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Characterization by LC-MS^n of Four New Classes of Chlorogenic Acids in Green Coffee Beans: Dimethoxycinnamoylquinic Acids, Diferuloylquinic Acids, Caffeoyl-dimethoxycinnamoylquinic Acids, and Feruloyl-dimethoxycinnamoylquinic Acids

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LC-MS^4 has been used to detect and characterize in green coffee beans 12 chlorogenic acids not previously reported in nature. These comprise three isomeric dimethoxycinnamoylquinic acids (**7**−**9**) (M_r 382), three caffeoyl-dimethoxycinnamoylquinic acids (**22**, **24**, and **26**) (M_r 544), three diferuloylquinic acids (**13**−**15**) (M_r 544), and three feruloyl-dimethoxycinnamoylquinic acids (**28**, **30**, and **32**) (M_r 558). Structures have been assigned on the basis of LC-MS^4 patterns of fragmentation and relative hydrophobicity and, in the case of the dimethoxycinnamoylquinic acids, by comparison with authentic standards. Several new structure-diagnostic fragmentations have been identified for use with diacyl-chlorogenic acids, for example, *m*/*z* 299 and 255 for C4 caffeoyl, *m*/*z* 313 and 269 for C4 feruloyl, nearly equal elimination of both cinnamoyl residues for *vic*-3,4-diacyl, and an increasing ratio of "dehydrated" ions to "non-dehydrated" ions at MS^2 with increasing methylation of those cinnamoyl residues. Possible mechanisms have been proposed to account for the fragmentations observed. The mass spectrometric resolution of six isomeric chlorogenic acids (M_r 544) in a crude plant extract by fragment-targeted LC-MS^2 and LC-MS^3 experiments illustrates the analytical power and advantage of ion trap mass spectroscopy.

KEYWORDS: Caffeoyl-dimethoxycinnamoylquinic acids; chlorogenic acids; coffee; diferuloylquinic acids; dimethoxycinnamoylquinic acids; feruloyl-dimethoxycinnamoylquinic acids; LC-MS^n

INTRODUCTION

Classically, chlorogenic acids are a family of esters formed between quinic acid and certain *trans*-cinnamic acids, most commonly caffeic, *p*-coumaric, and ferulic (*1*−*3*). Structures are shown in **Figure 1**. In the IUPAC system (−)-quinic acid is defined as 1L-1(OH),3,4/5-tetrahydroxycyclohexanecarboxylic acid, but Eliel and Ramirez (*4*) now propose 1α,3*R*,4α,5*R*-tetrahydroxycyclohexanecarboxylic acid. Chlorogenic acids are widely distributed in plants (*2*, *3*), but the coffee bean is remarkably rich, containing at least 18 chlorogenic acids that are not acylated at C1. These have been subdivided into five classes, that is, three caffeoylquinic acids, three *p*-coumaroyl-quinic acids, three feruloylquinic acids, three dicaffeoylquinic acids, and six caffeoyl-feruloylquinic acids (*5*). A considerable number of chlorogenic acid-like compounds have also been reported in coffee (*6*, *7*), and while some of these are cinnamic acid conjugates of amino acids (*8*), the possibility of additional quinic acid conjugates, for example, diferuloylquinic acids, could not be excluded. Accordingly, the structure-diagnostic LC-MS^n procedures previously developed (*5*, *8*, *9*) were applied to methanolic extracts of commercial green Robusta coffee beans.

MATERIALS AND METHODS

Methanolic Extracts of Coffee Beans. Methanolic extracts of green Robusta coffee beans were prepared as previously described (*5*). The extracts were treated with Carrez reagents (1 mL of reagent A plus 1 mL of reagent B) (*10*) to precipitate colloidal material, diluted to 100 mL with 70% v/v aqueous methanol, and filtered through a Whatman no. 1 filter paper. The methanol was removed by evaporation with nitrogen and the aqueous extract stored at −12 °C until required, thawed at room temperature, centrifuged (1360*g*, 10 min), and used directly for LC-MS.

As required, the methanolic extract of Robusta coffee beans was treated with tetramethylammonium hydroxide (TMAH) to interesterify and transesterify the diacyl-chlorogenic acids. Extract (200 μL) was treated with TMAH (20 μL) at room temperature for periods of 1, 3, and 5 min. The reaction was terminated by adding 3.5 M acetic acid

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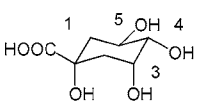
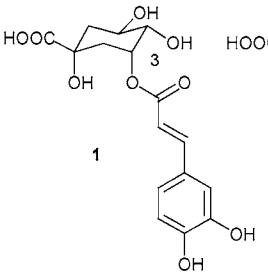


Quinic acid



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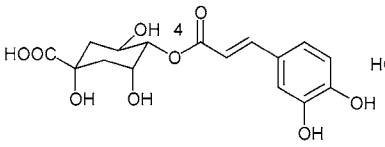

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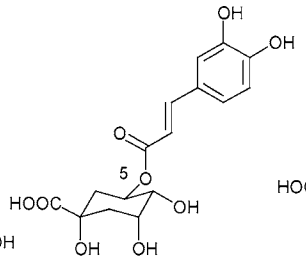

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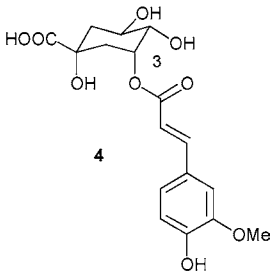

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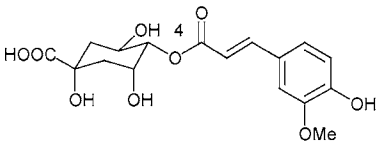

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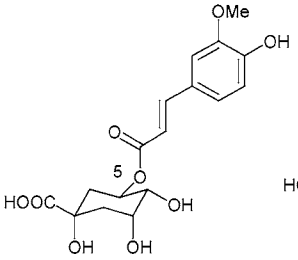

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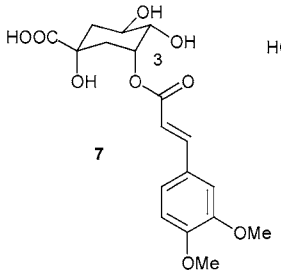

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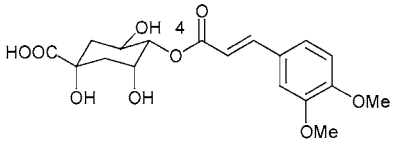

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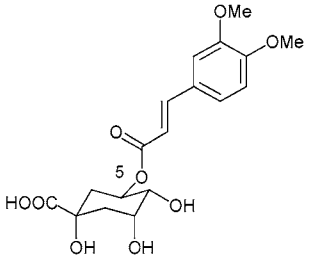

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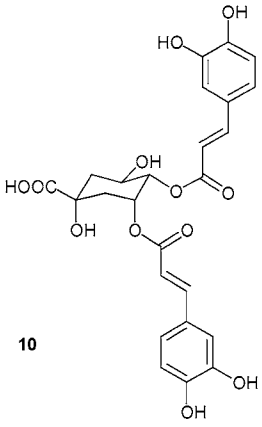

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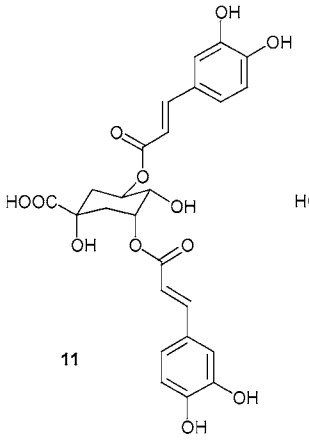

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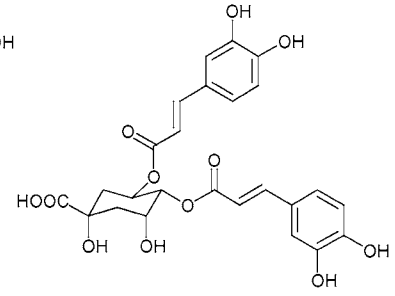

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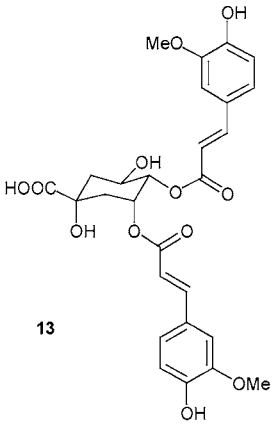

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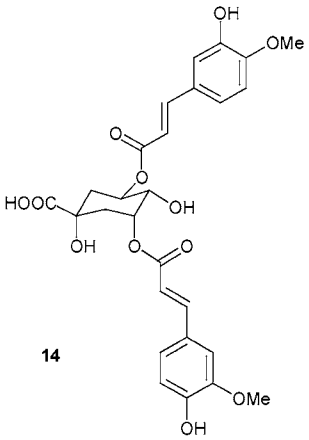

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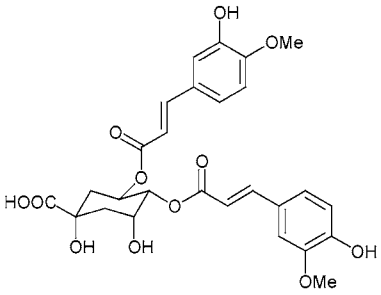

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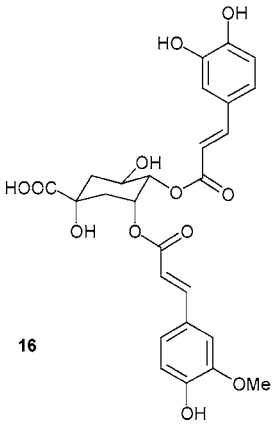

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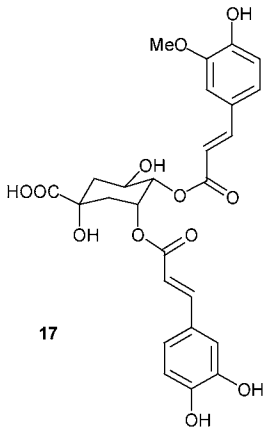

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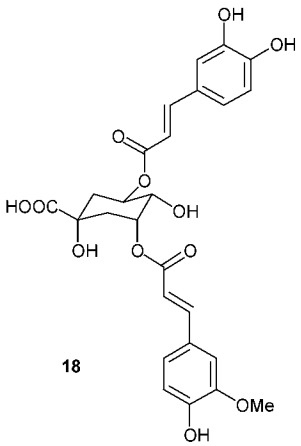

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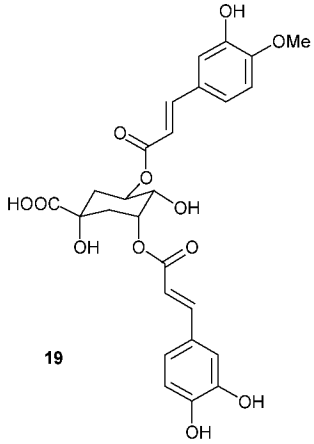

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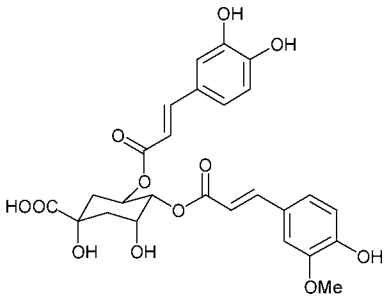

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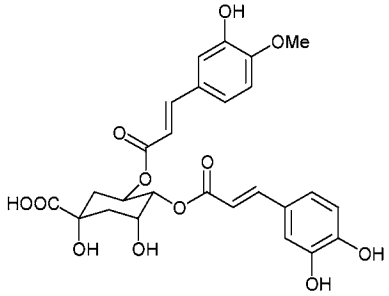

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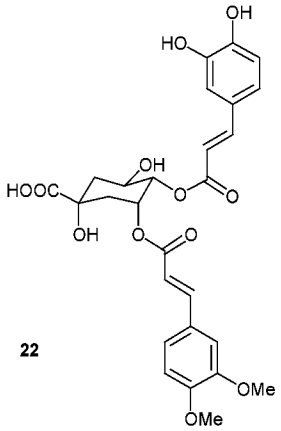

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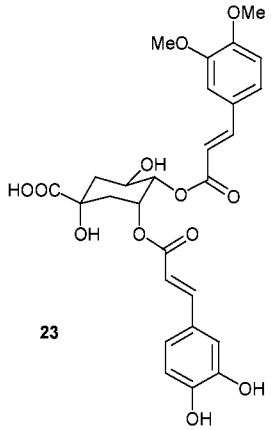

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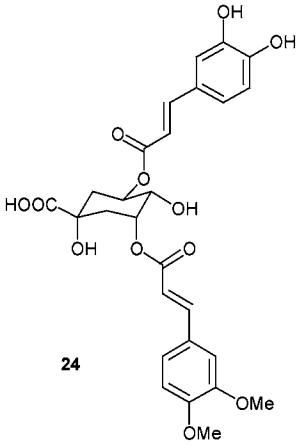

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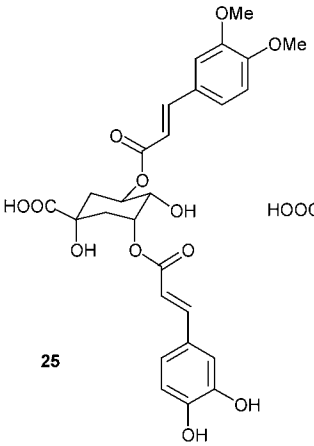

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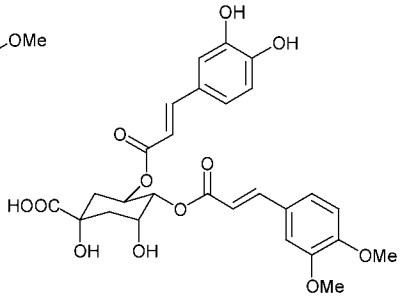

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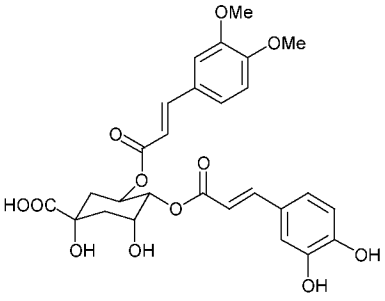

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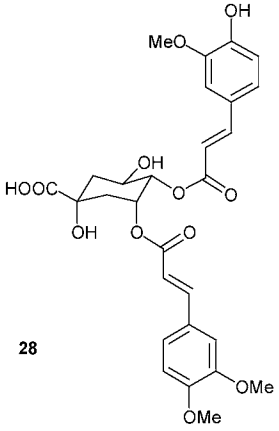

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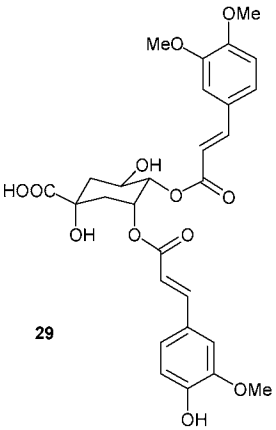

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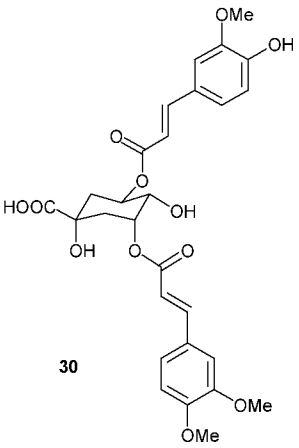

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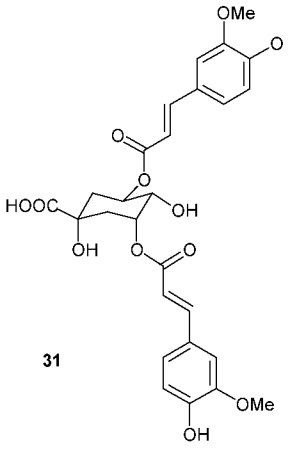

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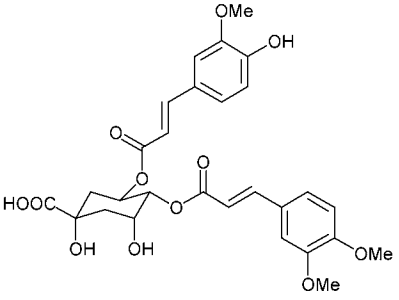

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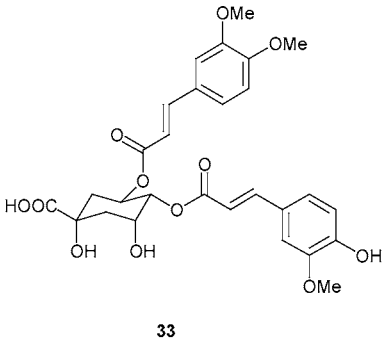

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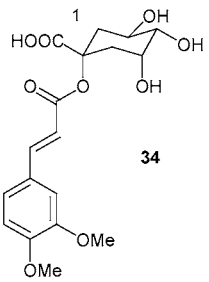

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Notes to Figure 1

Name and abbreviation	Number	R_1	R_3	R_4	R_5
3-*O*-caffeoylquinic acid	**1**	H	C	H	H
4-*O*-caffeoylquinic acid	**2**	H	H	C	H
5-*O*-caffeoylquinic acid	**3**	H	H	H	C
3-*O*-feruloylquinic acid	**4**	H	F	H	H
4-*O*-feruloylquinic acid	**5**	H	H	F	H
5-*O*-feruloylquinic acid	**6**	H	H	H	F
3-*O*-dimethoxycinnamoylquinic acid	**7**	H	D	H	H
4-*O*-dimethoxycinnamoylquinic acid	**8**	H	H	D	H
5-*O*-dimethoxycinnamoylquinic acid	**9**	H	H	H	D
3,4-di-*O*-caffeoylquinic acid	**10**	H	C	C	H
3,5-di-*O*-caffeoylquinic acid	**11**	H	C	H	C
4,5-di-*O*-caffeoylquinic acid	**12**	H	H	C	C
3,4-di-*O*-feruloylquinic acid	**13**	H	F	F	H
3,5-di-*O*-feruloylquinic acid	**14**	H	F	H	F
4,5-di-*O*-feruloylquinic acid	**15**	H	H	F	F
3-*O*-feruloyl, 4-*O*-caffeoylquinic acid	**16**	H	F	C	H
3-*O*-caffeoyl, 4-*O*-feruloylquinic acid	**17**	H	C	F	H
3-*O*-feruloyl, 5-*O*-caffeoylquinic acid	**18**	H	F	H	C
3-*O*-caffeoyl, 5-*O*-feruloylquinic acid	**19**	H	C	H	F
4-*O*-feruloyl, 5-*O*-caffeoylquinic acid	**20**	H	H	F	C
4-*O*-caffeoyl, 5-*O*-feruloylquinic acid	**21**	H	H	C	F
3-*O*-dimethoxycinnamoyl, 4-*O*-caffeoylquinic acid	**22**	H	D	C	H
3-*O*-caffeoyl, 4-*O*-dimethoxycinnamoylquinic acid	**23**	H	C	D	H
3-*O*-dimethoxycinnamoyl, 5-*O*-caffeoylquinic acid	**24**	H	D	H	C
3-*O*-caffeoyl, 5-*O*-dimethoxycinnamoylquinic acid	**25**	H	C	H	D
4-*O*-dimethoxycinnamoyl, 5-*O*-caffeoylquinic acid	**26**	H	H	D	C
4-*O*-caffeoyl, 5-*O*-dimethoxycinnamoylquinic acid	**27**	H	H	C	D
3-*O*-dimethoxycinnamoyl, 4-*O*-feruloylquinic acid	**28**	H	D	F	H
3-*O*-feruloyl, 4-*O*-dimethoxycinnamoylquinic acid	**29**	H	F	D	H
3-*O*-dimethoxycinnamoyl, 5-*O*-feruloylquinic acid	**30**	H	D	H	F
3-*O*-feruloyl, 5-*O*-dimethoxycinnamoylquinic acid	**31**	H	F	H	D
4-*O*-dimethoxycinnamoyl, 5-*O*-feruloylquinic acid	**32**	H	H	D	F
4-*O*-feruloyl, 5-*O*-dimethoxycinnamoylquinic acid	**33**	H	H	F	D
1-*O*-dimethoxycinnamoylquinic acid	**34**	D	H	H	H

C = caffeoyl; D = dimethoxycinnamoyl; F = feruloyl; H = hydrogen

Figure 1. Structures of selected coffee bean chlorogenic acids (IUPAC numbering) (*1*).

(40 μL) essentially as previously described (*5*, *11*, *12*). The reaction products were stored at −12 °C until required, thawed at room temperature, centrifuged (1360*g*, 10 min), and used directly for LC-MS.

Synthesis of Standards. *Methyl 3,4-Dimethoxycinnamate.* Acetyl chloride (0.1 mL) was added at 0 °C to 10 mL of methanol and stirred for 10 min. 3,4-Dimethoxycinnamic acid (300 mg; 1.44 mmol) was added to the solution, and the mixture was stirred for 20 h at room temperature. The solvent was removed under vacuum, and the residue was recrystallized from methanol and toluene to give the title compound as a white powder (266 mg, 83%): ^{1}H NMR ($CDCl_3$, 500 MHz) δ_H 7.64 (1H, d, J = 15.9 Hz, *H*C═C), 7.11 (1H, d, J = 8.3 Hz, ArH), 7.09 (1H, s, ArH), 6.87 (1H, d, J = 8.3 Hz, ArH), 6.31 (1H, d, J = 15.9 Hz, C═C*H*), 3.91 (6H, s, OCH_3), 3.80 (s, 3H, OCH_3); MS (ESI), *m*/*z* 221 $[M - H^+]^-$.

*1-(3,4-Dimethoxycinnamoyl)quinic Acid (**34**).* To a suspension of 214 mg (1 mmol) of 3,4-isopropylidenequinide and 12 mg of *N*,*N*-(dimethylamino)pyridine (0.1 mmol) in 5 mL of dichloromethane was added 110 mg of triethylamine followed by 250 mg (1.1 mmol) of 3,4-dimethoxycinnamoyl acid chloride, and the mixture was stirred at 20 °C for 48 h. HCl (1 M, 10 mL) and 10 mL of dichloromethane were added; the organic phase was separated, washed with 5 mL of brine, dried over Na_2SO_4, and filtered; and the solvent was removed under reduced pressure to give 302 mg of a crude ester. This crude

product [200 mg (≈0.50 mmol)] was dissolved in 8 mL of tetrahydrofuran and treated with 0.013 g of LiOH (0.54 mmol in 4 mL of H_2O). The solution was stirred for 3 days at room temperature and was then quenched by the addition of 2 M HCl. The reaction mixture was extracted with dichloromethane (3 × 30 mL) and 2 M HCl (10 mL). The organic phase was dried with $MgSO_4$ and filtered. The solvent was removed under reduced pressure to provide after recrystallization from ethanol the title compound (160 mg, 84% yield) as an off-white powder: R_f (TLC) 0.60 (MeCN); R_f (LC-MS, gradient as below) 32.3 min (UV λ_{max} 325 nm; MS, *m/z* 381.0 [M − H$^+$]$^-$); mp 140 °C; IR$_{\nu max}$ (Nujol)/cm^{-1} 3368 (OH), 2954 (COOH), 2854 (CH), 1797, 1712 (C═O), 1628 (C_{Ar}═C_{Ar}), 1075, 1156 (C−O); ^{1}H NMR (500 MHz, D_2O) δ 1.97 (1H, dd, J = 13.7, 10.9; H-6ax), 2.29 (1H, dd, J = 15.5, 3.4, H-2ax), 2.50 (1H, ddd, J = 13.7, 4.4, 3.4 Hz, H-6eq), 2.59 (1H, dt, J = 15.5, 3.4 Hz, 1H, H-2eq), 3.63 (1H, dd, J = 9.2, 3.4 Hz, H-4), 4.16, 3.86 (3H, s, OMe), 3.88 (3H, s, OMe) 4.16 (1H, ddd, J = 10.9, 9.2, 4.4 Hz, H-5), 4.27 (1H, q, J = 3.4 Hz, H-3), 6.43 (1H, d, J = 15.9 Hz; C═CH), 6.99 (1H, d, J = 8.3 Hz; CH), 7.18 (1H, d, J = 1.9 Hz, CH), 7.20 (1H, dd, J = 8.3, 1.9 Hz, CH), 7.63 (1H, d, J = 15.9 Hz; C═CH); ^{13}C NMR(125 MHz, D_2O) δ 34.3 (C-6), 38.51 (C-2), 55.64 (OMe), 55.73 (OMe), 66.13 (C-5), 68.62 (C-3), 74.46 (C-4), 80.93 (C-1), 110.26 (CH_{Ar}), 111.57 (CH_{Ar}), 114.77 (C═CH), 123.65 (C-9), 127.19 (C-12), 146.93 (C-10), 148.26 (C═C), 150.69 (C_{Ar}), 168.16 (C═O), 175.53 (C═O); accurate mass of $C_{18}H_{11}O_9$ requires 382.1264, found 382.1266.

*Synthesis of Mixture of Isomers of (3,4-Dimethoxycinnamoyl)quinic Acid (**7**−**9**, **34**).* To a suspension of 23 mg (0.1 mmol) of quinic acid in 1 mL of dichloromethane was added 10 mg of triethylamine followed by 12 mg (0.05 mmol) of 3,4-dimethoxycinnamoyl acid chloride, and the mixture was stirred at 20 °C for 48 h; the solvent was removed under reduced pressure, and the mixture was dried in a vacuum. The crude reaction mixture was dissolved in 1 mL of methanol, filtered and subjected to LC-MS conditions as described below to show in the SIM mode at *m/z* 381 four isomers of dimethoxycinnamoylquinic acids [estimated by TIC: 80% 5-dimethoxycinnamoylquinic acid (**9**), 10% 3-dimethoxycinnamoylquinic acid (**7**), 7% 4-dimethoxycinnamoylquinic acid (**8**), and 3% 1-dimethoxycinnamoylquinic acid (**34**)].

LC-MS^n. The LC equipment (ThermoFinnigan, San Jose, CA) comprised a Surveyor MS pump, an autosampler with a 50 μL loop, and a PDA detector with a light-pipe flow cell (recording at 320, 280, and 254 nm and scanning from 200 to 600 nm). This was interfaced with an LCQ Deca XP Plus mass spectrometer fitted with an ESI source (ThermoFinnigan) and operating in zoom scan mode for the accurate determination of parent ion *m/z*, and in data-dependent, full-scan, MS^n mode to obtain fragment ion *m/z*. For better discrimination of isomers having M_r 544, additional MS^2 and MS^3 experiments were performed that focused only on compounds producing a parent ion at *m/z* 543. MS operating conditions (negative ion) had been optimized using 5-caffeoylquinic acid (**3**) with a collision energy of 35%, an ionization voltage of 3.5 kV, a capillary temperature of 350 °C, a sheath gas flow rate of 65 arbitrary units, and an auxiliary gas flow rate of 10 arbitrary units.

Separations were achieved on 150 × 3 mm i.d. columns containing Luna 5μ phenylhexyl packing (Phenonemex, Macclesfield, U.K.). Solvent A was water/acetonitrile/glacial acetic acid (980:20:5, v/v, pH 2.68): solvent B was acetonitrile/ glacial acetic acid (1000:5, v/v). Solvents were delivered at a total flow rate of 300 μL/min. The gradient profile was 4% B to 33% B linearly in 90 min, a linear increase to 100% B at 95 min, followed by 5 min isocratic, a return to 4% B at 105 min, and 5 min isocratic to re-equilibrate.

RESULTS AND DISCUSSION

Preliminary Assessment of Data. All data for chlorogenic acids presented in this manuscript use the recommended IUPAC numbering system (*1*), and structures are presented in **Figure 1**. When necessary, previously published data have been amended to ensure consistency and avoid ambiguity.

The Robusta coffee extract gave a typical chromatogram in which the 18 previously reported chlorogenic acids were easily located (*5*). Selected ion monitoring at *m/z* 543 immediately located five chromatographic peaks eluting between 74 and 82 min, each with a UV spectrum typical of chlorogenic acids [λ_{max} 325 nm with a shoulder (85%) at 290 nm]. However, because coffee beans do not acylate quinic acid at C1 and produce only three dicaffeoylquinic acid isomers, that is, 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, and 4,5-dicaffeoylquinic acid (**10**−**12**), only three diferuloylquinic acids (**13**−**15**) were expected. The five peaks produced MS^3 fragment ions at either *m/z* 173 or 191 consistent with the presence of a quinic acid residue (*5*), and the absence of an MS^3 fragment ion at *m/z* 205 confirmed that none of these substances were derivatives of methyl quinate. Two of the five peaks produced MS^2 ions at *m/z* 367 ([feruloylquinic acid − H$^+$]$^-$) and *m/z* 349 ([feruloylquinic acid − H_2O − H$^+$]$^-$) and MS^3 ions at *m/z* 193 ([ferulic acid − H$^+$]$^-$) analogous to or identical with those produced by dicaffeoylquinic acids and caffeoyl-feruloylquinic acids (*5*) and, thus, totally consistent with the predictable behavior of diferuloylquinic acids.

The other three peaks variously produced MS^2 ions at *m/z* 335, 353, 363, and 381 and MS^3 ions at *m/z* 179 and 207. The MS^2 ions at *m/z* 335 and 353 and the MS^3 ion at *m/z* 179 are characteristic (*5*) of caffeic acid-derived diacyl-chlorogenic acids and can be assigned as [caffeoylquinic acid − H$^+$]$^-$, [caffeoylquinic acid − H_2O − H$^+$]$^-$, and [caffeic acid − H$^+$]$^-$, respectively. The remaining ions were tentatively assigned to a similar series of fragments 28 amu larger than the caffeic acid-related fragments (or 14 amu larger than the ferulic acid-related fragments), strongly suggesting that they might be derived from compounds in which both methyl residues are associated with the same cinnamic acid residue, that is, with quinic acid bearing one residue of caffeic acid and one residue of 3,4-dimethoxycinnamic acid, the dimethyl ether of caffeic acid. Although 3,4-dimethoxycinnamic acid had previously been reported in green Robusta coffee beans (*13*), its quinic acid esters had not. Those compounds of M_r 544 that produced MS^2 ions at *m/z* 363 or 381 were tentatively assigned as caffeoyl-dimethoxycinnamoylquinic acids. Although it is conceivable that a caffeoyl-dimethoxycinnamoylquinic acid might produce ions at *m/z* 349 or 367, diferuloylquinic acids would not produce ions at *m/z* 363 or 381, and there can be no doubt that both series of chlorogenic acids were present.

A post hoc search for ions at *m/z* 381 located an additional four peaks in the spectrum from the Robusta extract—one was a parent ion, and three were MS^2 fragments from *m/z* 557. These were tentatively assigned to a dimethoxycinnamoylquinic acid and three feruloyl-dimethoxycinnamoylquinic acids. These substances were further investigated using more sensitive and more specific LC-MS protocols: (i) MS^1 experiments targeting *m/z* 381, 543, and 557; (ii) MS^2 experiments targeting combinations of *m/z* 543 + 381, *m/z* 543 + 367, *m/z* 543 + 363, *m/z* 543 + 353, *m/z* 543 + 349, and *m/z* 543 + 335; and (iii) MS^2 experiments targeting combinations of *m/z* 557 + 381, *m/z* 557 + 367, *m/z* 557 + 363, and *m/z* 557 + 349.

Characterization of Putative Dimethoxycinnamoylquinic Acids (7−9). The Robusta extract contained three minor components with molecular ions at *m/z* 381 that eluted between 39 and 50 min. All three peaks had UV spectra typical of chlorogenic acids. MS^2 and MS^3 data are presented in **Table 1** and **Figure 2**.

Because the monoacyl chlorogenic acids so far examined on this phenylhexyl packing elute in the sequence 3-acyl, 5-acyl, and 4-acyl (*5*, *14*), the first dimethoxycinnamoylquinic acid was tentatively assigned as the 3-isomer (**7**). The MS^2 base peak at

Table 1. MS^3 Fragmentation Data for the Putative Dimethoxycinnamoylquinic Acids

			MS^2 base peak	MS^2 secondary ions						MS^3 base peak	MS^3 secondary ions							
compd	N[a]	parent ion *m/z*	*m/z*	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	*m/z*	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity
7	6	381.0	207.0	173.1	5	bp[b]	100			149.1	162.8	65	133.1	25				
8	6	381.0	173.1	bp	100	207.1	10			93.4	162.7	85			155.0	15	111.0	40
9	6	381.1	193.0					134.1	12	134.1	149.0	25						
34		381.1	173.1	143.1	15	298.9	10			93.4								

[a] Number of LC-MS analyses used to collect MS data. [b] Occurs as base peak.

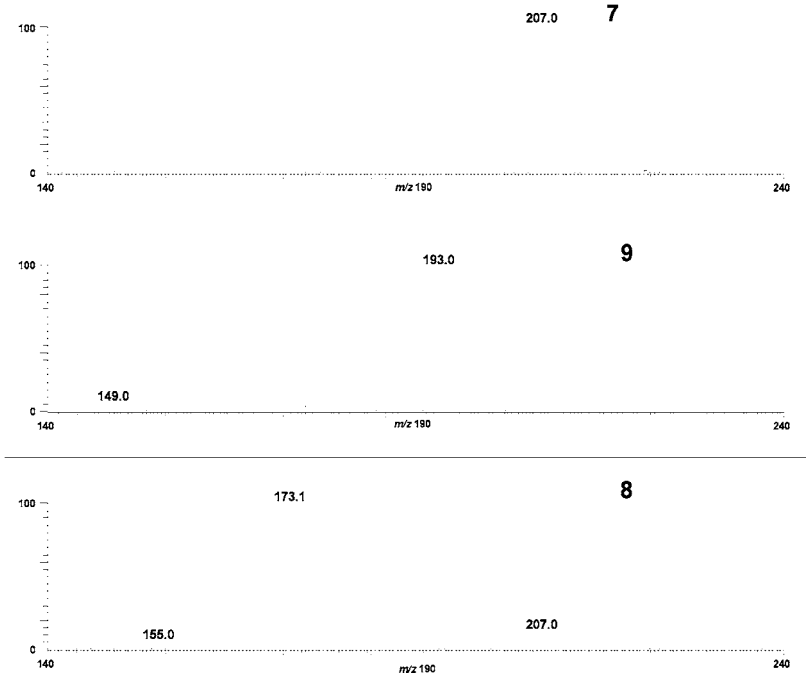

Figure 2. MS^2 spectra of the putative dimethoxycinnamoylquinic acids.

m/z 207 [dimethoxycinnamoylquinic acid − quinic acid − H^+]$^-$, which subsequently decarboxylates and demethylates at MS^3, is analogous in behavior to 3-feruloylquinic acid (**4**) (*5*). **T**he most strongly retained of the dimethoxycinnamoylquinic acid isomers has the fragmentation characteristic of a 4-acylchlorogenic acid (MS^2 and MS^3 base peaks at *m/z* 173 and 93, respectively) and can be reliably assigned as 4-dimethoxycinnamoylquinic acid (**8**). Peak 13, by a process of elimination and on the basis of its retention time, should be 5-dimethoxycinnamoylquinic acid (**9**). However, its fragmentation is atypical, giving an MS^2 base peak of *m/z* 193 (rather than the expected *m/z* 207), indicative of demethylation.

To gain further evidence to support this assignment, we synthesized pure 1-dimethoxycinnamoylquinic acid from a literature-known 3,4-acetal-protected quinide (*15*). The synthetic derivative displayed the expected NMR spectroscopic features and eluted under the same gradient conditions at 32 min. It showed the characteristic UV absorption and MS fragmentation pattern expected for 1-dimethoxycinnamoylquinic acid with a base peak in MS^2 at *m/z* 173.0. Furthermore, we obtained a mixture of the four possible isomers of dimethoxycinnamoylquinic acid by acylating quinic acid with the acid chloride of 3,4-dimethoxycinnamic acid. All four isomers could be readily detected by LC-MS using SIM at *m/z* 381.0 and, with the exception of 1-dimethoxycinnamoylquinic acid, were identical with respect to retention time, UV absorbance, and MS^2 fragmentation to the compounds detected in coffee.

We have proposed previously (*5*) that the elimination of a cinnamoyl residue from either C3 or C5 involves quinic acid chairs with a 1,3-syn diaxial conformation such that the C1 carboxyl protonates the C5 cinnamoyl residue or the C1 hydroxyl protonates the C3 cinnamoyl residue. For C3 the ease with which the cinnamoyl residue is lost and the nature of the subsequent base peak are functions of the identity of the cinnamoyl residue.

To explain the behavior of the 3,4-dimethoxycinnamoyl residue we must consider two alternative fragmentation mechanisms, but we are currently unable to distinguish between them. First, deprotonation at the most acidic site will produce an [M − H^+]$^-$ anion **A** (**Figure 3**). If the negative charge is localized at the COO^- group, this functionality can act as a nucleophile in an intermolecular acylation reaction to give bicyclic intermediate **B** via pathway A. Loss of the methyl cation can then lead to quinoid structure **C**. This assumption accounts for the ease of demethylation of 5-dimethoxycinnamoylquinic acid and its derivatives. In an alternative scenario the resulting anion is after proton transfer localized at an alcoholate oxygen, for example, in **D-1** or its resonant structure **D-2**. The [M − H^+]$^-$ anion **D-1** is in this case stabilized by resonance and an additional hydrogen bond from the carboxyl at C1 of the quinic acid moiety. This hydrogen-bonded species appears to be particularly stable and does not fragment easily in the case of the caffeoyl derivatives (see **D-2** for R = H in **Figure 3**). In the case of a 3,4-dimethoxycinnamoyl residue the hydrogen

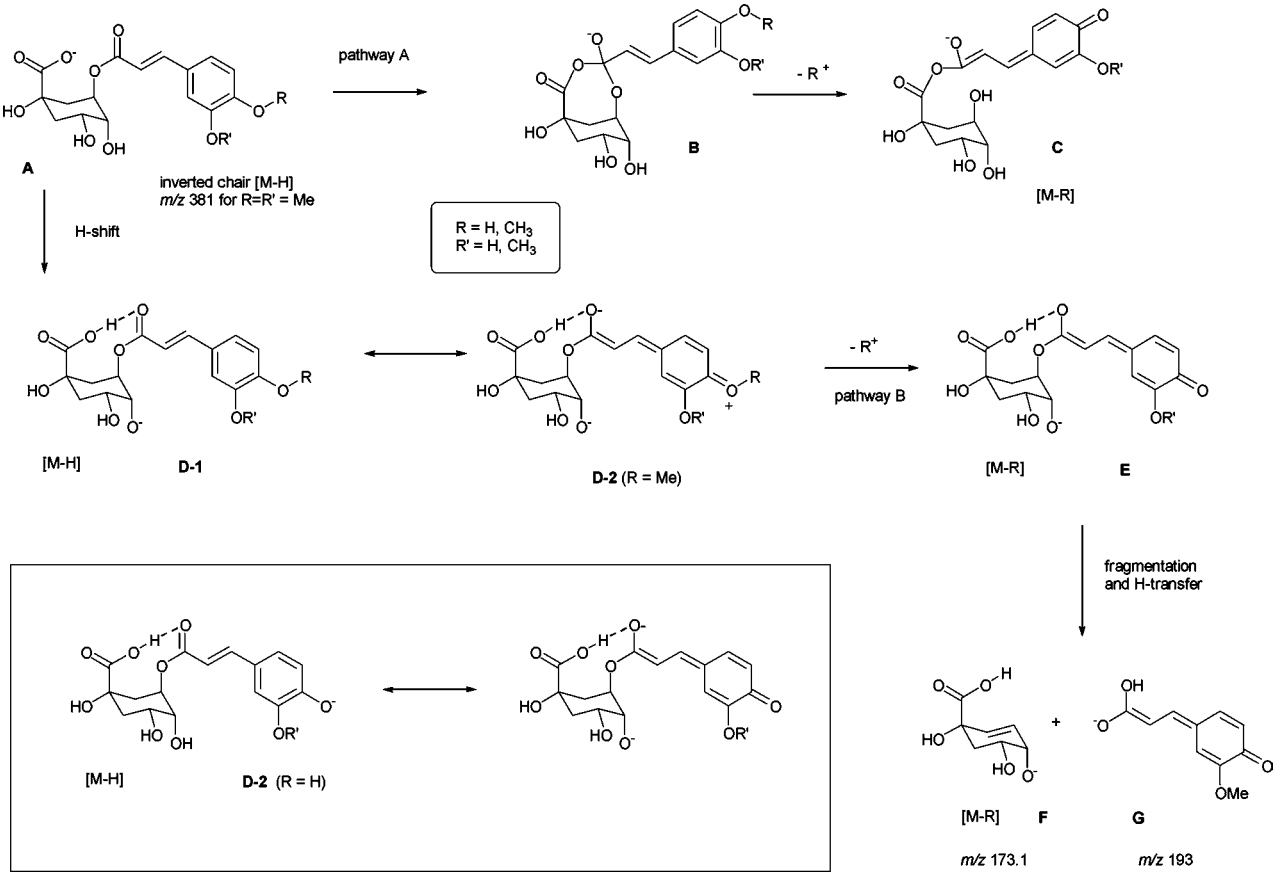

Figure 3. Fragmentation mechanism proposed for dimethoxycinnamoylquinic acids.

bond still occurs in **D-1**, the quinoid resonance structure of which is set up to demethylate easily to give via pathway B fragment ion **E**, a quinic acid fragment **F** at *m*/*z* 173, and a ketene quinide fragment at *m*/*z* 193 (**Figure 3**). Because all ESI spectra obtained in this investigation arise from highly acidic solvents, proton transfer should occur rapidly within the ESI droplet. Demethylation of a dimethoxycinnamoyl residue at C5, but not C3, can be attributed to the low p*K* of the protonating C1 carboxyl (p*K* $\sim$ 3.5) compared with the C1 hydroxyl (p*K* $>$ 15), which seems to reduce the stability of the hydrogen-bonded intermediate. Hence, a dimethoxycinnamoyl fragment at *m*/*z* 207 is observed instead of its demethylated counterpart at *m*/*z* 193.

Characterization of Diacyl-chlorogenic Acids with M_r 544. The LC-MS experiment targeted on compounds producing *m*/*z* 543 parent ions located six peaks. Four eluted at 75.2, 75.8, 76.6, and 77.8 min followed by an incompletely resolved pair at 80.3 and 80.7 min (**Figure 4**). The LC-MS experiments targeting subsequent fragmentation to either *m*/*z* 381, 367, 363, 353, 349, or 335 identified (**Figure 5**) three with MS^2 base peaks at *m*/*z* 381 (eluting at ca. 75.1, 76.6, and 79.6 min), and these were assigned tentatively as caffeoyl-dimethoxycinnamoyl quinic acids (**22**−**26**). The peaks eluting at ca. 74.4, 75.5, and 79.6 min (with MS^2 base peaks at *m*/*z* 349 or 367) were tentatively assigned as diferuloylquinic acids (**13**−**15**).

Characterization of the Putative Diferuloylquinic acids (13−**15). Table 2** and **Figure 6** compare the fragmentation patterns of the putative diferuloylquinic acids with data previously obtained for coffee bean dicaffeoylquinic acids (**10**−**12**). Peak **15** produces MS^2 and MS^3 base peaks that are either identical with those produced by 4,5-dicaffeoylquinic acid (**12**), or 14 amu larger, and it has been assigned as 4,5-diferuloylquinic acid (**15**).

On the basis of its intermediate hydrophobicity, **14** has been assigned tentatively as 3,5-diferuloylquinic acid despite a cinnamate-derived MS^3 base peak at *m*/*z* 193 in contrast to the quinic acid-derived *m*/*z* 191 produced by 3,5-dicaffeoylquinic acid (**11**). On the basis of the fragmentation (*5*, *9*) of 3,5-dicaffeoylquinic acid (**11**) and the two 3,5-caffeoyl-feruloylquinic acid isomers (**18**, **19**) it is anticipated that 3,5-diferuloylquinic acid (**14**) would lose the feruloyl residue at C5 before that at C3, producing [3-feruloylquinic acid − H^+]$^-$ (rather than [5-feruloylquinic acid − H^+]$^-$) as its MS^2 base peak. We have previously demonstrated (*5*) that whereas 3-caffeoylquinic acid (**1**) yields a quinic acid-derived *m*/*z* 191 base peak, 3-feruloylquinic acid (**4**) yields [ferulic acid − H^+]$^-$. Accordingly, the fragmentation of **14** is completely consistent with its assignment as 3,5-diferuloylquinic acid (**14**).

Peak 13, incompletely resolved from the first eluting caffeoyl-dimethoxycinnamoyl isomer (**22**), must logically be 3,4-diferuloylquinic acid (**13**). However, in contrast to 3,4-dicaffeoylquinic acid (**10**), **13** produces a "dehydrated" MS^2 base peak at *m*/*z* 349, and this yields *m*/*z* 175 as the MS^3 base peak. We have previously observed that 3-feruloyl-4-caffeoylquinic acid (**16**) produces a comparatively strong (45% of base peak) *m*/*z* 349 accompanied by *m*/*z* 175 at MS^3, and the fragmentation of **13** is thus not inconsistent with its assignment as 3,4-diferuloylquinic acid (**10**). The tendency of some chlorogenic acids to dehydrate during fragmentation, and the origin of the fragment ion *m*/*z* 175, are further discussed below.

Characterization of Putative Caffeoyl-dimethoxycinnamoylquinic Acids (22−**27).** Of the six peaks that yielded molecular ions at *m*/*z* 543, three produced an MS^2 base peak at *m*/*z* 381 with an MS^3 base peak or secondary fragment ion at *m*/*z* 207, suggestive of caffeoyl-dimethoxycinnamoylquinic acids. Theoretically, six caffeoyl-dimethoxycinnamoylquinic acid isomers

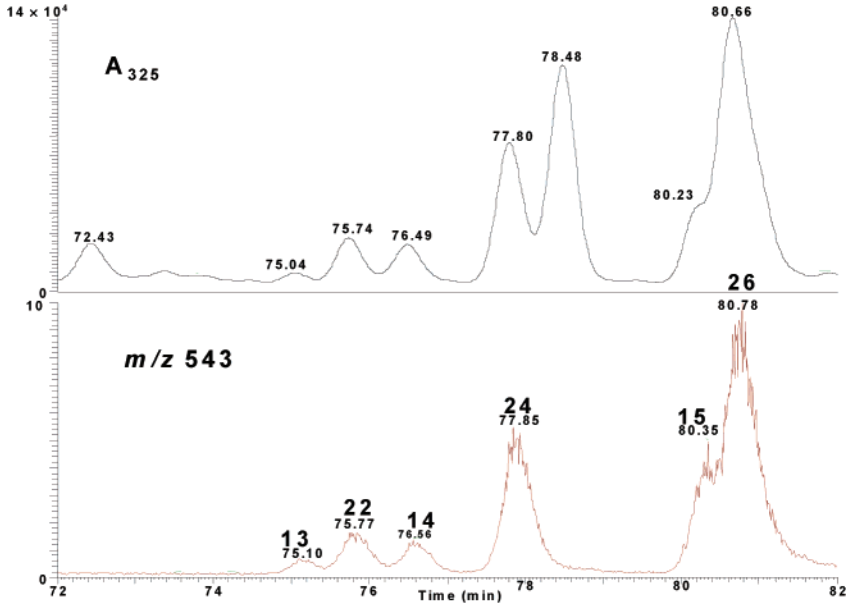

Figure 4. LC-MS of chlorogenic acids giving a parent ion at *m*/*z* 543.

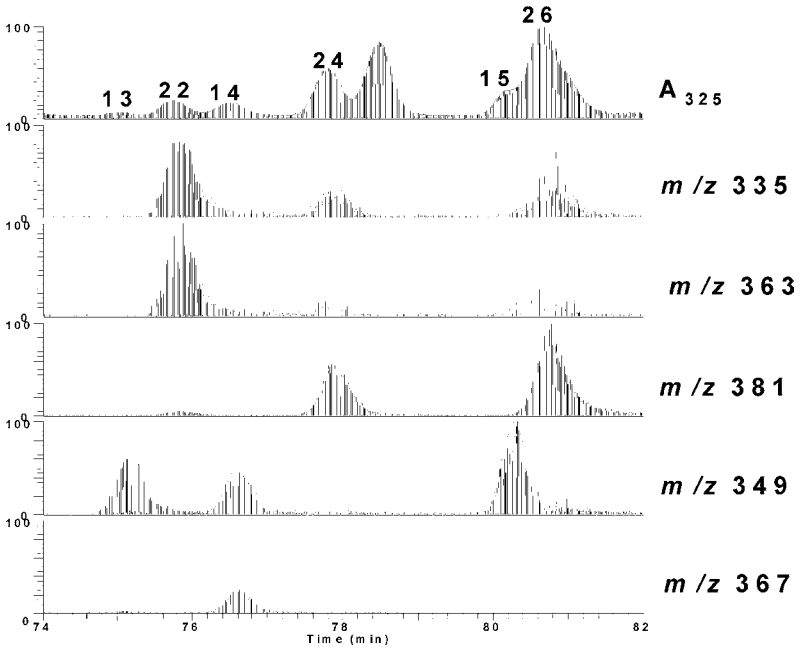

Figure 5. Fragment-targeted LC-MS^2 spectra of chlorogenic acids giving a parent ion at *m*/*z* 543.

(**22**−**27**) would have been expected, as seen previously (*5*) for the caffeoyl-feruloylquinic acids (**16**−**21**).

Table 3 and **Figure 6** compare the fragmentation patterns of the three putative caffeoyl-dimethoxycinnamoylquinic acids with data previously obtained for the six caffeoyl-feruloylquinic acids. The caffeoyl-feruloylquinic acids were assigned by comparing their fragmentation behavior at $MS^{(n+1)}$ with the MS^n fragmentation of caffeoylquinic acids and feruloylquinic acids and the $MS^{(n+1)}$ fragmentation of the dicaffeoylquinic acids (*5*). For chlorogenic acids not substituted at C1, it was observed that a caffeoyl residue at C5 was the most easily eliminated, whereas that at C4 was the most stable and that at C3 being of intermediate stability. For five of the six caffeoyl-feruloylquinic acids this resulted at MS^2 in one of the two cinnamoyl residues being eliminated almost exclusively (maximally 10% loss of the second cinnamoyl residue in 3-caffeoyl-4-feruloylquinic acid). In contrast, 3-feruloyl-4-caffeoylquinic acid lost both cinnamoyl residues with nearly equal facility, giving an MS^2 base peak (*m*/*z* 353) accompanied by a very intense (90% of base peak) secondary ion at *m*/*z* 367. Significant amounts of the corresponding dehydrated ions (*m*/*z* 335 and 349) were also produced (*5*).

By analogy with the relative hydrophobicity of the caffeoyl-feruloylquinic acids and dicaffeoylquinic acids (*5*, *9*, *14*) one would expect that the first caffeoyl-dimethoxycinnamoylquinic acid to elute would be one of the 3,4-caffeoyl-dimethoxy-cinnamoylquinic acid isomers. The first eluting caffeoyl-dimethoxycinnamoylquinic acid (**22**) loses its two cinnamoyl residues with nearly equal facility (slightly favoring the caffeoyl residue) and produces strong dehydrated ions at *m*/*z* 335 and 363, thus closely resembling 3-feruloyl-4-caffeoylquinic acid (**16**) (**Table 3**) (*5*). Its MS^3 base peak (*m*/*z* 207) is consistent with the MS^2 base peak being [3-dimethoxycinnamoylquinic acid − H^+]$^-$ rather than [4-dimethoxycinnamoylquinic acid −

Table 2. MS^4 Fragmentation Data for the Putative Diferuloylquinic Acids and Equivalent Dicaffeoylquinic Acids

			MS^2 base peak	MS^2 secondary Ions																	
		MS^2 parent ion																			
compd	*N*[a]	*m/z*	*m/z*	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity
13	6	543.1	349.0	bp[b]	100	367.0	17			299.0	2	255.1	5					193.0	10		
14	6	543.0	367.1	349.1	35	bp	100														
15	6	543.0	367.0	349.1	17	bp	100											193.0	5	173.1	10
10	3	515.1	353.0	335.1	16					299.0	3	255.1	3	203.1	10			179.1	15	173.1	18
11	3	515.0	353.1											203.9	10	191.8	20				
12	3	515.1	353.0					317.5	7	299.0	15	255.1	8	203.1	18			179.1	5	173.1	8

		MS^3 base peak	MS^3 secondary ions										MS^4 base peak	MS^4 secondary ions							
compd	*N*[a]	*m/z*	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	*m/z*	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity
13	6	173.1	193.0	27	bp[b]	100	161.1	12	155.0	10	134.1	5	134.1	149.1	80						
14	6	193.0	bp	100	173.1	7					134.1	5									
15	6	173.1	193.0	70	bp	100			155.0	5	134.1	7	93.2	111.1	65						
10	3	173.5	179.5	91	bp	100	191.7	53			135.6	14	93.4	128.1	3	172.9	2	111.1	50	bp	100
11	3	191.5	179.5	53	173.1	5	bp	100			135.6	12	85.5	127.0	95	172.9	90	110.7	60	93.4	80
12	3	173.5	179.4	80	bp	100	191.6	27			135.7	12	93.3	127.5	3	172.9	15	111.4	38	bp	100

[a] Number of LC-MS analyses used to collect MS data. [b] Occurs as base peak.

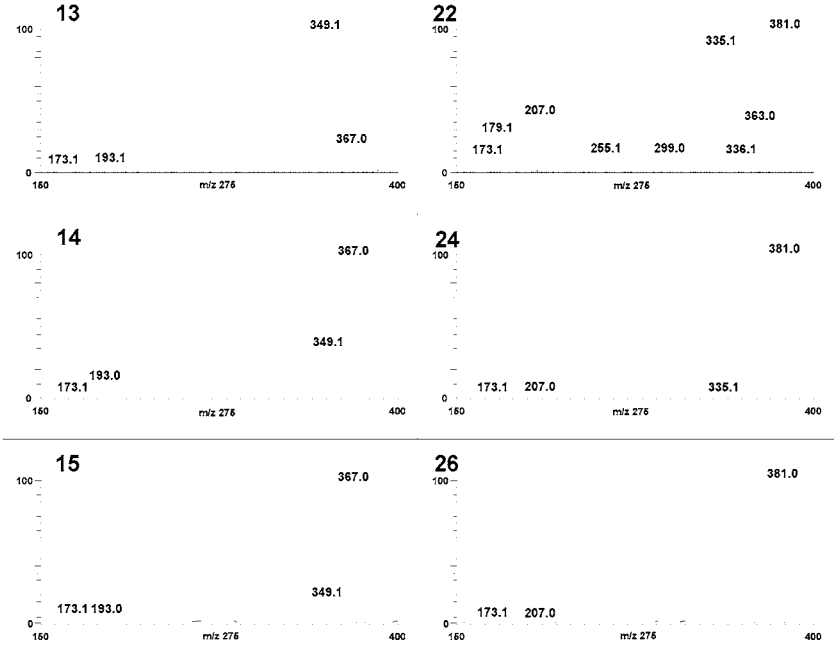

Figure 6. LC-MS^2 spectra of the putative diferuloylquinic acids and caffeoyl-dimethoxycinnamoylquinic acids.

$H^+]^-$ that by analogy with the fragmentation of 4-dimethoxycinnamoylquinic acid would be expected to produce the dehydrated *m/z* 173 at MS^3. A distinctive feature of the MS^2 spectrum is ions (~15% of base peak) at *m/z* 299 and 255. This combination of ions has previously been seen only in the MS^2 spectra of dicaffeoylquinic acids and caffeoyl-feruloylquinic acids with a C4 caffeoyl residue (**Table 3**) (*5*, *9*). They have been attributed respectively to full aromatization ($-3H_2O$) of the quinic acid moiety in a caffeoylquinic acid fragment and its subsequent decarboxylation. Such ions would be expected in the MS^2 spectrum of 3-dimethoxycinnamoyl-4-caffeoylquinic acid (**22**) but not 3-caffeoyl-4-dimethoxycinnamoylquinic acid (**23**). Accordingly, it is assigned as 3-dimethoxycinnamoyl-4-caffeoylquinic acid (**22**).

The next eluting caffeoyl-dimethoxycinnamoylquinic acid (**24**) also loses its caffeoyl residue before its dimethoxycinnamoyl residue, but does not produce a dehydrated MS^3 base peak, a fragmentation consistent with a 3,5-caffeoyl-dimethoxycinnamoylquinic acid rather than a *vic*-caffeoyl-dimethoxycinnamoylquinic acid. It has been shown that a caffeoyl residue at C5 is easily eliminated (*5*). Such an elimination would produce [3-dimethoxycinnamoylquinic acid $-H^+]^-$ as the MS^2 base peak, and as argued above for **22**, the MS^3 base peak at *m/z* 207 is consistent with such an assignment, suggesting that the second eluting caffeoyl-dimethoxycinnamoylquinic acid is 3-dimethoxycinnamoyl-5-caffeoylquinic acid (**24**). Its fragmentation pattern more closely resembles 3-feruloyl-5-caffeoylquinic acid (**18**) than 3-caffeoyl-5-feruloylquinic acid (**19**) (**Table 3**) (*5*).

By analogy with the sequence of elution and relative hydrophobicity observed for dicaffeoylquinic acids and caffeoyl-feruloylquinic acids (*5*, *9*, *14*), the most hydrophobic caffeoyl-dimethoxycinnamoylquinic acid should be one of the 4,5-isomers. Peak 26 (**Table 3**) has the characteristic fragmentation

Table 3. MS^4 Fragmentation Data for the Putative Caffeoyl-dimethoxycinnamoylquinic Acids, Putative Feruloyl-dimethoxycinnamoylquinic Acids, and Equivalent Caffeoyl-feruloylquinic Acids

			MS^2 base peak	MS^3 secondary ions													
compd	N[a]	parent ion *m/z*	*m/z*	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity
22	6	543.0	381.1			335.1	92	363.1	32	349.1	8	298.7	12	254.9	16	203.1	22
23																	
24	6	543.0	381.1			335.0	2										
25																	
26	6	543.0	381.0			335.1	2										
27																	
28	6	557.0	349.1					363.1	75	bp	100						
29																	
30	6	557.0	381.2							349.2	75						
31																	
32	6	557.0	381.0							349.1	30						
33																	
16	6	529.1	353.0	367.1	90	335.1	50	bp[b]	100	349.1	45	298.9	6	255.1	6	203.1	8
17	6	529.0	367.0	bp	100	335.1	10	353.0	5	349.3	5						
18	6	529.0	367.0	bp	100	335.1	5										
19	6	529.0	353.0	367.0	55	335.1	7	bp	100	349.1	7						
20	6	529.0	367.0	bp	100	335.1	2										
21	6	529.0	353.0	367.0	25							299.0	8	255.1	5	203.1	8

			MS^3 base peak	MS^3 secondary ions					MS^4 base peak	MS^4 secondary ions									
compd	N[a]	*m/z*	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	*m/z*	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity
22	6	207.1	173.3	10					149.1	bp[b]	100								
23																			
24	6	207.1	173.0	25					149.0	bp	100	162.7	80			133.1	22		
25																			
26	6	173.1	207.0	55					93.2					155.1	20			111.1	35
27																			
28	6	175.0	193.1	80	269.0	55	313.0	25	160.1										
29																			
30	6	207.1	173.1	30					149.0	bp	100	162.6	70			133.1	50		
31																			
32	6	173.1	207.3	47					93.2					155.1	10			111.1	40
33																			
16	6	173.1	179.1	85	191.5	15	135.8	11	134.1	148.9	32								
17	6	173.0	193.0	30					93.5									111.3	73
18	6	193.3	173.4	45					134.0	149.0	48								
19	6	191.5	179.5	56					127.2	172.4	68							111.2	57
20	6	173.0	193.0	70					93.4									111.2	40
21	6	173.1	179.1	84	191.1	28	135.2	17	93.1										

[a] Number of LC-MS analyses. [b] Occurs as base peak.

of a *vic*-diacyl-chlorogenic acid (MS^3 and MS^4 base peaks at *m/z* 173 and 93, respectively), and it clearly loses its caffeoyl residue before its dimethoxycinnamoyl residue [MS^2 base peak at *m/z* 381 and a strong MS^3 secondary ion (55% of base peak) at *m/z* 207]. The MS^2 base peak must be either [4-dimethoxycinnamoylquinic acid $- H^+]^-$ or [5-dimethoxycinnamoylquinic acid $- H^+]^-$, and the subsequent fragmentation to the dehydrated ion *m/z* 173 indicates [4-dimethoxycinnamoylquinic acid $- H^+]^-$ and thus favors assignment as 4-dimethoxycinnamoyl-5-caffeoylquinic acid (**26**). The absence of the MS^2 ions at *m/z* 299 and 255, suggesting that **26** does not have a caffeoyl residue at C4, is consistent. Its fragmentation pattern more closely resembles 4-feruloyl-5-caffeoylquinic acid (**20**) than 4-caffeoyl-5-feruloylquinic acid (**21**) (**Table 3**) (*5*).

Characterization of Putative Feruloyl-dimethoxycinnamoylquinic Acids (28−33). The LC-MS data for the feruloyl-dimethoxycinnamoylquinic acids (**28**−**33**) are summarized in **Table 3** and **Figure 7**. The fragmentation pattern of the slowest eluting isomer (**32**) is identical to that of caffeoyl-dimethoxycinnamoylquinic acid (**26**) if allowance is made for the weak caffeic acid-derived ion at *m/z* 335 being replaced by the analogous, but more intense, *m/z* 349, suggesting that logically it can be assigned as 4-dimethoxycinnamoyl-5-feruloylquinic acid (**32**). An identical argument can be made for assigning the preceding feruloyl-dimethoxycinnamoylquinic acid as 3-dimethoxycinnamoyl-5-feruloylquinic acid (**30**).

The fragmentation of the most rapidly eluting feruloyl-dimethoxycinnamoylquinic acid (**28**) resembles the analogous caffeoyl-dimethoxycinnamoylquinic acid (**22**) in that both lose their two cinnamoyl residues with almost equal facility. Whereas 3-dimethoxycinnamoyl-4-caffeoylquinic acid (**22**) slightly favors elimination of the caffeoyl residue, **28** shows a slight preference for eliminating the dimethoxycinnamoyl residue. Fragmentation of the MS^2 base peak (*m/z* 349) produces *m/z* 175, an ion previously seen in the fragmentation of 3,4-diferuloylquinic acid (**13**), 3-feruloyl-4-caffeoylquinic acid (**16**), and 3-dimethoxycinnamoyl-4-caffeoylquinic acid (**22**). Because this ion is not seen during the fragmentation of 3,4-dicaffeoylquinic acid (**10**) or 3-caffeoyl-4-feruloylquinic acid (**17**), it is associated clearly with a methylated cinnamoyl residue at C3, but for a feruloyl-dimethoxycinnamoylquinic acid it is necessary to decide whether the feruloyl or the dimethoxycinnamoyl residue is at C3.

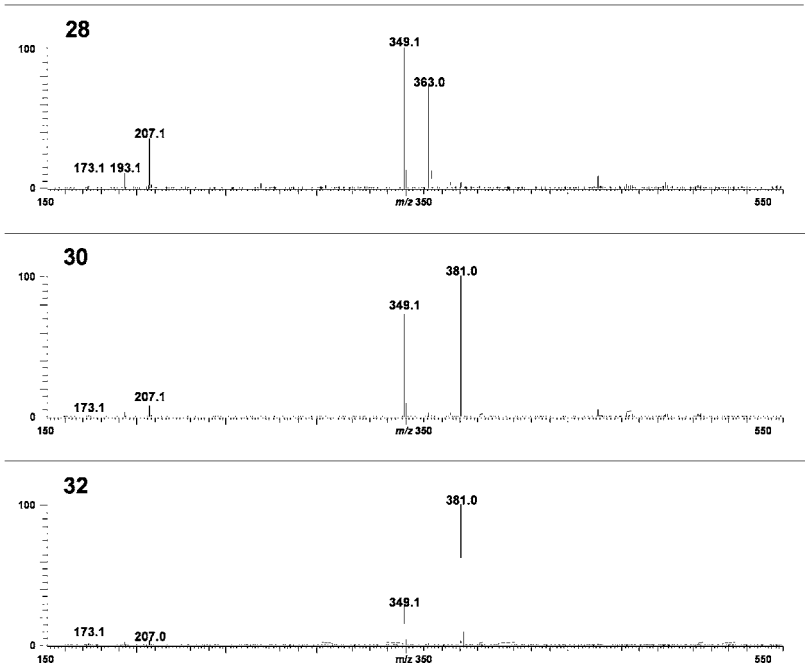

Figure 7. LC-MS^2 spectra of the putative feruloyl-dimethoxycinnamoylquinic acids.

The MS^2 secondary fragment ions at *m*/*z* 299 and 255 (associated with aromatization and decarboxylation of a 4-caffeoylquinic acid moiety) are replaced at MS^3 by the analogous *m*/*z* 313 and 269, suggesting that **28** is 3-dimethoxycinnamoyl-4-feruloylquinic acid. The strong MS^2 secondary ion (*m*/*z* 363) fragmenting to *m*/*z* 207, suggesting that it is derived from [3-dimethoxycinnamoylquinic acid − H_2O − H^+]$^-$ (rather than [4-dimethoxycinnamoylquinic acid − H_2O − H^+]$^-$), is consistent with this suggestion. Accordingly, **28** has been assigned tentatively as 3-dimethoxycinnamoyl-4-feruloylquinic acid rather than 3-feruloyl-4-dimethoxycinnamoylquinic acid (**29**).

3-Dimethoxycinnamoyl-4-feruloylquinic acid (**28**) differs from 3-dimethoxycinnamoyl-4-caffeoylquinic acid (**22**) in that at MS^2 the dehydrated ions are seen without the associated "non-dehydrated" ions. In the caffeoyl residue-containing series 3,4-dicaffeoylquinic acid (**10**), 3-feruloyl-4-caffeoylquinic acid (**16**), 3-dimethoxycinnamoyl-4-caffeoylquinic acid (**22**), the ratio of dehydrated to non-dehydrated MS^2 ions increases in the sequence 0.16, 0.6, and 1.8 with progressively increasing methylation of the parent molecule. The equivalent ratios for 3,4-diferuloylquinic acid (**13**) and 3-dimethoxycinnamoyl-4-feruloylquinic acid (**28**) (increasing methylation without a caffeoyl residue) are 6.3 and >100, respectively, reinforcing the relationship between extent of cinnamoyl methylation and tendency to produce dehydrated ions.

The observed preference of the methylated derivatives for dehydration is surprising. We have postulated a 1,2-acyl migration mechanism for the dehydration step. In this mechanism an inverted chair conformation allows facile loss of H_2O by 1,2-acyl participation using the acyl substituent in the 4-position of the quinic acid, leading to an oxonium ion intermediate (*4*). Further H-transfer, presumably via an exo-elimination, will give the dehydrated molecular ion. Because neither the basicity of the OH functionality nor the C−O bond strength is expected to change if OH is replaced by OMe, the only rationale available is that increased nucleophilicity of the ester carbonyl increases the rate of dehydration or the stability of the dehydrated ion. In solution chemistry Hammet parameters are frequently used to describe the electronic effects of aromatic substituents on a particular reaction mechanism. However, both σ_p^0 [σ_p^0 (OH) = −0.22 and σ_p^0 (OMe) = −0.12] and σ_p^+ [σ_p^+ (OH) = −0.92 and σ_p^+ (OMe) = −0.78] indicate that OH should stabilize a positive charge in an oxonium ion intermediate better than OMe (*16*), and therefore it should marginally increase the nucleophilicity of the ester substituent in comparison with OMe. In the gas-phase reaction observed, it appears that this trend is reversed.

Relative Retention Time of Diacyl-CGA. It has previously been noted (*11*, *12*) that a feruloylquinic acid isomer elutes from a C_{18} HPLC column packing some 32% later than the equivalent caffeoylquinic acid isomer and that a caffeoyl-feruloylquinic acid elutes some 16% later than the equivalent dicaffeoylquinic acid. On the phenylhexyl packing used in this study, the retention times of the newly characterized diacyl-chlorogenic acids, relative to the dicaffeoylquinic acids, are 1.19−1.20 (caffeoyl-feruloylquinic acids), 1.38−1.41 (diferuloylquinic acids), 1.38−1.43 (caffeoyl-dimethoxycinnamoylquinic acids), and 1.60−1.66 (feruloyl-dimethoxycinnamoylquinic acids), an increase of ∼20% for each additional methyl group.

Treatment with Tetramethylammonium Hydroxide. The ability of TMAH to facilitate isomerization of chlorogenic acids, along with the partial formation of the pertinent methyl cinnamate(s), has been used to characterize various classes of chlorogenic acids [including caffeoyl-feruloylquinic acids (*11*)] and to synthesize 1,4-dicaffeoylquinic acid (*9*). This procedure was applied to the crude methanolic extracts of Robusta coffee beans for two reasons. One was to generate methyl dimethoxycinnamate from dimethoxycinnamoylquinic acids, caffeoyl-dimethoxycinnamoylquinic acids, and feruloyl-dimethoxycinnamoylquinic acids, and another was to confirm by spiking with an authentic sample of methyl 3,4-dimethoxycinnamate (data not shown) that TMAH treatment did, indeed, produce the 3,4-dimethoxy isomer.

Whereas TMAH facilitates the acyl migration of a cinnamoyl residue from C5 to C4 to C3 (or vice versa), it would not be expected to move a residue from C3 to C5 (or vice versa) if C4 were occupied. For example, 3-dimethoxycinnamoyl-4-caffeoylquinic acid (**22**) could be converted stepwise to 3-dimethoxycinnamoyl-5-caffeoylquinic acid (**24**) and 4-dimethoxy-

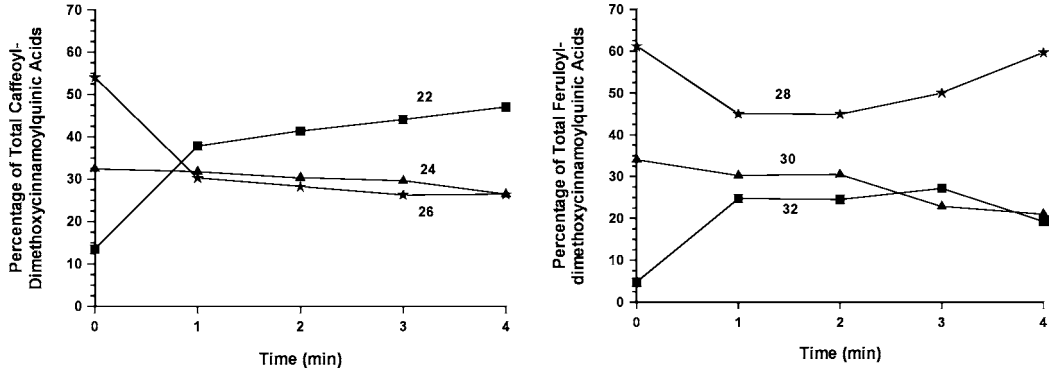

Figure 8. Effect of interesterification with tetramethylammonium hydroxide on the relative proportions of caffeoyl-dimethoxycinnamoylquinic acids and feruloyl-dimethoxycinnamoylquinic acids.

cinnamoyl-5-caffeoylquinic acid (**26**), but not to 4-caffeoyl-5-dimethoxycinnamoylquinic acid (**27**). The structures assigned tentatively in this study to these hetero-diacyl-chlorogenic acids all have the dimethoxycinnamoyl residue at the numerically "low" position and the caffeoyl or feruloyl residue at the "high" position and should, therefore, be interconvertible. Treatment with TMAH for up to 4 min demonstrated (**Figure 8**) that the three caffeoyl-dimethoxycinnamoylquinic acids and the three feruloyl-dimethoxycinnamoylquinic acids interconvert, providing further evidence that they do indeed belong to one "numerical" group as assigned on the basis of their fragmentation.

Missing Isomers. Because all six theoretical isomers of the caffeoyl-feruloylquinic acids (**16**−**21**) are easily located in the Robusta extract (*5*), it is a little surprising that only three caffeoyl-dimethoxycinnamoylquinic acids (**22**, **24**, and **26**) and three feruloyl-dimethoxycinnamoylquinic acids (**28**, **30**, and **32**) have been observed. This could reflect the failure of the coffee plant to synthesize all six isomers, but because all three dimethoxycinnamoylquinic acids (**7**−**9**) are produced, there is no obvious impediment to this. It is possible that these other isomers are present, but below the limits of detection. If that is the case, then the most concentrated of the "missing" caffeoyl-dimethoxycinnamoylquinic acids and feruloyl-dimethoxycinnamoylquinic acids must be present at a concentration considerably below that of the weakest isomer found, because analysis of deliberately (×10) concentrated extracts still did not allow them to be located. A third possibility is that they are present at low concentration and not resolved chromatographically. However, not only can the six caffeoyl-feruloylquinic acids (**16**−**20**) be resolved chromatographically, but critical pairs, for example, 3-feruloyl-4-caffeoylquinic acid (**16**) and 3-caffeoyl-4-feruloylquinic acid (**17**), can be discriminated also by their fragmentation patterns at MS^2, MS^3, and MS^4 (*5*). At MS^2 the feruloyl-dimethoxycinnamoylquinic acid peaks appear to be homogeneous, as do the caffeoyl-dimethoxycinnamoylquinic acid peaks once an allowance is made for the coeluting diferuloylquinic acids.

Inspection of the fragmentation data in **Table 3** suggested that if any of the missing compounds with a dimethoxycinnamoyl residue at C5 (**25**, **27**, **31**, or **33**) form a [5-dimethoxycinnamoylquinic acid − H^+]$^-$ MS^2 base peak and it fragments to *m*/*z* 193 [as observed for 5-dimethoxycinnamoylquinic acid (**9**) itself], then their MS^3 spectra should be distinguishable from those isomers with either a caffeoyl (**24** or **26**) or feruloyl (**30** or **32**) residue at C5. Similarly, if 3-caffeoyl-5-dimethoxycinnamoylquinic acid (**25**) fragments in the same manner as 3-caffeoyl-5-feruloylquinic acid (**19**), then it should be locatable by MS^4 ions at *m*/*z* 127 and 172 that should be absent from 3-dimethoxycinnanmoyl-5-caffeoylquinic acid (**26**). Accordingly, fragment-targeted MS^2 (*m*/*z* 543 + 381 and *m*/*z* 557 + 381) and MS^3 (*m*/*z* 543 + 353 + 191, *m*/*z* 543 + 381 + 173, *m*/*z* 543 + 381 + 207, *m*/*z* 557 + 349 + 193, and *m*/*z* 557 + 381 + 207) experiments were performed. A suggestion of heterogeneity in **28** was observed as weak MS^4 signals (not exceeding 10^3) detected by variation in the ratios of *m*/*z* 155 to 111 and *m*/*z* 149 to 163, in the *m*/*z* 543 + 381 + 173 and *m*/*z* 543 + 381 + 207 spectra, respectively. It was not possible to detect any heterogeneity in **24** and **26** or in **28**, **30**, and **32**. One must therefore conclude that if the missing isomers are present, then they are present in only trace amounts and lack a distinctive pattern of fragmentation

As for the other mono-acyl chlorogenic acids, 5-dimethoxycinnamoylquinic acid (**9**) dominates. For the diferuloylquinic acids (**13**−**15**), caffeoyl-dimethoxycinnamoylquinic acids (**22**, **24**, and **26**) and feruloyl-dimethoxycinnamoylquinic acids (**28**, **30**, and **32**) there is within each group a progressive increase in concentration (ca. ×6) with increasing hydrophobicity, and this pattern remained essentially constant over five Robusta samples of different origin (Angola, Madagascar, Sierra Leone, Sri Lanka, and Tanzania) previously observed to have different chromatographic profiles (*6*). In contrast, the caffeoyl-feruloylquinic acids (**16**−**21**) increase, by a factor of ca. ×50, in the sequence 3-caffeoyl-5-feruloylquinic acid (**19**), 3-feruloyl-4-caffeoylquinic acid (**16**), 4-feruloyl-5-caffeoylquinic acid (**20**), 3-feruloyl-5-caffeoylquinic acid (**18**), 4-caffeoyl-5-feruloylquinic acid (**21**), and 3-caffeoyl-4-feruloylquinic acid (**17**).

Although the generation of quantitative data was not a primary objective of this investigation, crude estimates of the content of these newly reported chlorogenic acids were obtained from the relative absorbance of individual peaks at 325 nm relative to 5-caffeoylquinic acid (**3**), assigned unit absorbance. Dimethoxycinnamoylquinic acids (**7**−**9**) ranged from 0.005 to 0.020 unit, caffeoyl-dimethoxycinnamoylquinic acids (**22**, **24**, and **26**) from 0.008 to 0.028 unit, feruloyl-dimethoxycinnamoylquinic acids (**28**, **30**, and **32**) from 0.004 to 0.020 unit, and diferuloylquinic acids (**13**−**15**) from 0.004 to 0.010 unit, similar to or slightly lower than the caffeoyl-feruloylquinic acids (**16**−**21**) (0.005−0.030 unit). Assuming ∼5% dry mass basis (dmb) as a typical 5-caffeoylquinic acid (**3**) content (*17*−*19*), this suggests that none of these new chlorogenic acids are likely individually to exceed ∼0.15% dmb, or ∼0.7% dmb in total.

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