograms for the methanolic extracts of var. *crispum* and var. *latifolium*, respectively. Figure 3 shows the TIC chromatogram for the methanolic extracts of both varieties.

Characterization of Free Small Organic Acids. Two free small organic acids were identified by comparing their UV−vis spectra and MS fragmentation pattern with standard compounds. Peak 1, eluting at approximately 2 min, produced the MS^2 base peak at m/z 115, corresponding to the loss of a water molecule $[M - H - H_2O]^-$ and was assigned to malic acid (MW 134). Peak 3 with a retention time of 6.3 min and an $[M - H]^-$ at m/z 191, with MS^2 fragments at m/z 173 $[M - H - H_2O]^-$ and at m/z 127 $[M - H - CO - 2H_2O]^-$, was identified as quinic acid.[20]

Characterization of Hydroxycinnamic Acid Derivatives. The presence of free and esterified phenolic acids is not surprising in vegetables of the *Cichorium* genus.[21−23] Peak 2, *p*-coumaric acid (MW 164), showed maximum absorption at 311 nm and produced the MS^2 base peak at m/z 145, corresponding to the loss of a water molecule $[M - H - H_2O]^-$, while peak 4, absent in var. *latifolium* and identified as caffeic acid (MW 180), yielded a base peak at m/z 135, corresponding to the loss of a carboxyl group $[M - H - CO_2]^-$.

Several other derivatives containing a quinic acid unit were identified in this work. Product ion scan experiments of all of these compounds disclosed characteristic fragmentations involving cleavage of one or two caffeoyl moieties or a feruloyl residue. The linkage position of acyl groups on quinic acid could be determined based on the MS^2 fragmentation behavior of the $[M - H]^-$ ions; literature data reported a base peak at m/z 191 for the monocaffeoylquinic acid $[M - H - \text{caffeoyl}]^-$ ion when the acyl group is linked to the 3-OH or 5-OH position, which involved that these isomers could be differentiated solely on the basis of the relative intensity of m/z 179

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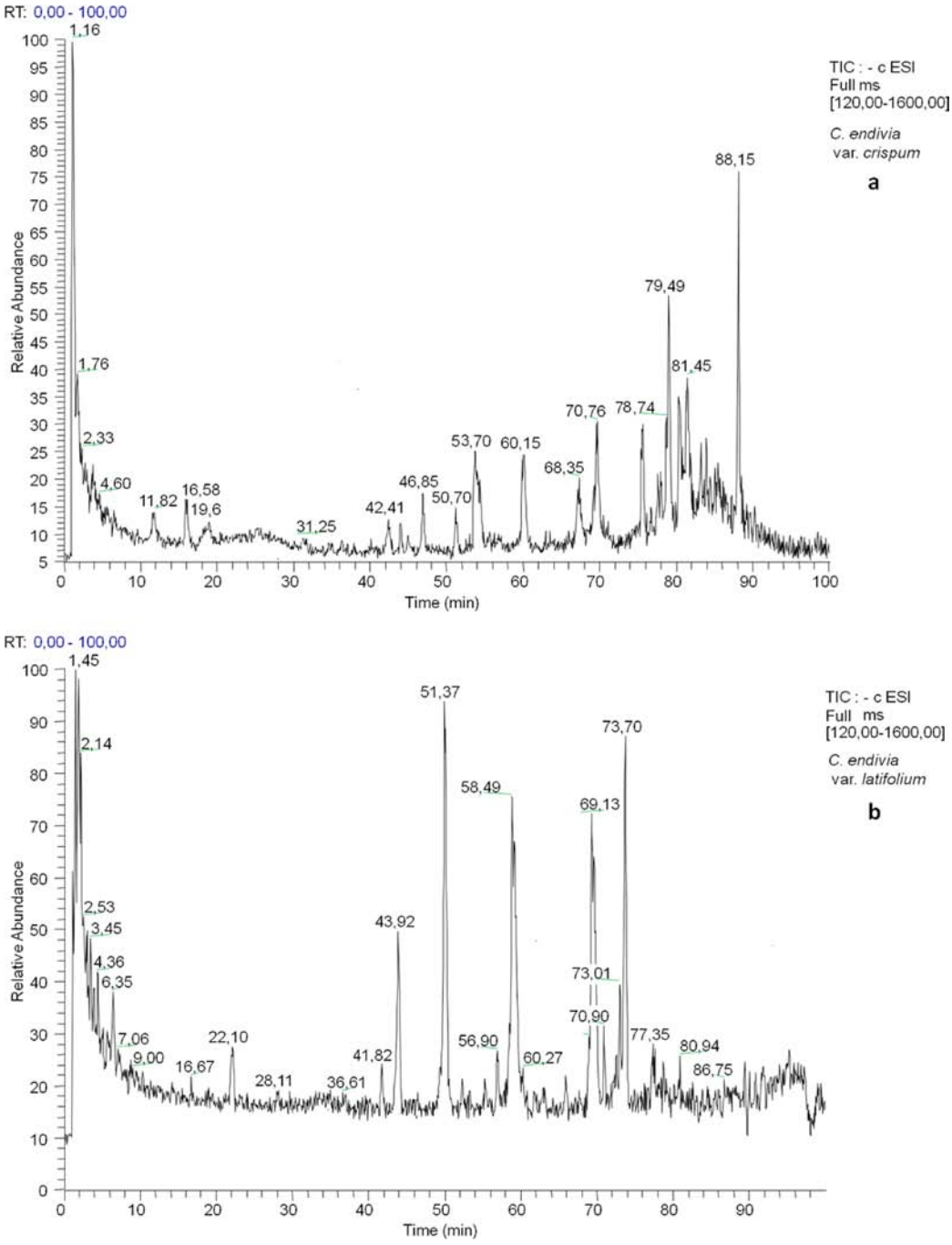

Figure 3. Total ion current (TIC) chromatogram of *C. endivia* var. *crispum* (a) and var. *latifolium* (b).

$[M - H -$ quinic acid$]^-$, which is more significant for the 3-OH compound.[13,24] On the basis of these assumptions and on the comparison of retention time, UV−vis exhibiting absorption maxima at 246 and 324 nm and a shoulder at 310 nm, and MS^n spectra with the commercial standard, compound **15** was identified as 5-CQA, commonly known as chlorogenic acid, and compound **14**, found only in var. *crispum*, eluting earlier than compound **15**, was recognized as 3-*O*-caffeoylquinic acid.[25]

We describe for the first time three different isomers of dicaffeoylquinic acids in chicory in addition to monocaffeoylquinic acids. Compounds **23**, **24**, **26**, and **28**, eluting between 69.9 and 72.2 min, gave $[M - H]^-$ at m/z 515; their fragmentation in MS^2 spectra gave m/z 353 as the base peak, corresponding to the loss of a caffeoyl moiety $[M - H - 162]^-$. Moreover, MS^2 fragments of compounds **24** and **26** were seen to be unique to 4-acyl dicaffeoylquinic acids such as m/z 299 (intensity 35%), the less intense fragments at m/z 255, 203, 173, and 317 found only in the MS^2 spectrum of compound **24**. The ion at m/z 335 was absent in MS^2 spectra of compound **28**. In addition, the MS^3 spectra of the four compounds are quite different; compounds **23** and **28** showed a base peak at m/z 191 characteristic of a 3-OH-substituted quinic acid and a fragment ion at m/z 179 (50% of the base peak). Compounds **24** and **26**, conversely, showed a base peak at m/z 173, characteristic of a 4-OH substitution, and a fragment ion at m/z 179, showing an intensity higher than 70% (Figure 4). On the basis of the fragmentation pattern, the intensity of MS^n ions,

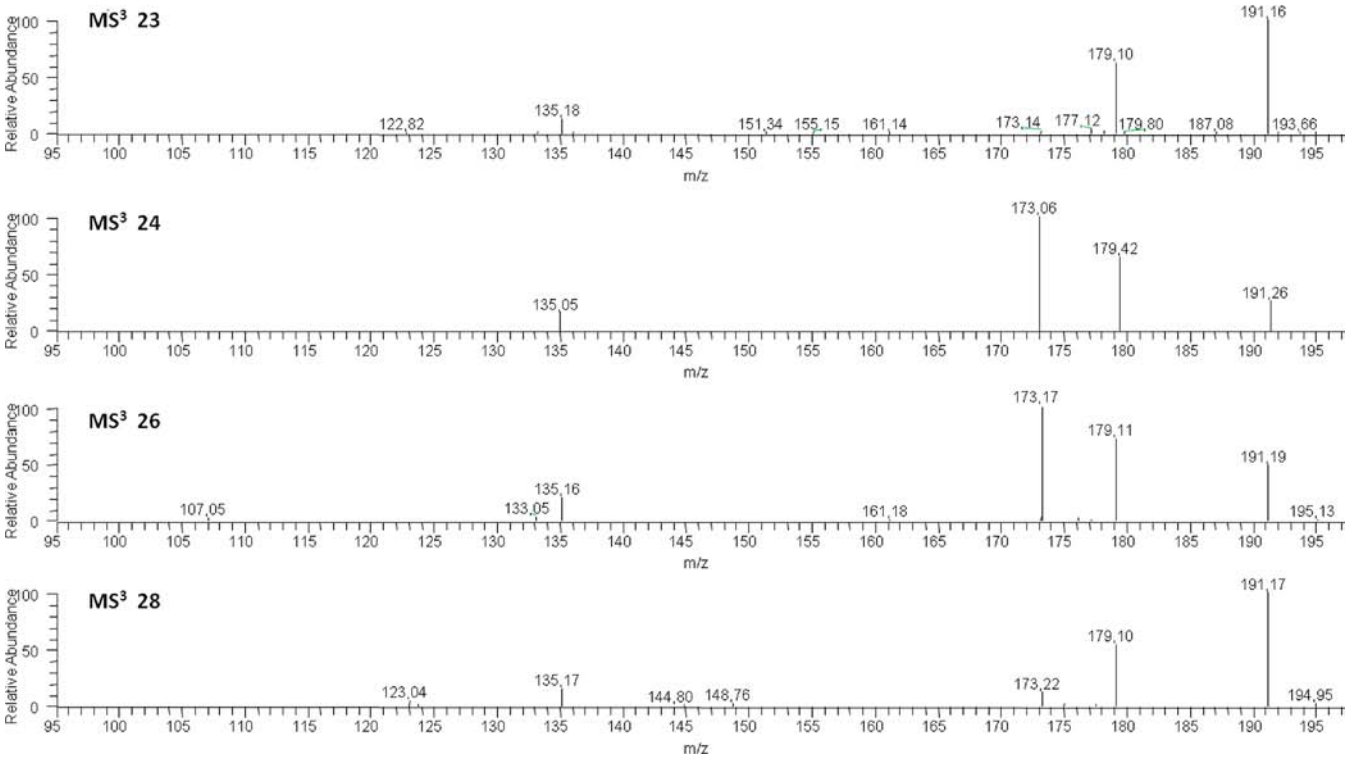

Figure 4. MS^3 spectra of 1,3-dicaffeoylquinic acid (**23**), 1,4-dicaffeoylquinic acid (**24**), 3,4-dicaffeoylquinic acid (**26**), and 3,5-dicaffeoylquinic acid (**28**) in *C. endivia* var. *crispum*.

and the hydrophilic features, compound **24**, which was found only in var. *crispum*, was assigned to 1,4-dicaffeoylquinic acid, while compounds **23**, **26**, and **28** were identified, respectively, as 1,3-dicaffeoylquinic acid, 3,4-dicaffeoylquinic acid, and 3,5-dicaffeoylquinic acid (isochlorogenic acid, found only in var. *crispum*).[25−27]

Compound **6** produced the MS^2 base peak at m/z 149 after losing a caffeoyl residue, and secondary peaks at m/z 179 $[M - H - \text{tartaric acid}]^-$ and m/z 135 due to the subsequent loss of a CO_2 molecule. However, a signal in the mass spectrum at m/z 623, resulting from adduct formation of two individual molecules, was predominant as compared to the ion at m/z 311. On the basis of these and the UV data, the compound was identified as *trans*-caffeoyltartaric acid (*trans*-caftaric acid).[13,28]

Compound **31** eluted at approximately 75 min and exhibited absorption maxima at 241 and 327 nm, with a shoulder at 305 nm. It displayed $[M - H]^-$ at m/z 473 and $[2M - H]^-$ at m/z 947 as well as MS^2 fragmentation with loss of 162 amu, corresponding to the caffeoyl moiety, producing a base peak at m/z 311. The MS^2 fragmentation produced m/z at 293 $[M - H - \text{caffeoyl} - H_2O]^-$, at 179 $[M - H - \text{caffeoyltartaric}]^-$ and at 149 $[M - H - \text{dicaffeoyl}]^-$, leading to identification of chicoric acid. The occurrence of chicoric acid has previously been reported in different plants of the *Asteraceae* family,[28] in particular in vegetables of the *Chicorium* genus.[21−23]

Two peaks (compounds **18** and **21**) eluting, respectively, at 57.8 and 64.5 min, produced a molecular ion at m/z 367. Both peaks displayed identical UV−vis spectra and fragmentation patterns producing the base peak at m/z 191, due to the loss of a feruloyl moiety, and the secondary peak at m/z 173. These data are consistent with the *cis* and *trans* isomers of 5-feruloylquinic acid. Acyl quinic acids normally occur with the acyl acid residue(s) predominantly in the *trans* configuration, but when exposed to natural UV light, they undergo *trans-cis* isomerization.[28] The *cis*-5-acyl isomers were appreciably more hydrophobic than their *trans* counterparts, as reported by Clifford et al.;[27] compound **18** was therefore attributed to the *trans* isomer, and compound **21** was attributed to the *cis* isomer. In *C. endivia* var. *latifolium*, only the *cis* isomer was present.

Characterization of Flavonol Derivatives. Compounds **32** and **38** showed a molecular ion at m/z 447. They were characterized by retention times of 76.2 and 81.3 min, respectively. They led to the same fragmentation pattern, albeit with different intensities; the relative abundance of the radical aglycone ion closely correlated with the glycosylation position: in fact, in MS^2 compound **32** showed the base peak at m/z 285 due to loss of 162 amu, deriving from the fragmentation process of heterolytic cleavage of deprotonated flavonoid glycosides;[17,29] in contrast, compound **38** produced as the base peak at m/z 284 the radical aglycone ion, deriving from a homolytic cleavage. These features are characteristic of 7-*O*-glycosylation and 3-*O*-glycosylation, respectively. In addition, the MS^2 spectrum of kaempferol-3-*O*-glucoside disclosed an ion at m/z 255 that was more abundant than the one at m/z 257, whereas the opposite was noted in kaempferol-7-*O*-glucoside. Furthermore, compound **32** produced a fragment $[M - H - 120]^-$ at m/z 327 that was not found in the corresponding spectrum of compound **38**, indicating a different glycosidic linkage, as reported by Ablajan et al.[17] The presence of kaempferol-monoglucoside has previously been reported in *Cichorium* vegetables,[30] but the presence of the two different isomers in var. *crispum* is shown here for the first time.

Compound **30** was assigned to kaempferol-monoglucuronide; it showed two absorption maxima at 265 and 346 nm in the UV−vis spectrum and produced a base peak at m/z 287, corresponding to the loss of the monoglucuronic residue (176 amu) in the MS^2 spectrum (set in positive ionization mode).

This compound, which was not found in var. *latifolium*, was identified in fresh *C. endivia* extracts by DuPont et al.,[30] who noted its degradation in extracts stored at −20 °C. Analysis of our extracts frozen at −40 °C for 3 months did not evidence degradation of this derivative.

Two peaks, eluting at 78.0 and 79.5 min, respectively, were detected at m/z 535 (compounds **35** and **36**) in positive ionization mode. Also, in this case, they led to the same fragmentation pattern (m/z 287, m/z 286, and m/z 449), albeit with different intensities; the relative abundance of the radical aglycone ion made it possible to identify the glycosylation position; compound **35** was identified as kaempferol 7-*O*-(6′′-malonyl)-glucoside and compound **36** as kaempferol 3-*O*-(6′′-malonyl)-glucoside. The latter compound had previously been observed in endive by Heimler[21] and DuPont.[30]

Compound **17**, found only in var. *crispum*, produced an ion at m/z 611 in positive ionization mode. The ion produced the MS^2 base peak at m/z 287 and a secondary peak at m/z 449 (20% of the base peak) that was due to a glucosyl loss from the $[M + H]^+$. On the basis of literature data and on the low relative intensity at m/z 449, the compound was assigned to kaempferol *O*-disaccharide with two sugar moieties linked to the same phenolic position.[31]

Another kaempferol derivative found in var. *crispum* was compound **20**, producing in positive ionization mode a molecular ion at m/z 697. Its fragmentation led to m/z at 449 due to the loss of a malonyl-glucose moiety and to secondary peaks, respectively, at m/z 535, due to loss of a glucose residue, and at m/z 287, corresponding to the aglycone. This fragmentation pattern was observed in 3,7-di-*O*-glycoside; the fact that the relative abundance of m/z 449 was higher than that of m/z 535 suggested that neutral loss of the 7-*O*-(6′′-malonyl)-glucoside residue is more favorable than the loss of 3-*O*-glucoside. Therefore, the compound was identified as kaempferol 3-*O*-glycosil-7-*O*-(6″-malonyl)-glucoside.[17]

A quercetin derivative (compound **19**), found only in var. *crispum*, was detected as a monoglucoside derivative. It showed a MS fragmentation characteristic of flavonol, with a hexoside at position 7. The base peak was at m/z 301; a secondary peak, deriving from a homolytic cleavage, was at m/z 300. These findings and two absorption maxima at λ = 255 and 351 nm in the UV−vis spectrum allowed identification of the compound as quercimeritrin (quercetin 7-*O*-glucoside).

Compound **29**, eluting at 72.9 min, was assigned to quercetin-3-*O*-(6″-malonyl)-glucoside through comparison of its MS fragmentation, retention time, and UV−vis spectrum with literature data. Its fragmentation yielded a base peak at m/z 303 $[M + H - 248]^+$, corresponding to the loss of a malonyl-glucose residue, and a secondary ion at m/z 389, corresponding to the loss of a glucose residue.

A peak at 85.1 min (compound **40**) produced a molecular ion at m/z 625. Its MS fragmentation led to ion $[M - H - 162]^-$ as the base peak and to secondary peaks, respectively, at m/z 462, deriving from homolytic cleavage, and at m/z 301 ($[M - H - 162 - 162]^-$), indicating a di-*O*-glycosylation. Comparison with the commercial standard of MS and MS^2 spectra, retention time, and UV−vis spectra with maxima at 255 and 349 nm and a shoulder at 268 nm, compound **40** was assigned to quercetin 3,7-di-*O*-glucoside.

A peak at 66.0 min (compound **22**) produced a molecular ion at m/z 481 in positive ionization mode. The MS/MS spectrum showed the base peak $[M + H]^+$ at m/z 319 due to loss of 162 amu (a glucose residue) and a secondary ion at m/z 335. The UV−vis spectrum showed two maxima at 255 and 355 nm. The compound, absent in var. *latifolium*, was assigned to myricetin 3-*O*-glucoside.

Putative Characterizations and Unidentified Compounds. Compound **5** produced two absorption maxima at 223 and 279 nm and showed a pseudomolecular ion at m/z 299 in the negative ionization mode; in the MS^2 spectrum, it gave a base peak at m/z 203 and secondary ions at m/z 281 and 248, respectively. Its chemical structure remains unknown.

Two further unknown compounds (**7** and **8**, found only in var. *crispum*), eluting at 28.8 and 30.6 min, shared the same pseudomolecular ion at m/z 313 and the same fragmentation pattern, despite different abundances.

Compound **9** was tentatively identified as a dimethyl ester of caffeoyltartaric acid. The loss of a caffeoyl residue in the MS^2 spectrum produced the m/z at 177. The further loss of two methyl groups and a carboxylic group gave m/z at 149 and 133, respectively, with very low intensity.

Compound **10**, eluting at 39.9 min, produced in the MS/MS spectrum a pseudomolecular ion at m/z 331 and a fragment ion at m/z 151, corresponding to vanillin after the loss of 162 amu. It could therefore be vanillin hexoside or caffeoylvanillin. Compounds **9** and **10** were found only in var. *crispum*.

Compound **11**, eluting at 40.2, min showed a pseudomolecular ion at m/z 439; its fragmentation gave the base peak at m/z 421 and secondary ions at m/z 259 and 215, respectively. Its chemical structure remains unknown.

Compound **12**, eluting at about 41 min, produced a pseudomolecular ion at m/z 307 and was tentatively assigned to the malic ester of quinic acid due to the presence of an ion at m/z 191 (loss of the malic residue) and at m/z 133 (loss of the quinic residue) in the MS^2 spectrum.[32]

Compound **16**, found only in var. *crispum* and eluting at 55.3 min, was tentatively assigned to quercetin-monoglucuronil-(6″-malonyl)-glucoside; it produced a pseudomolecular ion at m/z 727 in the positive ion mode. Its fragmentation yielded a base peak at m/z 479, due to the loss of 248 amu, corresponding to a malonyl-glucose residue, and a secondary ion at m/z 303 assigned to the aglycone.

Compound **25**, eluting at 71.0 min, produced a pseudomolecular ion at m/z 483 and the base peak at m/z 285. It was not identified.

Compound **27**, eluting at 72.0 min, was tentatively identified as a galloyl derivative. It produced a pseudomolecular ion at m/z 411 and showed in MS^2 spectrum an ion at m/z 259 due to the loss of a gallic acid residue.

Compound **33** was tentatively assigned to quercetin−monoglucuronide; it produced, respectively, two absorption maxima at 265 and 346 nm and a pseudomolecular ion at m/z 479 in the positive ion mass spectrum. Its fragmentation yielded the base peak at m/z 435 due to the loss of CO_2 (44 amu) and secondary ions at m/z 303 (corresponding to the aglycone) and m/z 289.

Peak 34, eluting at ∼77 min, produced a pseudomolecular ion at m/z 499 and exhibited two absorption maxima around 240 and 330 nm in the UV−vis spectrum. Its fragmentation gave an ion at m/z 353 indicating the loss of 146 amu (corresponding to the rhamnose moiety), an ion at m/z 191 corresponding to the quinic acid residue, and another at m/z 179 corresponding to the caffeic acid residue. The compound was therefore tentatively identified as caffeoylquinic acid rhamnoside.

Compound **37**, eluting at 79.9 min, gave a pseudomolecular ion at *m/z* 529 and produced an ion at *m/z* 379 due to the loss of a vanillic acid residue in the MS^2 spectrum. It could be a vanillic acid derivative.

Compound **39**, eluting at 84.6 min, was tentatively assigned to a kaempferide derivative. It produced a pseudomolecular ion at *m/z* 579 in the positive ion mode. Its fragmentation yielded the base peak at *m/z* 301 due to the loss of 278 amu and a secondary ion at *m/z* 392. This compound was not found in var. *latifolium*.

Compound **41**, again found only in var. *crispum*, eluted at 86.2 min and belonged to the flavanone compounds; it was tentatively identified as pinocembrin-*O*-rhamnoside. It gave a pseudomolecular ion in positive ion mode at *m/z* 403 that after fragmentation produced the base peak at *m/z* 257, corresponding to pinocembrin. Its UV−vis spectrum exhibited absorption maxima at 287 and 312 nm.

In conclusion, in this study, the data-dependent acquisition approach was applied to the identification of unknown compounds by MS. The approach led to identification of a large number of phenolic acid and flavonol derivatives in *C. endivia* var. *crispum* and var. *latifolium*. Two free small organic acids (malic and quinic acids), two free hydroxycinnamic acids (*p*-coumaric and caffeic acids), 16 hydroxycinnamic acid derivatives, 14 flavonols (kaempferol, quercetin, and myricetin derivatives), a flavanone derivative, a quinic ester, and a galloyl derivative were identified.

Some of these molecules, for example, hydroxycinnamic acid derivatives, flavonoids, and stilbenes, have been reported to protect against several diseases, such as cancer, cardiovascular disease, inflammation, and chronic neurodegenerative conditions. Other molecules, including catechin and proanthocyanidins, seem to be protective against several bacterial pathogens; as yet unpublished in vitro studies by our group suggest that quinic acid may be active against oral cavity pathogens. Further studies, focusing especially on the adsorption and metabolism of these compounds, are needed to define the significance of polyphenols in improving human health. Greater attention should be devoted to the activity of the different isomers and their synergism, as indicated by our preliminary studies involving the different activities of monocaffeoylquinic acid isomers against oral bacteria.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; ESI-MS, electrospray ionization mass spectrometry; MW, molecular weight; MS, mass spectrometry; PDA, photodiode array detector; amu, atomic mass unit

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