Separation and Characterization of Phenolic Compounds in Fennel (*Foeniculum vulgare*) Using Liquid Chromatography–Negative Electrospray Ionization Tandem Mass Spectrometry

Irene Parejo,[†] Olga Jauregui,[‡] Ferran Sánchez-Rabaneda,[†] Francesc Viladomat,[†] Jaume Bastida,[†] and Carles Codina*,[†]

Departament de Productes Naturals, Biologia Vegetal i Edafologia, Facultat de Farmàcia, Universitat de Barcelona, Avinguda Joan XXIII s/n, 08028 Barcelona, Catalunya, Spain, and Serveis Científico-Tècnics, Universitat de Barcelona, Josep Samitier 1-5, 08028 Barcelona, Catalunya, Spain

Liquid chromatography (LC) diode array detection (DAD) coupled to negative electrospray ionization (ESI) tandem mass spectrometry (MS/MS) was used for the rapid and sensitive identification of water-soluble phenolic compounds in fennel waste. The plant material was first extracted and then chromatographed on Sephadex LH-20 to afford seven fractions, each of them being subjected to LC-MS analysis. Identification of the compounds was carried out by interpretation of UV, MS, and MS/MS spectra. Forty-two phenolic substances were identified, 27 of which had not previously been reported in fennel, including hydroxycinnamic acid derivatives, flavonoid glycosides, and flavonoid aglycons.

INTRODUCTION

Fennel (*Foeniculum vulgare*) is a widespread plant species commonly used as a spice and flavoring and in folk medicine as a carminative, digestive, lactagoge, and diuretic (*1*). Despite being widely studied for its essential oils by gas chromatography−mass spectrometry (GC-MS) (*2*), little information is available on the nonvolatile constituents of fennel. Thus, only some phenolic compounds, such as chlorogenic acid, quercetin-3-*O*-rutinoside, quercetin-3-*O*-glucuronide, kaempferol-3-*O*-glucuronide, isoquercitrin, and isorhamnetin-3-*O*-glucoside have been previously described as occurring in fennel (*3−6*). To our knowledge, only one work on the identification of phenolics by liquid chromatography−mass spectrometry (LC-MS) has been carried out in fennel teas and decoctions of fennel seeds with the detection of only quercetin-3-*O*-glucuronide and chlorogenic acid, together with two other nonphenolic substances, *trans*-anethole and *p*-anisaldehyde (*1*).

In a former study, fennel waste proceeding from essential oil distillation was found to exhibit high antioxidant activity and total phenolic content (*7*). The activity of that material free of essential oil was thought to be due to its nonvolatile compounds, mainly to the phenolic substances remaining in the waste. On the basis of these previous results, and the potential use of fennel as a source of antioxidant extracts of commercial

interest, the identification of as many compounds as possible was performed in the present work.

Plant extracts usually contain complex mixtures of phenolic substances, and LC-MS/MS has been proved to be a powerful tool for rapid analysis of these polar, nonvolatile, and thermally labile constituents (*8*). Although the direct identification of the compounds by comparison of both retention times and UV spectra with those of reference substances or with data reported in the literature is possible, the on-line combination of LC-DAD and MS techniques provides useful information for peak characterization. Thus, in this work, a liquid chromatography diode array detection (DAD) coupled to negative electrospray ionization (ESI) tandem mass spectrometry (MS/MS) method for the separation and identification of phenolic compounds in fennel waste was used.

MATERIALS AND METHODS

Chemicals. Methanol, ethyl acetate, acetonitrile, and hexane (HPLC grade) were from SDS (Peypin, France), formic acid (analytical grade) was from Probus (Badalona, Spain), and acetic acid (analytical grade) was from Merck (Darmstadt, Germany). Ultrapure water (Milli-Q, Waters, Milford, MA) was used. Phenolic standards were purchased as follows: chlorogenic acid, quercetin-3-*O*-rutinoside, and kaempferol from Sigma (St. Louis, MO); rosmarinic acid, luteolin, isorhamnetin, naringenin, kaempferol-3-*O*-glucoside, luteolin-7-*O*-glucoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-galactoside, kaempferol-3-*O*-rutinoside, kaempferol-7-*O*-neohesperidoside, eriodictyol-7-*O*-neohesperidoside, and isorhamnetin-3-*O*-rutinoside from Extrasynthese (Genay, France). Neochlorogenic acid, cryptochlorogenic acid, quercetin-3-*O*-

---

* Corresponding author (telephone +34-93-4024493; fax +34-93-4029043; e-mail ccodina@farmacia.far.ub.es).
† Departament de Productes Naturals.
‡ Serveis Científico-Tècnics.

Table 1. Phenolic Compounds Identified in Fennel Waste by LC-ESI-MS/MS

| | | | | ions full scan MS | | MS/MS experiments | | |
compound	peak	t_R (min)	fraction	[M − H]⁻	fragments	neutral loss scan	precursor ion scan	product ion scan
p-hydroxybenzoic acid-*O*-glucoside[a]	1	6.01	C	299	137	162		299
3-caffeoylquinic acid (neochlorogenic acid)	2	7.39	A, B	353	191, 179	162		353
5-caffeoylquinic acid (chlorogenic acid)	3	8.22	A, B	353	191, 179	162		353
esculetin-*O*-glucoside (esculin)[a]	4	8.38	C	339	177	162		339
1-caffeoylquinic acid[a]	5	11.06	A, B	353	191	162		353
3-feruloylquinic acid[a]	6	11.73	A, B	367	193			367
4-caffeoylquinic acid (cryptochlorogenic acid)	7	12.00	A, B	353	191, 173	162		353
isorhamnetin-*O*-dihexoside 1[a]	8	12.60	C	639	477, 315, 300	162	315	
isorhamnetin-*O*-dihexoside 2[a]	9	13.20	C	639	477, 315, 300	162	315	
6, 8-*C*-dihexosylapigenin[a]	10	13.60	C	593	503, 473, 383, 353			593
4-coumaroylquinic acid[a]	11	13.87	A, B	337	191	162		337
5-coumaroylquinic acid[a]	12	14.47	A, B	337	191, 163	162		337
5-feruloylquinic acid[a]	13	15.10	A, B	367	191			367
quercetin-*O*-dihexoside 1	14	15.22	F	625	301, 151	162	301	625
1-feruloylquinic acid[a]	15	15.54	A, B	367	193, 173			367
quercetin-*O*-dihexoside 2[a]	16	15.79	F	625	463, 301, 151	162	301	625
4-feruloylquinic acid[a]	17	16.04	A, B	367	193			367
quercetin-3-*O*-rutinoside (rutin)	18	16.80	D, E	609	301, 151	308	301	
eriodictyol-7-*O*-rutinoside (eriocitrin)	19	17.19	D, E, F	595	459, 287, 151, 135	308	287	595
luteolin-7-*O*-rutinoside[a]	20	17.91	E	593	285	308	285	
quercetin-3-*O*-galactoside (hyperoside)	21	18.30	G	463	301, 271, 255, 151	162	301	
naringerin-7-*O*-rutinoside (narirutin)[a]	22	18.58	F	579	271, 119	308	271	
quercetin-3-*O*-glucoside (isoquercitrin)	23	18.60	G	463	301, 271, 255, 151	162	301	
kaempferol-3-*O*-rutinoside	24	18.90	E	593	285, 255, 227	308	285	
kaempferol-3-*O*-glucoside	25	19.79	G	447	285, 255, 227	162	285	
isorhamnetin-3-*O*-rutinoside[a]	26	20.05	E	623	315, 300, 271, 151	308	315	
quercetin-3-*O*-glucuronide (miquelianin)	27	20.28	D, E, F	477	301, 151, 135	176	301	
luteolin-7-*O*-glucuronide[a]	28	20.40	D	461	285, 216	176	285	285
isorhamnetin-3-*O*-galactoside[a]	29	20.47	G	477	315, 300, 271	162	315	
isorhamnetin-3-*O*-glucoside[a]	30	20.87	G	477	315, 300, 271	162	315	
1,3-dicaffeoylquinic acid[a]	31	21.09	F, E	515	353, 191, 179	162	353	515, 353
dicaffeoylquinic acid[a,b]	32	21.20	F, E	515	353, 191, 173	162	353	515, 353
1,5-dicaffeoylquinic acid	33	21.69	F	515	353, 191	162	353	515, 353
kaempferol-3-*O*-glucuronide	34	22.00	D	461	285, 257, 229, 135	176	285	285
isorhamnetin-3-*O*-glucuronide[a]	35	22.30	C	481	315, 300, 271, 135	176	315	491, 315
rosmarinic acid	36	22.45	C	359	197, 179, 161, 135	162		359
apigenin-7-*O*-glucuronide[a]	37	22.96	C	445	269	176	269	
acacetin-7-*O*-rutinoside[a]	38	25.53	F	591	283, 268	308	283	591
acacetin[a]	39	25.60	F	283	268, 240, 151			283
kaempferol	40	29.07	C	285	257, 229, 216			285
naringenin[a]	41	30.34	D	271	151			271
isorhamnetin[a]	42	32.76	C	315	300, 271, 255, 135			315

[a] Compounds identified for the first time in fennel. [b] Could be either 1,4-dicaffeoylquinic acid or 3,4-dicaffeoylquinic acid (as described in the text).

glucuronide, eriodictyol-3-*O*-rutinoside, and 1,5-*O*-dicaffeoylquinic acid were isolated from fennel in our laboratory. The purity of the standards was >98%, and all of them were prepared as stock solutions at 1 g/L in methanol. Working standard solutions (1 mg/L) were made by diluting the stock solutions with the LC mobile phase.

Plant Material. Bitter fennel (*F. vulgare* Mill. var. *vulgare*, Apiaceae) was collected during the flowering period from cultures established in an experimental plot (Cetina, Zaragoza, Spain) under agronomically controlled conditions. The plant material was first distilled for essential oils by steam distillation at pilot plant scale ("La Alfranca" Experimental Farm, Diputación General de Aragón) under a standard operation protocol of the Spanish Ministerio de Agricultura, Pesca y Alimentación. After distillation, the remaining material was air-dried and then powdered with a mill.

Extraction and Fractionation. One kilogram (dry weight) of fennel waste was extracted by water decoction (15 L × 2) for 15 min and then chromatographed by consecutive passage over two 70 × 5 cm Amberlite columns packed with XAD-7 and XAD-16. The phenolic compounds were eluted with methanol/water 1:1 (2 L), methanol (4 L), and finally acetone (3 L) until the eluate was colorless. This eluate was concentrated under vacuum to dryness and then redissolved in water and partitioned with ethyl acetate, affording a water soluble fraction and an ethyl acetate soluble fraction. An aliquot of the water fraction was dissolved in methanol/water (1:1) and fractionated by gel filtration

on a 50 × 5 cm column of Sephadex LH-20 (Pharmacia, MeOH/H₂O, 1:1, 1 mL/min) to afford 96 fractions (10 mL each). The fractions were chromatographed by thin-layer chromatography (TLC) on Alugram silica gel plates (Macherey-Nagel) with EtOAc/AcOH/H₂O (10:2:3) and monitored under UV light at 254 and 365 nm and by spraying with a DPPH• methanolic solution (20 g/L) and with 1% diphenylboric acid in methanol for UV enhancement of phenolic compounds. Seven final active fractions (A−G) were obtained and analyzed by HPLC-DAD-ESI-MS/MS to determine the phenolic composition.

Analytical Conditions. LC analyses were carried out using an 1100 Agilent quaternary pump system (Waldbronn, Germany) equipped with autosampler and DAD. A Hewlett-Packard Chemstation was used for data acquisition and processing. A 150 × 2.1 mm i.d., 5 μm, Luna C$_{18}$ column (Phenomenex, Torrance, CA) was used for the separation of phenolics. Gradient elution was performed with water/0.1% formic acid (solvent A) and acetonitrile/0.1% formic acid (solvent B) at a constant flow rate of 400 μL/min. An increasing linear gradient (v/v) of solvent B was used [*t* (min), % B]: 0, 5; 10, 15; 30, 35; 40, 80; 45, 5. Detection was carried out at 280 and 365 nm, with peak scanning between 200 and 600 nm. An API 3000 triple-quadrupole mass spectrometer (Perkin-Elmer Sciex, Concord, ON, Canada) was used. All of the analyses were carried out using a Turbo Ionspray source in the negative mode with the following settings: capillary voltage, 3500 V; nebulizer gas (N₂),
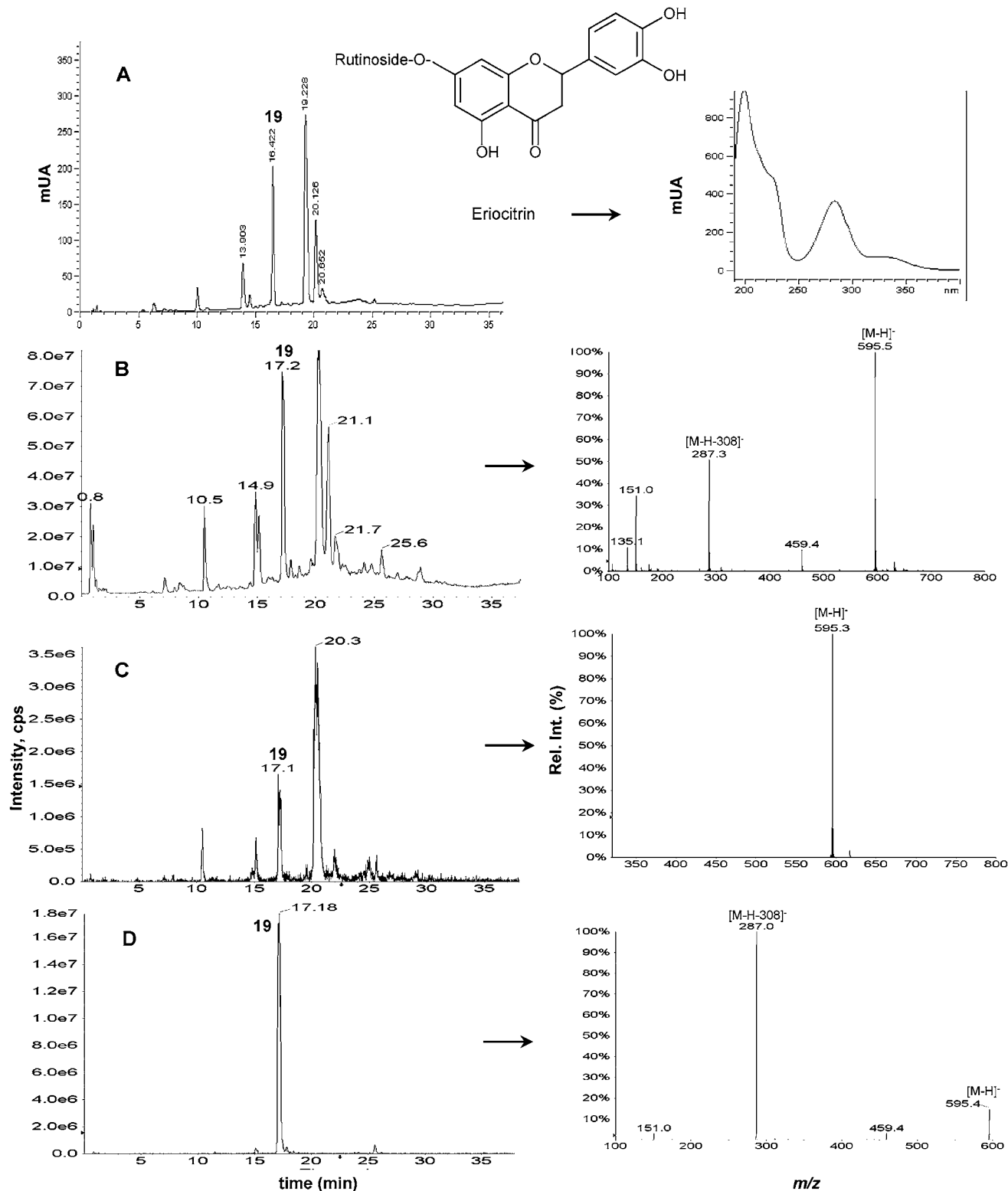
Figure 1. Sequence of identification of eriodictyol-7-*O*-rutinoside (eriocitrin, **19**) in fraction F: (**A**) UV chromatogram at 280 nm and 200–600 nm UV spectrum of peak 19; (**B**) TIC chromatogram in full scan mode from *m/z* 100 to 800 u and MS spectrum; (**C**) TIC chromatogram in neutral loss scan of 308 and 176 u and MS/MS spectrum in neutral loss of 308; (**D**) TIC chromatogram in product ion scan of *m/z* 595 and MS/MS spectrum in product ion scan of *m/z* 595.

10 (arbitrary units); curtain gas (N_2), 12 (arbitrary units); collision gas (N_2), 10 (arbitrary units); focusing potential, -200 V; entrance potential, 10 V; drying gas (N_2), heated to 400 °C and introduced at a flow rate of 8000 mL/min. The declustering potential (DP) was 60 and the collision energy (CE), 35.

Qualitative LC-DAD-MS/MS. Full scan acquisition was performed by scanning from *m/z* 100 to 800 u in profile mode at a cycle time of

2 s with a step size of 0.1 u and a pause between each scan of 2 ms. MS/MS product ions were produced by collision-activated dissociation (CAD) of the selected precursor ions in the collision cell of the triple-quadrupole mass spectrometer and analyzed using the second analyzer of the instrument. In all of the experiments, both quadrupoles (Q_1 and Q_3) were operated at unit resolution. Different MS/MS experiments, neutral loss scan, product ion scan, and precursor ion scan of selected
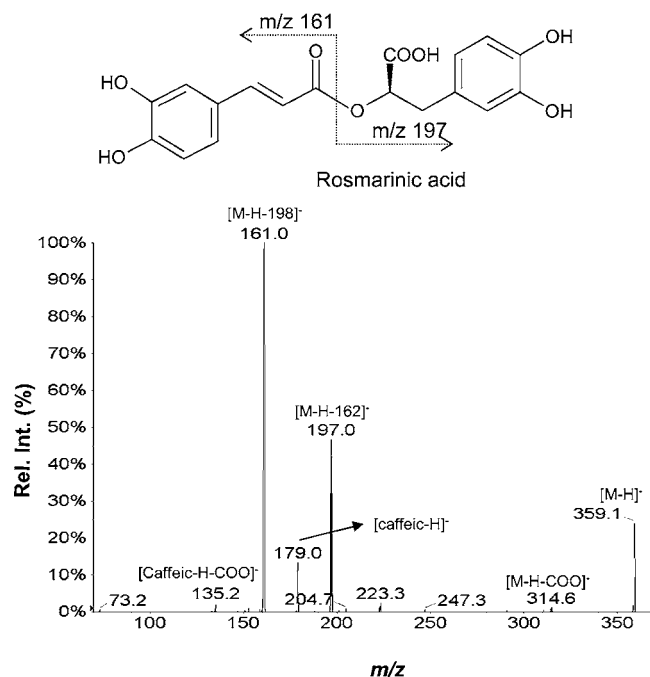
Figure 2. Identification of rosmarinic acid (peak 36) in fraction C: MS spectrum in product ion scan of *m*/*z* 359.

molecules were carried out to confirm the structure of the compounds previously identified by full scan mode.

RESULTS AND DISCUSSION

The seven fractions obtained (A−G) were analyzed by LC-DAD-ESI-MS/MS. Sample peaks were compared with those of available reference compounds analyzed under the same LC conditions in order to compare the UV and mass spectra, as well as the retention time. For those compounds not commercially available, full scan mode analyses followed by MS/MS experiments in negative mode appeared to be a powerful tool for their characterization. The 42 compounds identified in fennel waste are listed in **Table 1**. **Figure 1** shows an example of the steps followed in peak identification, that of eriodictyol-7-*O*-rutinoside (**19**). First, the UV spectrum (**Figure 1A**) gives information about the family of phenolic compounds (peak 19 showed a UV spectrum typical of flavanone glycoside with absorption bands at 200 and 284 nm). Second, the mass spectrum in full scan mode (**Figure 1B**) displayed a first approach to the compound structure, where the [M − H]⁻ ion

corresponds to the deprotonated aglycon *m*/*z* 287 due to the loss of 308 u (**Figure 1C**), which indicated the presence of a rhamnoglucose moiety. Finally, MS/MS experiments in product ion scan of *m*/*z* 595 (**Figure 1D**) were carried out for sugar elucidation. Eriodictyol-7-*O*-rutinoside was also identified by comparison of its retention time and MS/MS experiment in product ion scan of *m*/*z* 595 with those of a reference compound.

UV Identification. A preliminary study of the UV spectrum of the peaks gave a first indication of the family of phenolic compounds. Thus, the UV profile of fractions A, B, and C showed the occurrence of compounds with absorption bands at 322−324 and 216−218 nm and a shoulder at 290−300 nm, typical of phenolics containing a hydroxycinnamoyl moiety. Moreover, fraction C was found to contain a compound with absorption bands at 271 and 336 nm, typical of a *C*-glycoside. Fractions from D to G showed the occurrence of quercetin derivatives with absorption bands at 257 and 354 nm and a shoulder at 256 nm, kaempferol derivatives with absorption bands at 266 and 348 nm and a shoulder at 290 nm, luteolin derivatives with absorption bands at 256 and 348 nm and a shoulder at 266 nm, isorhamnetin derivatives with absorption bands at 265 and 354 nm and a shoulder at 282 nm, and a flavanone derivative with absorption bands at 200 and 284 nm (*9*).

Simple Phenolic Acid Derivatives. *p*-Hydroxybenzoic acid-*O*-glucoside (**1**) and esculetin-*O*-glucoside (esculin) (**4**) were identified in fraction C by MS/MS experiments. A characteristic fragmentation involving cleavage of the intact sugar and the ion corresponding to the phenolic acid fragment was observed. The MS/MS experiment in neutral loss scan of 162 u revealed the hexoside nature of these compounds, which was confirmed by the product ion scan of *m*/*z* 299 and 337, respectively, for compounds **1** and **4**. These results are consistent with those reported by Fang et al. (*10*), who determined phenolic glycosides in dried plums by multiple ion trap mass spectrometry using an electrospray ionization source in negative mode (LC-ESI-ITD-MS).
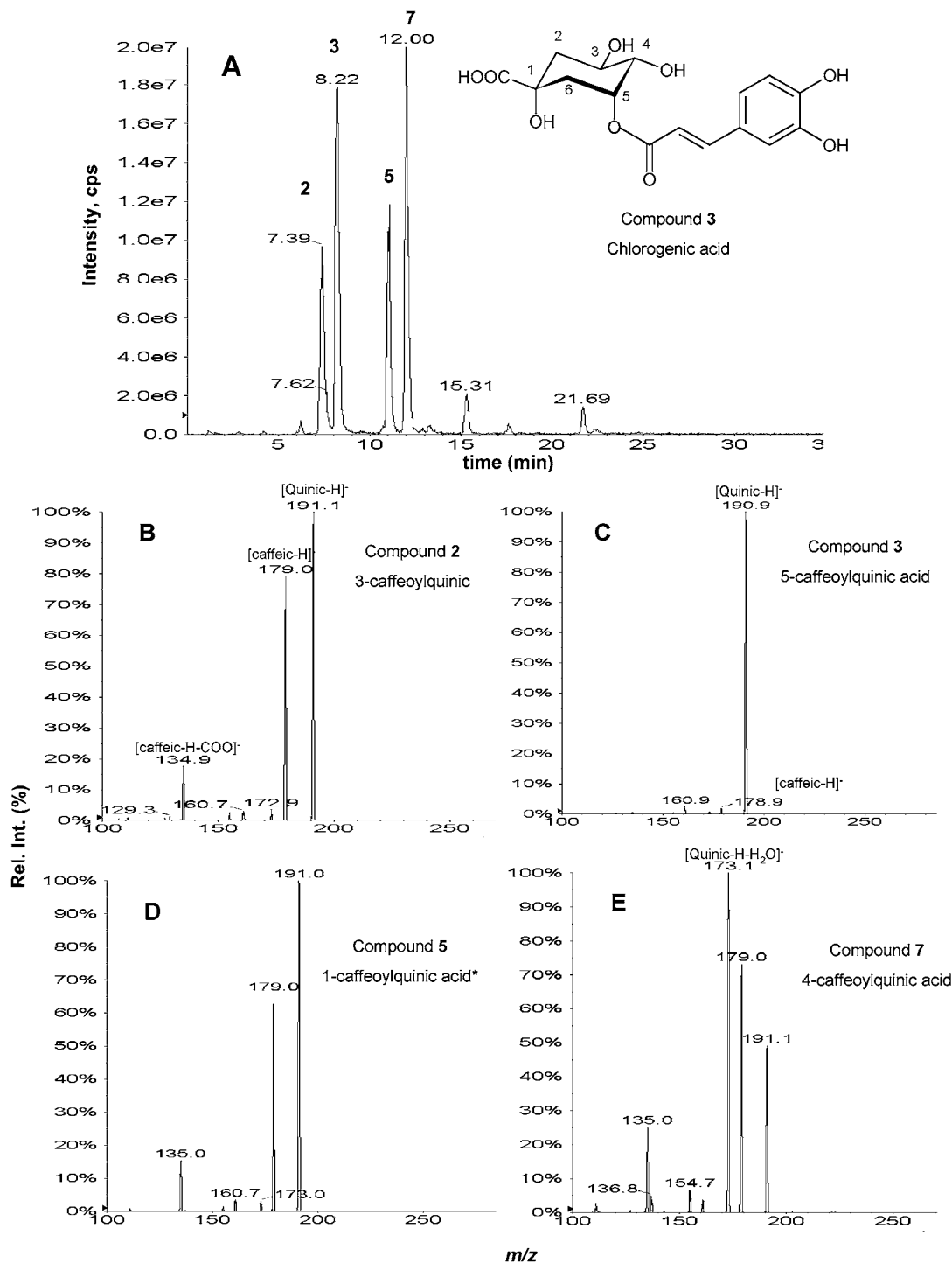
Rosmarinic acid (**36**) was also identified in fraction C by comparison of its retention time and MS spectrum in full scan mode and MS/MS experiments in neutral loss scan of 162 u and product ion scan of *m*/*z* 359 with those of a reference compound. **Figure 2** shows the MS/MS product ion scan spectrum of this compound, with characteristic fragment ions at *m*/*z* 197 and 161.

Hydroxycinnamoylquinic Acid Derivatives. Peaks of *m*/*z* 353, 367, and 337 in fraction B revealed the presence of four

Table 2. Product Ion Scan Data for Hydroxycinnamoyl Quinic Acids

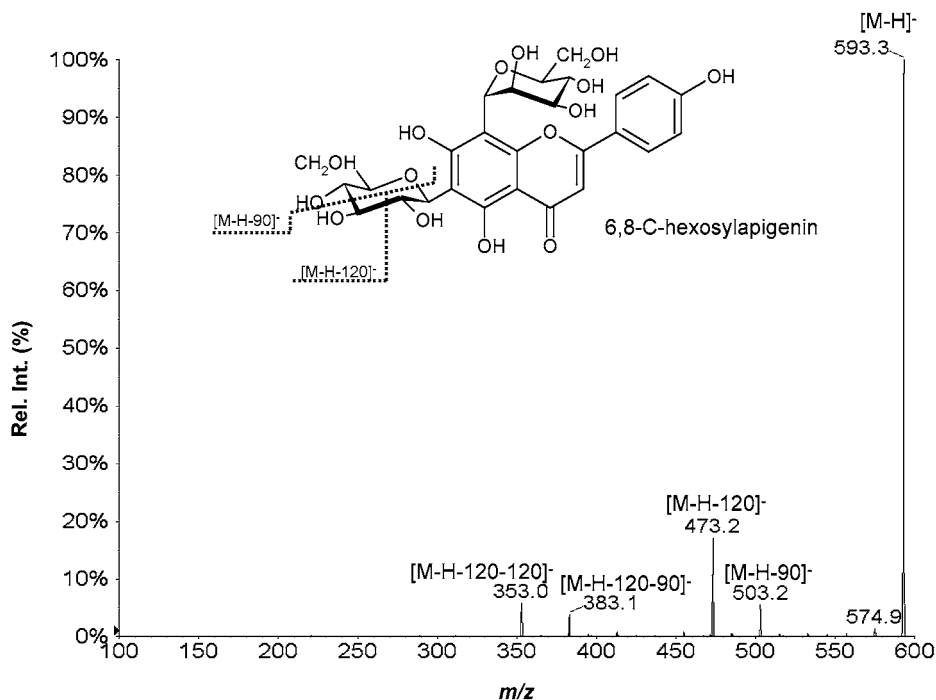|	product ion scan of [M − H]⁻ ions		product ion scan of [M − H − caffeic]⁻ ions	
compound	[M − H]⁻	fragments *m*/*z* (% intensity)	[M − H − caffeic]⁻	fragments *m*/*z* (% intensity)
1-caffeoylquinic acid[a]	353	191 (100), 179 (65)		
3-caffeoylquinic acid	353	191 (100), 179 (76), 135 (19), 161 (10)		
4-caffeoylquinic acid	353	173 (100), 179 (82), 191 (42), 135 (28) (100), 179 (<5)		
5-caffeoylquinic acid	353			
1-feruloylquinic acid[a]	367	191 (100), 173 (<5)		
3-feruloylquinic acid	367	193 (100), 191 (<2)		
4-feruloylquinic acid	367	173 (100), 191 (88)		
5-feruloylquinic acid	367	191 (100), 173 (57)		
4-coumaroylquinic acid	337	173 (100), 163 (<5)		
5-coumaroylquinic acid	337	191 (100), 173 (16)		
1,3-dicaffeoylquinic acid[a]	515	515 (7), 353 (100), 335 (13), 191 (36), 179 (56)	353	353 (6), 191 (100), 179 (91), 161 (6), 155 (18)
dicaffeoylquinic acid[a,b]	515	515 (100), 353 (43)	353	353 (25), 173 (100), 179 (75), 191 (51)
1,5-dicaffeoylquinic acid	515	515 (60), 353 (100), 335 (7), 191 (5)	353	353 (14), 191 (100), 179 (13)

[a] Tentatively identified. [b] Could be either 1,4-dicaffeoylquinic acid or 3,4-dicaffeoylquinic acid (as described in the text).

LC-MS/MS Identification of Phenolics in Fennel

J. Agric. Food Chem., Vol. 52, No. 12, 2004 **3683**

Figure 3. Identification of caffeoylquinic acid isomers in fraction B (peaks 2, 3, 5, and 7): **(A)** TIC chromatrogram in product ion scan of *m/z* 353; **(B–E)** MS/MS spectra in product ion scan of *m/z* 353 showing the different relative intensities of the ion fragments for the different caffeoylquinic acid isomers (*, tentatively assigned).

isomers of chlorogenic acid (compounds **2**, **3**, **5**, and **7**), four feruloylquinic acid derivatives (compounds **6**, **13**, **15**, and **17**), and two isomers of the coumaroylquinic acid (compounds **11** and **12**), respectively, whereas a peak of *m/z* 515 in fractions E and F showed three dicaffeoylquinic acid derivatives (compounds **31**−**33**). Peak identification was accomplished by comparison of MS/MS fragmentation with data reported using LC-ESI-MS (*10*, *11*) and LC-ESI-ITD-MS (*12*), both in negative mode, as well as by comparison with MS/MS spectra of standards when available. Product ion scan experiments of all these compounds revealed characteristic fragmentations involv-

ing cleavage of a caffeoyl, feruloyl, or coumaroyl moiety (respectively for caffeoylquinic, feruloylquinic, or coumaroylquinic acid isomers) or two caffeoyl moieties (dicaffeoylquinic acid isomers). However, the relative intensities of the fragment ions were quite different depending on the quinic acid substitution. Thus, it was possible to differentiate the isomers on the basis of fragments and relative intensities in MS/MS spectra in product ion scan mode (**Table 2**), according to the method of Clifford et al. (*11*). The 4-hydroxycinnamoylquinic acids gave *m/z* 173 [quinic − H − H_2O]⁻, quinic acid dehydrated, as a base peak in product ion scan mass spectrum of [M − H]⁻

3684 *J. Agric. Food Chem.,* Vol. 52, No. 12, 2004

Parejo et al.

Figure 4. Identification of 6,8-*C*-dihexosylapigenin (**10**) in fraction C. The MS/MS spectrum in product ion scan of *m/z* 593 shows the charateristic fragment ions of a *C*-diglycoside flavonoid.

(**Figure 3E**), whereas both 5-hydroxycinnamoylquinic and 3-hydroxycinnamoylquinic acids gave *m/z* 191 [M − H − 162]⁻ corresponding to quinic acid as a base peak in the product ion scan mass spectrum of [M − H]⁻. Thus, for example, 3-caffeoylquinic acid and 5-caffeoylquinic acid could be differentiated from each other by the different intensities of the fragment ion *m/z* 179, which was of 80 and <5%, respectively (**Figure 3B,C**). Product ion scan experiment also allowed distinction between 3-coumaroylquinic acid and 3-feruloylquinic acid on the basis of the base peaks *m/z* 163 and 193, corresponding to coumaric acid and ferulic acid, respectively. Retention times and MS/MS spectra in product ion scan mode of compounds **2**, **3**, **7**, and **33** were identical to those of standard neochlorogenic, chlorogenic, cryptochlorogenic, and 1,5-dicaffeoylquinic acids, respectively. As these compounds were assigned by comparison with reference substances, compound **5** (**Figure 3D**) was tentatively identified as 1-caffeoylquinic acid, although it might also be a stereoisomer of the neochlorogenic acid, chlorogenic acid, or chyptochlorogenic acid (*10*). Identification of dicaffeoylquinic acids **31** and **32** was carried out tentatively as neither reference compounds nor literature data were available. Compound **31** differs from 1,5-dicaffeoylquinic acid (**33**) in the intensity of the fragment ion *m/z* 179 observed in the product ion scan experiment of [M − H − caffeic]⁻, which was 91 and 13% for compounds **31** and **33**, respectively. As was mentioned above for the monocaffeoylquinic acid isomers, this difference in relative intensity allowed us to distinguish between the 3-caffeoylquinic and the 5-caffeoylquinic acid positional isomers. The isomer 3,5-dicaffeoylquinc acid was discarded because the fragment ion *m/z* 335 was not detected, and the intensity of fragment ion *m/z* 179 was half of that observed for compound **31**. Therefore, this compound was tentatively identified as the isomer 1,3-dicaffeoylquinic acid. Compound **32** showed the fragment ion *m/z* 173 as base peak in the product ion scan spectrum of [M − H − caffeic]⁻, which was also previously observed for 4-caffeoylquinic acid. It could be tentatively identified as 1,4- or 3,4-dicaffeoylquinic acid, but

the complete identification was not possible due to the lack of standard.

Flavonoid Diglycosides. Four *O*-dihexosides, two isorhamnetin-*O*-dihexosides (**8** and **9**), two quercetin-*O*-dihexosides (**14** and **16**), and one *C*-dihexoside, 6,8-*C*-dihexosylapigenin (**10**), were found to occur in different fractions of this fennel waste (**Table 1**). Both standard flavonoid *C*-glycosides and flavonoid *O*-glycosides were found to exhibit different fragmentation patterns in MS/MS experiments using a triple-quadrupole mass spectrometer in the negative mode (*13*). In this work, the product ion scan of *C*-diglycosides did not reveal the deprotonated aglycon [M − H]⁻ ions but characteristic ions due to the fragmentation in the *C*-diglycosidic unit. Losses of 90 and 120 u corresponding to cross-ring cleavages in the sugar unit were observed. **Figure 4** shows the product ion spectra of *m/z* 593 of 6,8-*C*-dihexosylapigenin displaying the losses of 90 and 120 u from *m/z* 593 and 473, respectively, which confirmed the presence of two hexose (glucose or galactose) units. Compound **10** was then identified as 6,8-*C*-dihexosylapigenin on the basis of the ion fragments obtained, which were fully in agreement with those reported in *Ocinum gratissimum* var. *gratissimum* using LC-APCI-ITD-MS in negative mode (*14*). Substituents in compound **10** were assigned to positions C-6 and C-8 because to date C-glycosylation has been found in only these positions of the flavonoid nucleus, as reported by Cuyckens and Claeys (*15*). The fragmentation of flavonoid *O*-diglycosides in the product ion scan mode involves the cleavage of the glycosidic bond and the loss of the sugar moieties, thus showing the deprotonated aglycon [A − H]⁻ and, in some cases, the intermediate flavonoid *O*-hexoside [M − H − 162]⁻ ion fragment. Assignment of the position of the hexose (glucose or galactose) unit in compounds **8**, **9**, **14**, and **16** was not possible due to the lack of both standards and reported data.

Flavonoid *O*-Rhamnoglucosides. The presence of flavonoid *O*-rhamnoglucosides was checked by MS/MS experiments in neutral loss scan mode of 308 u, which has been revealed to be

LC-MS/MS Identification of Phenolics in Fennel

*J. Agric. Food Chem.,* Vol. 52, No. 12, 2004 **3685**

useful for the fast screening of these compounds. As for glucosides and glucuronides, the glucosidic bond of *O*-rhamnoglucosides is easily cleaved in the collision cell in the negative mode to generate product ions of [M − H − 308]⁻, which correspond to the fragments resulting from the neutral loss of a rhamnoglucosyl moiety from the deprotonated molecule. Quercetin-3-*O*-rutinoside (rutin) (**18**), eriodictyol-7-*O*-rutinoside (eriocitrin) (**19**), kaempferol-3-*O*-rutinoside (**24**), and isorhamnetin-3-*O*-rutinoside (**26**) were thus identified by comparison of their MS spectrum and MS/MS experiments with those of reference substances. As an example of the characterization of a flavonoid rhamnoglucoside, **Figure 1** shows the identification pattern of eriocitrin (**19**).

Rutinosides can be differentiated from the neohesperidosides by the different relative intensities of the ion fragments in product ion scan mass spectrum as described previously (*13*). Thus, flavonoid rutinosides did not reveal a high degree of fragmentation in opposition to flavonoid neohesperidosides. Furthermore, a high relative intensity of the aglycon fragment (usually 100%) was typical of rutinosides, because in neohesperidosides this fragment was difficult to produce.

Other flavonoid rhamnoglucosides identified were naringenin-7-*O*-rutinoside (narirutin) (**22**) and acacetin-7-*O*-rutinoside (**38**), showing ion fragments at *m/z* 579 and 271 and at *m/z* 591 and 283, respectively, in full scan mode. To elucidate the aglycon, a product ion scan of *m/z* 271 and 283 was performed. These two compounds exhibited different fragment ions: *m/z* 119 for compound **22** and *m/z* 268 and 211 for compound **38**, in agreement with previously reported data (*13*, *16*, *17*). Finally, two peaks showing *m/z* 593 and 285 appeared at retention times of 17.91 (compound **20**) and 18.90 (compound **24**) minutes. Compound **24** was identified as kaempferol-3-*O*-rutinoside by comparison with a standard, whereas compound **20** did not match with the retention time of standard kaempferol-3-*O*-neohesperidoside and showed a high relative intensity of the aglycon fragment, and it was tentatively identified as luteolin-7-*O*-rutinoside, as its MS/MS spectrum in product ion scan mode of *m/z* 285 showed characteristic fragmentation of luteolin, with ions at *m/z* 199, 151, and 133.

Flavonoid Glucuronides. Flavonoid-*O*-glucuronides, such as quercetin-3-*O*-glucuronide (miquelianin) (**27**), luteolin-7-*O*-glucuronide (**28**), kaempferol-3-*O*-glucuronide (**34**), isorhamnetin-3-*O*-glucuronide (**35**), and apigenin-7-*O*-glucuronide (**37**), were identified by neutral loss scan of 176 u, as described in *Erigeron breviscapus* using LC-ESI-MS/MS in negative mode in a triple-quadrupole mass spectrometer (*18*). Quercetin-3-*O*-glucuronide (**27**) and luteolin-7-*O*-glucuronide (**28**) were confirmed by comparison of their MS/MS spectra with those of reference substances. Owing to the lack of standards, compounds **34**, **35**, and **37** were identified after product ion scan of the aglycons, *m/z* 285, 315, and 269, respectively. **Figure 5** shows the different steps in the characterization of isorhamnetin-3-*O*-glucuronide (**35**) as an example of the identification of flavonoid glucuronides.

Flavonoid Glycosides. Flavonoid *O*-glycosides, such as quercetin-3-*O*-galactoside (hyperoside) (**21**), quercetin-3-*O*-glucoside (isoquercitin) (**23**), and kaempferol-3-*O*-glucoside (**25**), were identified by comparison of their retention times (18.30, 18.60, and 19.79 min, respectively) with those of reference substances and also by MS/MS analysis in neutral loss scan of 162 u, which corresponds to the loss of a glucose unit under CAD conditions in the collision cell (*18*). Compounds **29** and **30** were tentatively identified as isorhamnetin-3-*O*-galactoside and isorhamnetin-3-*O*-glucoside, respectively, by
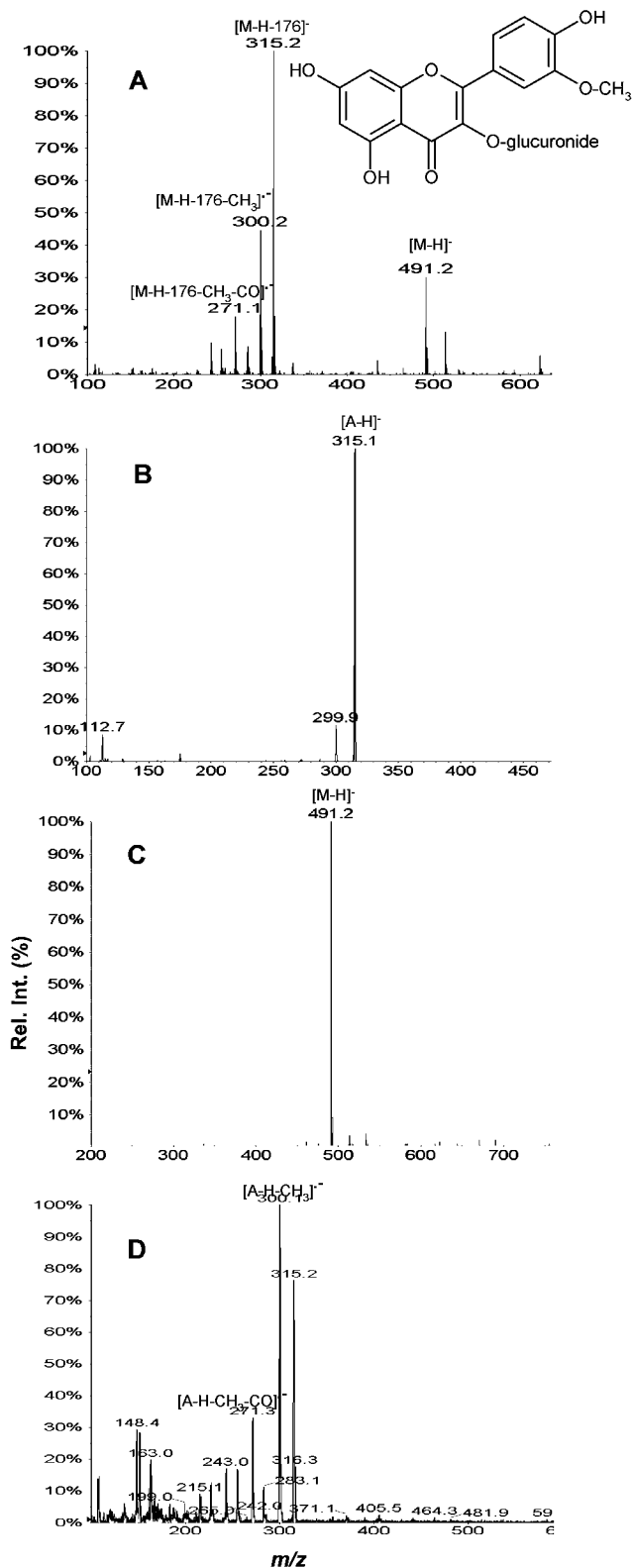
Figure 5. Sequence of identification of isorhamnetin-3-*O*-glucuronide (**35**) in fraction C: (**A**) MS spectrum in full scan mode; (**B**) MS/MS spectrum in product ion scan of *m/z* 491; (**C**) MS/MS spectrum in neutral loss of 176 u; (**D**) MS/MS spectrum in product ion scan of *m/z* 285.

neutral loss scan of 162 u and precursor ion scan of *m/z* 315. Because both compounds exhibited the same mass spectrum, they were differentiated from each other by the retention time, as the galactoside of a given flavonol aglycon elutes prior to the glucoside (*19*), which is also in agreement with the elution

3686 *J. Agric. Food Chem.,* Vol. 52, No. 12, 2004

Parejo et al.
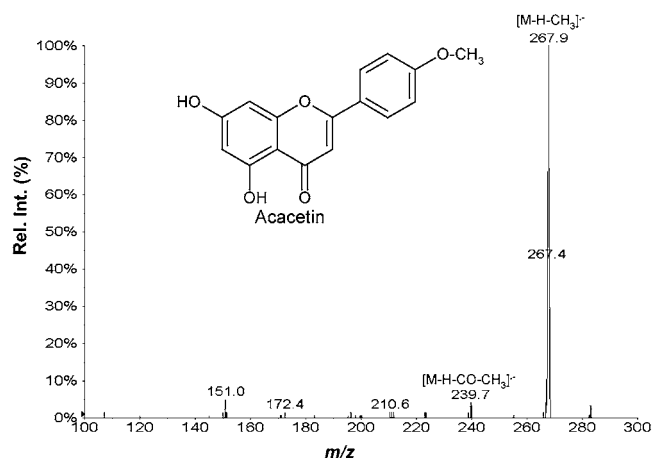
Figure 6. Identification of acacetin (**39**) from fraction F: MS/MS spectrum in product ion scan of *m/z* 283.

of quercetin-3-*O*-galactoside (**21**) prior to quercetin-3-*O*-glucoside (**23**) found in this work.

Flavonoid Aglycons. The flavonoid aglycons acacetin (**39**), kaempferol (**40**), naringenin (**41**), and isorhamnetin (**42**) were identified in full scan and product ion scan mode of the [A − H]⁻ ion. Naringenin, kaempferol, and isorhamnetin were identified by comparison of their retention times with those of reference compounds. Acacetin was identified on the basis of its product ion scan spectrum, showing the [A − CH₃]•⁻ *m/z* 268, [A − COCH₃]•⁻ *m/z* 240, and *m/z* 211 (**Figure 6**). The absence of the fragment ion *m/z* 117 discarded the other posssible methoxylated isomers of acacetin, which was in agreement with previously reported data using LC-APCI-MS/MS (*14*) and LC-API-ESI-MS/MS in negative mode (*17*). All of the aglycons gave the characteristic ions due to a retro-Diels−Alder fragmentation described by Fabre et al. (*20*).

The LC-DAD-MS/MS system used in this work appeared to be an excellent tool for the screening of phenolic substances in plant samples. Its application to fennel waste has allowed the identification of 42 phenolic compounds among the different fractions, including hydroxycinnamic acid and one *p*-hydroxybenzoic acid derivative, flavonoid glycosides, and aglycons in a short time of analysis. No other analytical method has formerly been found to give so much detailed information on the phenolic composition of fennel. Twenty-seven of these compounds are here described for the first time in fennel (**Table 1**).

The fractionation of the initial crude extract was found to be useful not only to remove possible interfering substances but also for the separation of the phenolics by different families of compounds. Furthermore, it has allowed not only the identification of the major components of the fennel waste but also that of minor compounds and/or coeluting substances, which could probably be not identified in the crude extract due to a high extent of ion suppression and matrix effects in MS analysis.

The identification of these phenolic compounds in fennel, some of them having exhibited antioxidant activity, can constitute a new step in the understanding of the health benefits of this plant as a spice or as a medicinal preparation.

LITERATURE CITED

(1) Bilia, A. R.; Fumarola, M.; Gallori, S.; Mazzi, G.; Vincieri, F. F. Identification by HPLC-DAD and HPLC-MS analyses and quantification of constituents of fennel teas and decoctions. *J. Agric. Food Chem.* **2000**, *48*, 4734−4738.

(2) Guillén, M. D.; Manzanos, M. J. A study of several parts of the plant *Foeniculum vulgare* as a source of compounds with industrial interest. *Food Rev. Int.* **1996**, *29*, 85−88.

(3) Harborne, J. B.; Saleh, N. A. M. Flavonol glycoside variation in fennel, *Foeniculum vulgare. Phytochemistry* **1971**, *10*, 399−400.

(4) Kunzemann, J.; Herrmann, K. Isolation and identification of flavon(ol)-*O*-glycosides in caraway (*Carvum carvi* L.), fennel (*Foeniculum vulgare* Mill.), anise (*Pimpinella anisum* L.), and coriander (*Coriandrum sativum* L.), and of flavon-*C*-glycosides in anise. I. Phenolics of species. *Z. Lebensm. Unters. Forsch.* **1977**, *164*, 194−200.

(5) Harborne, J. B.; Boardley, M. Use of high-performance liquid chromatography in the separation of flavonol glycosides and flavonol sulfates. *J. Chromatrogr.* **1984**, *299*, 377−385.

(6) Soliman, F. M.; Shehata, A. H.; Khaleel, A. E.; Ezzat, S. M. An acetylated kaempferol glycoside from flowers of *Foeniculum vulgare* and *F. dulce. Molecules* **2002**, *7*, 245−251.

(7) Parejo, I.; Viladomat, F.; Bastida, J.; Rosas-Romero, A.; Flerlage, N.; Burillo, J.; Codina, C. Comparison between the radical scavenging activity and antioxidant activity of six distilled and nondistilled Mediterranean herbs and aromatic plants. *J. Agric. Food Chem.* **2002**, *50*, 6882−6890.

(8) Xian-Guo, H. On-line identification of phytochemical constituents in botanical extracts by combined high-performance liquid chromatographic-diode array detection-mass spectrometric techniques. *J. Chromatogr. A* **2000**, *880*, 203−232.

(9) Carini, M.; Aldini, G.; Furlanetto, S.; Stefani, R.; Maffei-Facino, R. LC coupled to ion-trap MS for the rapid screening and detection of polyphenol antioxidants from *Helichrysum stoechas. J. Pharm. Biomed.* **2001**, *24*, 517−526.

(10) Fang, N.; Yu, S.; Prior, R. L. LC/MS/MS characterization of phenolic constituents in dried plums. *J. Agric. Food Chem.* **2002**, *50*, 3579−3585.

(11) Clifford, M. N.; Johnston, K. L.; Knight, S.; Kuhnert, N. Hierarchical scheme for LC-MS identification of chlorogenic acids. *J. Agric. Food Chem.* **2003**, *51*, 2900−2911.

(12) Tomás-Barberán, F.; Gil, M. I.; Cremin, P.; Waterhouse, A. L.; Hess-Pierce, B.; Kader, A. A. HPLC-DAD-ESIMS analysis of phenolic compounds in nectarines, peaches, and plums. *J. Agric. Food Chem.* **2001**, *49*, 4748−4760.

(13) Sánchez-Rabaneda, F.; Jáuregui, O.; Casals, I.; Andrés-Lacueva, C.; Izquierdo-Pulido, M.; Lamuela-Raventós, R. Liquid chromatographic/electrospray ionization tandem mass spectrometric study of the phenolic composition of cocoa (*Teobroma cacao*). *J. Mass Spectrom.* **2003**, *38*, 35−42.

(14) Grayer, R. J.; Kite, G. C.; Abou-Zaid, M.; Archer, L. J. The application of atmospheric chemical ionisation liquid chromatography−mass spectrometry in the chemotaxonomic study of flavonoids: Characterisation of flavonoids from *Ocinum gratissimum* var. *gratissimum. Phytochem. Anal.* **2000**, *11*, 257−267.

(15) Cuyckens, F.; Claeys, M. Mass spectrometry in the structural analysis of flavonoids. *J. Mass Spectrom.* **2004**, *39*, 1−15.

(16) Justesen, U. Negative atmospheric pressure chemical ionisation low-energy collision activation mass spectrometry for the characterisation of flavonoids in extracts of fresh herbs. *J. Chromatogr. A* **2000**, *902*, 369−379.

(17) Justesen, U. Collision-induced fragmentation of deprotonated methoxylated flavonoids, obtained by electrospray ionization mass spectrometry. *J. Mass Spectrom.* **2001**, *36*, 169−178.

LC-MS/MS Identification of Phenolics in Fennel

J. Agric. Food Chem., Vol. 52, No. 12, 2004 **3687**

(18) Qu, J.; Wang, Y.; Luo, G.; Wu, Z. Identification and determination of glucuronides and their aglycones in *Erigeron breviscapus* by liquid chromatography-tandem mass spectrometry. *J. Chromatogr. A* **2001**, *928*, 155−162.

(19) Schieber, A.; Keller, P.; Streker, P.; Klaiber, I.; Carle, R. Detection of isorhamnetin glycosides in extracts of apples (*Malus domestica* cv. "Brettacher") by HPLC-PDA and HPLC-APCI-MS/MS. *Phytochem. Anal.* **2002**, *13*, 87−94.

(20) Fabre, N.; Rustan, I.; De Hoffmann, E.; Quetin-Leclercq, J. Determination of flavone, flavonol, and flavanone aglycones by negative ion trap mass spectrometry. *J. Am. Soc. Mass Spectrom.* **2001**, *12*, 707−715.