JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. 2006, 54, 4095−4101 4095

# Characterization by LC-MS$^n$ of Four New Classes of *p*-Coumaric Acid-Containing Diacyl Chlorogenic Acids in Green Coffee Beans

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LC-MS$^4$ has been used to detect and characterize in green coffee beans 15 quantitatively minor *p*-coumaric acid-containing chlorogenic acids not previously reported in nature. These comprise 3,4-di-*p*-coumaroylquinic acid, 3,5-di-*p*-coumaroylquinic acid, and 4,5-di-*p*-coumaroylquinic acid ($M_r$ 484); 3-*p*-coumaroyl-4-caffeoylquinic acid, 3-*p*-coumaroyl-5-caffeoylquinic acid, 4-*p*-coumaroyl-5-caffeoylquinic acid, 3-caffeoyl-4-*p*-coumaroyl-quinic acid, 3-caffeoyl-5-*p*-coumaroyl-quinic acid; and 4-caffeoyl-5-*p*-coumaroyl-quinic acid ($M_r$ 500); 3-*p*-coumaroyl-4-feruloylquinic acid, 3-*p*-coumaroyl-5-feruloylquinic acid and 4-*p*-coumaroyl-5-feruloylquinic acid ($M_r$ 514); and 4-dimethoxycinnamoyl-5-*p*-coumaroylquinic acid and two isomers ($M_r$ 528) for which identities could not be assigned unequivocally. Structures have been assigned on the basis of LC-MS$^4$ patterns of fragmentation. Forty-five chlorogenic acids have now been characterized in green Robusta coffee beans.

**KEYWORDS: Chlorogenic acids; coffee; *p*-coumaroylquinic acids; *p*-coumaroyl-caffeoylquinic acids; *p*-coumaroyl-dimethoxycinnamoylquinic acids; *p*-coumaroyl-feruloylquinic acids; di-*p*-coumaroylquinic 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-tetrahydroxycyclohexane carboxylic acid, but Eliel and Ramirez (*4*) recommend 1α,3*R*,4α,5*R*-tetrahydroxycyclohexane carboxylic acid. Chlorogenic acids are widely distributed in plants (*2*, *3*), but the coffee bean is remarkably rich, containing at least 30 chlorogenic acids that are not acylated at C1 of the quinic acid moiety. These have been subdivided into nine classes, that is, three caffeoylquinic acids, three *p*-coumaroylquinic acids, three dimethoxycinnamoylquinic acids, three feruloylquinic acids, three dicaffeoylquinic acids, three diferuloylquinic acids, six caffeoyl-feruloylquinic acids, three caffeoyl-dimethoxycinnamoylquinic acids, and three feruloyl-dimethoxycinnamoylquinic acids (*5*, *6*). Several chlorogenic acid-like compounds previously observed in coffee (*7*, *8*) have yet to be assigned. This study exploits the structure−diagnostic LC-MS$^n$ procedures previously developed (*5*, *6*, *9*).

## MATERIALS AND METHODS

**Methanolic Extracts of Coffee Beans.** Methanolic extracts of green Robusta coffee beans from Tanzania 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 to investigate the inter-relationship of individual isomers, the methanolic extract of Robusta coffee beans was treated with tetramethylammonium hydroxide (TMAH) to interesterify and trans-esterify 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 (40 μL) essentially as previously described (*5*, *6*, *9*, *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.

**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*. As necessary, MS$^2$ and MS$^3$ fragment-targeted experiments were performed to focus only on compounds producing a parent ion at *m*/*z* 483, 499, 513, or 527. MS operating conditions (negative ion) had been optimized using 5-caffeoylquinic acid (**31**) 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.

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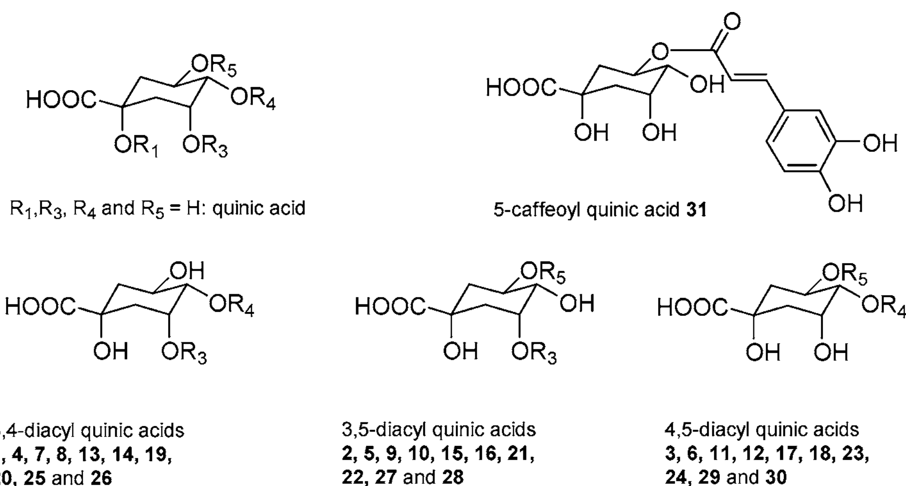
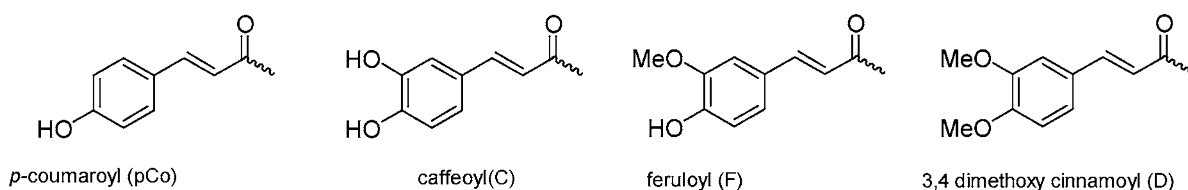
10.1021/jf060536p CCC: $33.50 © 2006 American Chemical Society
Published on Web 05/23/2006




Structures and abbreviations of ester substituents




| Name | Number | $R_1$ | $R_3$ | $R_4$ | $R_5$ |
|---|---|---|---|---|---|
| 3,4-di-*O*-*p*-coumaroylquinic acid | **1** | H | *p*Co | *p*Co | H |
| 3,5-di-*O*-*p*-coumaroylquinic acid | **2** | H | *p*Co | H | *p*Co |
| 4,5-di-*O*-*p*-coumaroylquinic acid | **3** | H | H | *p*Co | *p*Co |
| 3,4-di-*O*-feruloylquinic acid | **4** | H | F | F | H |
| 3,5-di-*O*-feruloylquinic acid | **5** | H | F | H | F |
| 4,5-di-*O*-feruloylquinic acid | **6** | H | H | F | F |
| 3-*O*-feruloyl, 4-*O*-caffeoylquinic acid | **7** | H | F | C | H |
| 3-*O*-caffeoyl, 4-*O*-feruloylquinic acid | **8** | H | C | F | H |
| 3-*O*-feruloyl, 5-*O*-caffeoylquinic acid | **9** | H | F | H | C |
| 3-*O*-caffeoyl, 5-*O*-feruloylquinic acid | **10** | H | C | H | F |
| 4-*O*-feruloyl, 5-*O*-caffeoylquinic acid | **11** | H | H | F | C |
| 4-*O*-caffeoyl, 5-*O*-feruloylquinic acid | **12** | H | H | C | F |
| 3-*O*-*p*-coumaroyl, 4-*O*-caffeoylquinic acid | **13** | H | *p*Co | C | H |
| 3-*O*-caffeoyl, 4-*O*-*p*-coumaroylquinic acid | **14** | H | C | *p*Co | H |
| 3-*O*-*p*-coumaroyl, 5-*O*-caffeoylquinic acid | **15** | H | *p*Co | H | C |
| 3-*O*-caffeoyl, 5-*O*-*p*-coumaroylquinic acid | **16** | H | C | H | *p*Co |
| 4-*O*-*p*-coumaroyl, 5-*O*-caffeoylquinic acid | **17** | H | H | *p*Co | C |
| 4-*O*-caffeoyl, 5-*O*-*p*-coumaroylquinic acid | **18** | H | H | C | *p*Co |

| | | | | | |
|---|---|---|---|---|---|
| 3-*O*-*p*-coumaroyl, 4-*O*-feruloylquinic acid | **19** | H | *p*Co | F | H |
| 3-*O*-feruloyl, 4-*O*-*p*-coumaroylquinic acid | **20** | H | F | *p*Co | H |
| 3-*O*-*p*-coumaroyl, 5-*O*-feruloylquinic acid | **21** | H | *p*Co | H | F |
| 3-*O*-feruloyl, 5-*O*-*p*-coumaroylquinic acid | **22** | H | F | H | *p*Co |
| 4-*O*-*p*-coumaroyl, 5-*O*-feruloylquinic acid | **23** | H | H | *p*Co | F |
| 4-*O*-feruloyl, 5-*O*-*p*-coumaroylquinic acid | **24** | H | H | F | *p*Co |
| 3-*O*-*p*-coumaroyl, 4-*O*-dimethoxycinnamoylquinic acid | **25** | H | *p*Co | D | H |
| 3-*O*-dimethoxycinnamoyl, 4-*O*-*p*-coumaroylquinic acid | **26** | H | D | *p*Co | H |
| 3-*O*-*p*-coumaroyl, 5-*O*-dimethoxycinnamoylquinic acid | **27** | H | *p*Co | H | D |
| 3-*O*-dimethoxycinnamoyl, 5-*O*-*p*-coumaroylquinic acid | **28** | H | D | H | *p*Co |
| 4-*O*-*p*-coumaroyl, 5-*O*-dimethoxycinnamoylquinic acid | **29** | H | H | *p*Co | D |
| 4-*O*-dimethoxycinnamoyl, 5-*O*-*p*-coumaroylquinic acid | **30** | H | H | D | *p*Co |
| 5-*O*-caffeoylquinic acid | **31** | H | H | H | C |

C = caffeic acid; D = dimethoxycinnamic acid; F = ferulic acid; *p*Co = *p*-coumaric acid; QA = quinic acid.

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

Separations were achieved on 150 × 3 mm i.d. columns containing either Luna 5 $\mu$m phenylhexyl packing or Kromasil $C_{18}$ 5 $\mu$m (Phenonemex, Macclesfield, U.K.). Solvent A was water/acetonitrile/glacial acetic acid (980:20:5, v/v, pH 2.68), and solvent B was acetonitrile/glacial acetic acid (1000:5, v/v). Solvents were delivered at a total flow rate of 300 $\mu$L/min. The gradient profile was from 4% B to 33% B linearly in 90 min, a linear increase to 100% B at 95 min, followed by 5 min isocratic, and a return to 4% B at 105 min and 5 min isocratic to re-equilibrate.

## RESULTS AND DISCUSSION

**General LC-MS Chromatographic and Spectroscopic Data.** All data for chlorogenic acids presented in this paper use the recommended IUPAC numbering system (*1*), and structures are presented in **Figure 1**. Generally, peak assignments have been made on the basis of the structure−diagnostic hierarchical keys previously developed, supported by examination of the UV spectrum and retention time relative to 5-caffeoylquinic acid (**31**) (*5*, *9*). However, for quantitatively minor components, the tabulated data are derived from more sensitive and more selective fragment-targeted MS$^n$ experiments (*6*). For certain chlorogenic acids such experiments generate spectra that differ slightly from those used in the original diagnostic, for example, 4-caffeoylquinic acid yields near equal *m/z* 173 and 179 at MS$^2$ compared with an *m/z* 173 base peak and *m/z* 179 at ∼70% of base peak (*13*).

The Robusta coffee extract gave a typical chromatogram in which the 30 previously reported chlorogenic acids were easily located on both HPLC column packings (*5*, *6*). To search for novel *p*-coumaric acid containing diacyl chlorogenic acids, negative ion MS$^2$ experiments were targeted on *m/z* 483 (di-*p*-coumaroylquinic acids), 499 (*p*-coumaroyl-caffeoylquinic acids), 513 (*p*-coumaroyl-feruloylquinic acids), and 527 (*p*-coumaroyl-dimethoxycinnamoylquinic acids). This strategy located four series of isomers that yielded various chlorogenic acid-related fragments (for example, *m/z* 319, 335, 337, 349, 363, 367, or 381) at MS$^2$ and that eluted from the phenylhexyl packing from 71 to 78 min, from 60 to 66 min, from 73 to 80 min, or from 85 to 92 min, respectively. When the $C_{18}$ packing was used, these same groups of isomers eluted from 62 to 73 min, from 52 to 63 min, from 63 to 75 min, and from 75 to 84 min, but with better resolution of individual peaks within each group. Accordingly, the $C_{18}$ packing was chosen for the more detailed investigations reported below. Some additional peaks producing identical molecular ions, but that did not yield recognizable chlorogenic acid-related MS$^2$ fragments, were not examined further in this study.

**Characterization of Putative Di-*p*-coumaroylquinic Acids (1−3).** Three peaks were detected at *m/z* 483. Their fragmentation is illustrated in **Figure 2** and **Table 1**, where these data are compared with those previously reported for the diferuloylquinic acids (*6*). The most hydrophilic di-*p*-coumaroylquinic acid isomer (**1**) produced a 'dehydrated' MS$^2$ base peak at *m/z* 319 that yielded *m/z* 145 at MS$^3$. In contrast, the second di-*p*-coumaroylquinic acid isomer (**2**) produced an MS$^2$ base peak at *m/z* 337 and a cinnamate-derived MS$^3$ base peak (*m/z* 163), whereas the most hydrophobic di-*p*-coumaroylquinic (**3**) yielded MS$^2$ and MS$^3$ base peaks at *m/z* 337 and 173. As illustrated in **Table 1**, these fragments are either identical or analogous (that is, 30 amu smaller than) to those produced by the diferuloylquinic acids (**4−6**) (*6*). Accordingly, these compounds were assigned as 3,4-di-*p*-coumaroylquinic acid (**1**), 3,5-di-*p*-coumaroylquinic acid (**2**), and 4,5-di-*p*-coumaroylquinic acid (**3**), respectively.

**Characterization of Putative *p*-Coumaroyl-caffeoylquinic Acids (13−18).** The fragment-targeted MS$^2$ experiments (*m/z* 499 + 337, *m/z* 499 + 319, *m/z* 499 + 353, and *m/z* 499 + 335) located a total of six peaks with a molecular ion at *m/z* 499 (**Figure 3** and **Table 2**). The first-eluting *p*-coumaroyl-caffeoylquinic acid (**13**) produced an MS$^2$ base peak at *m/z* 353 and secondary ions at *m/z* 337, 335, and 319, indicating significant losses of the caffeoyl and *p*-coumaroyl residues, and significant dehydration, behavior characteristic of a 3,4-*vic*-chlorogenic acid (*5*, *6*, *9*). The ratio of "dehydrated" ions to "non-dehydrated" ions (∼0.30) at MS$^2$ is consistent with a 3,4-

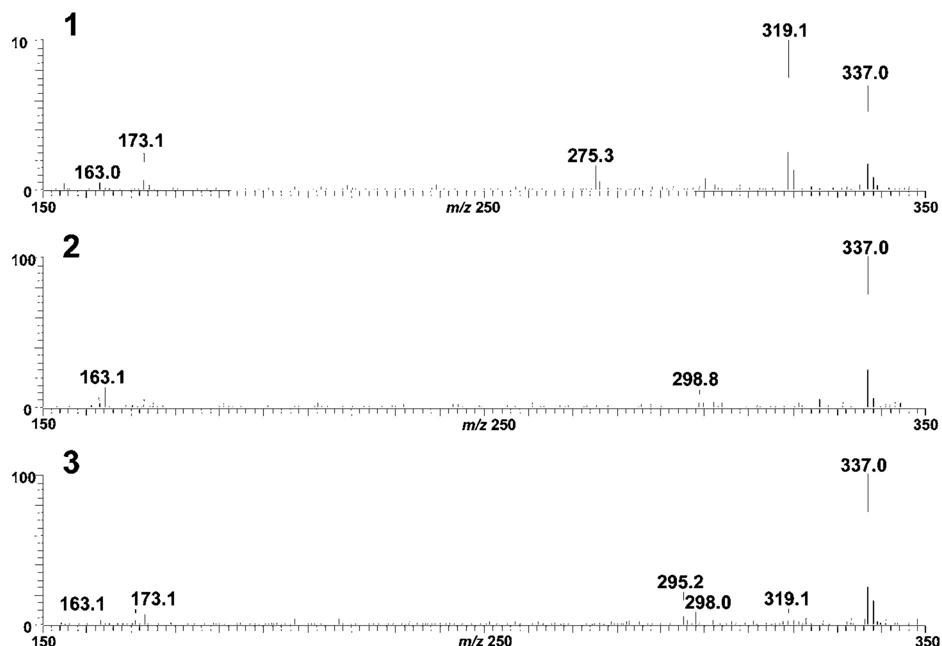



**Figure 2.** Negative ion MS² spectra of the putative di-*p*-coumaroylquinic acids.

**Table 1.** Negative Ion MS³ Fragmentation Data for the Putative Di-*p*-coumaroylquinic Acids and the Previously Characterized Diferuloylquinic Acids

| | | parent ion | MS² base peak | MS² secondary ions | | | | MS³ base peak | MS³ secondary ions | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| compd | *N*[a] | *m/z* | *m/z* | *m/z* | intensity | *m/z* | intensity | *m/z* | *m/z* | intensity | *m/z* | intensity |
| **1** | 6 | 483.0 | 319.0 | 336.9 | 50 | bp[b] | 100 | 145.0 | 163.0 | 55 | 173.1 | 90 |
| **2** | 6 | 483.1 | 337.0 | bp | 100 | 319.0 | 33 | 163.1 | bp | 100 | 173.0 | 20 |
| **3** | 6 | 483.0 | 336.9 | bp | 100 | 319.0 | 10 | 173.1 | 163.0 | 40 | bp | 100 |
| **4** | 6 | 543.1 | 349.0 | 367.0 | 17 | bp | 100 | 175.1 | 193.0 | 27 | | |
| **5** | 6 | 543.0 | 367.1 | bp | 100 | 349.1 | 35 | 193.0 | bp | 100 | 173.1 | 7 |
| **6** | 6 | 543.0 | 367.0 | bp | 100 | 349.1 | 17 | 173.1 | 193.0 | 70 | bp | 100 |

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

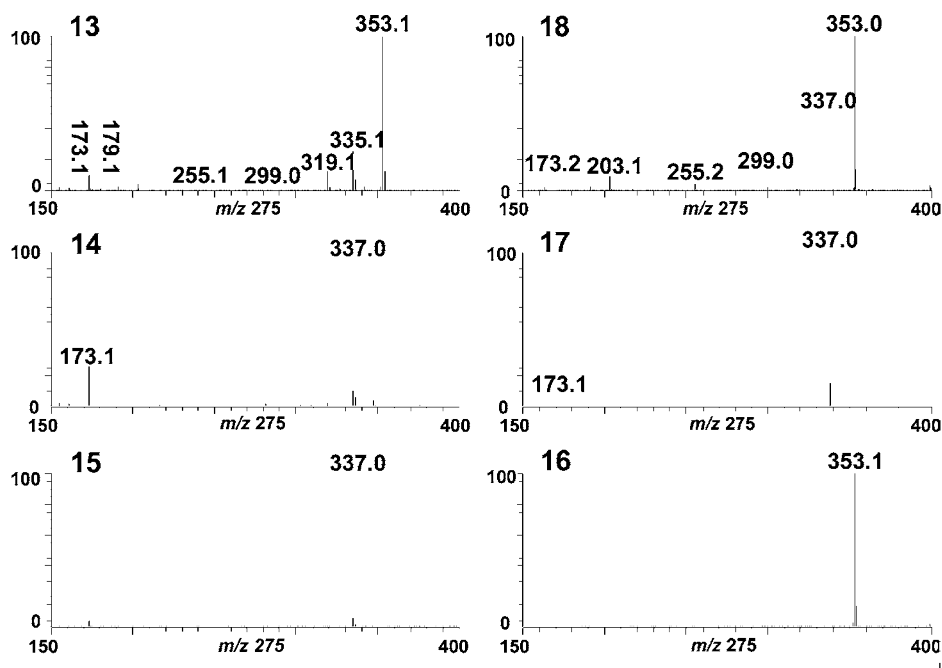



**Figure 3.** Negative ion MS² spectra of the putative *p*-coumaroyl-caffeoylquinic acids.

*vic*-chlorogenic acid in which neither cinnamoyl residue is methylated, and secondary fragment ions at *m/z* 299 and 255 are consistent with a caffeoyl residue at C4 (*6*). Accordingly, it has been assigned as 3-*p*-coumaroyl-4-caffeoylquinic acid (**13**). The second-eluting *p*-coumaroyl-caffeoylquinic acid loses its caffeoyl residue more extensively than its *p*-coumaroyl residue. The MS² base peak (*m/z* 337) yields *m/z* 173 at MS³, consistent with assignment as 3-caffeoyl-4-*p*-coumaroylquinic acid (**14**).

Peaks **17** and **18** both produce an MS³ base peak at *m/z* 173, indicating that these are the *vic*-4,5-*p*-coumaroyl-caffeoylquinic

**Table 2.** Negative Ion MS$^3$ Fragmentation Data for the Putative *p*-Coumaroyl-caffeoylquinic Acids and the Previously Characterized Caffeoyl-feruloylquinic Acids

| | | parent ion | MS$^2$ base peak | MS$^2$ secondary ions | | | | | | | | | | MS$^3$ base peak | MS$^3$ secondary ions | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| compd | *N*[a] | *m/z* | *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 |
| **13** | 6 | 499.0 | 353.0 | 337.1 | 87 | bp[b] | 100 | 319.1 | 20 | 335.1 | 30 | 299.0 | 6 | 173.1 | 179.1 | 67 | 191.1 | 25 |
| **14** | 6 | 499.0 | 337.0 | bp | 100 | | | 319.1 | 5 | 335.1 | 12 | | | 173.1 | 163.0 | 40 | | |
| **15** | 6 | 499.0 | 337.0 | bp | 100 | | | | | | | | | 163.1 | 173.0 | 35 | | |
| **16** | 6 | 499.0 | 353.1 | | | bp | 100 | | | | | | | 191.0 | | | bp | 100 |
| **17** | 6 | 499.0 | 337.0 | bp | 100 | | | | | 335.0 | 2 | | | 173.1 | 163.1 | 25 | | |
| **18** | 6 | 499.0 | 353.0 | 337.0 | 25 | bp | 100 | | | | | 299.0 | 10 | 173.1 | | | | |
| **7** | 6 | 529.1 | 353.0 | 367.1 | 90 | bp | 100 | 349.1 | 45 | 335.1 | 50 | 298.9 | 6 | 173.1 | 179.1 | 85 | 191.1 | 15 |
| **8** | 6 | 529.0 | 367.0 | bp | 100 | 353.0 | 5 | 349.3 | 5 | 335.1 | 10 | | | 173.0 | 193.0 | 30 | | |
| **9** | 6 | 529.0 | 367.0 | bp | 100 | | | | | 335.1 | 5 | | | 193.3 | 173.1 | 45 | | |
| **10** | 6 | 529.0 | 353.0 | 367.0 | 55 | bp | 100 | 349.1 | 7 | 335.1 | 7 | | | 191.5 | 179.5 | 56 | 191.1 | 100 |
| **11** | 6 | 529.0 | 367.0 | bp | 100 | | | | | 335.1 | 2 | | | 173.0 | 193.0 | 70 | | |
| **12** | 6 | 529.0 | 353.0 | 367.0 | 25 | bp | 100 | | | | | 299.0 | 8 | 173.1 | 179.1 | 84 | 191.1 | 28 |

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

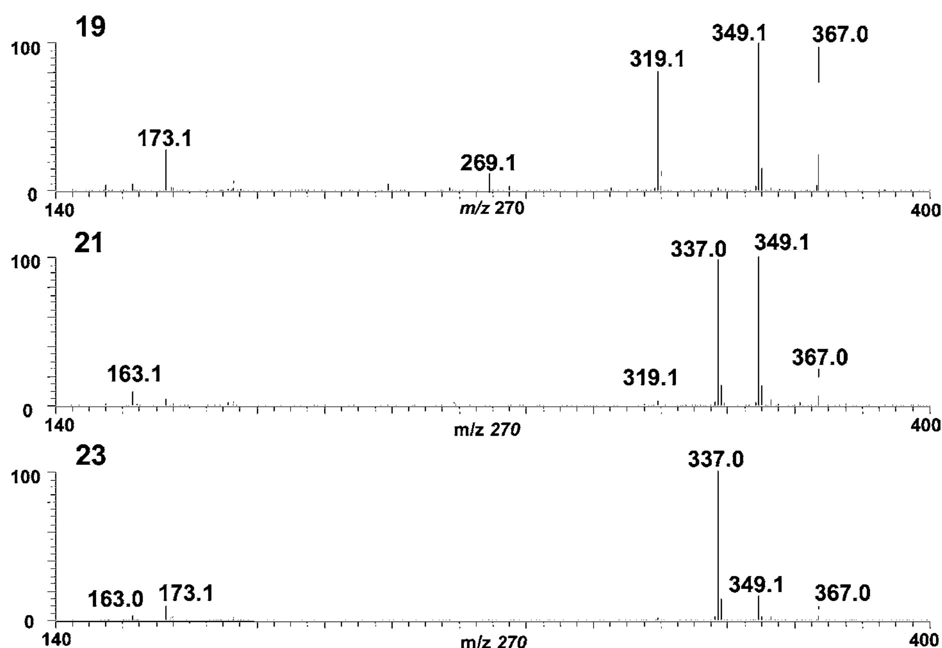



**Figure 4.** Negative ion MS$^2$ spectra of the putative *p*-coumaroyl-feruloylquinic acids.

acids. The former loses its caffeoyl residue at MS$^2$, whereas the latter loses its *p*-coumaroyl residue. Because cinnamoyl residues at C5 are lost more easily than those at C4 (*5*, *6*, *9*) and because peak 18 has significant MS$^2$ secondary ions at *m/z* 255 and 299 indicative of a caffeoyl residue at C4, these can be assigned as 4-*p*-coumaroyl-5-caffeoylquinic acid (**17**) and 4-caffeoyl-5-*p*-coumaroylquinic acid (**18**), respectively.

The remaining pair of isomers (**15**, **16**) produced comparatively weak signals that were increased in magnitude by treatment of the methanolic extract with TMAH for 1 min at room temperature. Their elution between two pairs of *vic*-*p*-coumaroyl-caffeoylquinic acids and the absence of a strong *m/z* 173 fragment ion at MS$^3$ are consistent with their being 3,5-*p*-coumaroyl-caffeoylquinic acids. Peak 15 lost its *p*-coumaroyl residue before its caffeoyl residue and produced an *m/z* 191 MS$^3$ base peak, whereas peak 16 lost its caffeoyl residue first and produced *m/z* 163 and 119 as the MS$^3$ and MS$^4$ base peaks, respectively. Accordingly, these were assigned as 3-caffeoyl-5-*p*-coumaroylquinic acid (**16**) and 3-*p*-coumaroyl-5-caffeoylquinic acid (**15**). These six isomers behaved in a manner completely analogous to the caffeoyl-feruloylquinic acids (**7**−**12**) previously characterized (*5*).

**Characterization of Putative *p*-Coumaroyl-feruloylquinic Acids (19−24).** The fragment-targeted MS$^2$ experiments located three peaks with a molecular ion at *m/z* 513 that subsequently yielded MS$^2$ base peaks at *m/z* 337, 349, or 367 (**Figure 4** and **Table 3**). The most hydrophobic isomer, peak 23, produced MS$^2$, MS$^3$, and MS$^4$ base peaks at *m/z* 337, 173, and 93, respectively. Because it clearly lost its feruloyl residue before its *p*-coumaroyl residue, it was assigned as 4-*p*-coumaroyl-5-feruloylquinic acid (**23**). Peak 21 lost its feruloyl residue before its *p*-coumaroyl residue and produced *m/z* 163 as the MS$^3$ base peak, behavior analogous to that of 3-caffeoyl-5-*p*-coumaroylquinic acid (**16**) and, accordingly, was assigned as 3-*p*-coumaroyl-5-feruloylquinic acid (**21**). The first-eluting *p*-coumaroyl-feruloylquinic acid (**19**) produced a high yield of dehydrated fragment ions relative to non-dehydrated fragment ions (∼1.8:1), consistent with a 3,4-*vic* isomer in which one of the cinnamoyl residues is methylated (*6*, *9*). Both cinnamoyl residues are lost, the *p*-coumaroyl residue more extensively than the feruloyl residue, suggesting that this is 3-*p*-coumaroyl-4-feruloylquinic acid (**19**). A weak MS$^2$ fragment ion at *m/z* 269, indicative of C4 feruloyl residue, is consistent with this assignment.

**Table 3.** Negative Ion MS$^3$ Fragmentation Data for the Putative *p*-Coumaroyl-feruloylquinic Acids

| | | parent ion | MS$^2$ base peak | MS$^2$ secondary ions | | | | | | | | | | MS$^3$ base peak | MS$^3$ secondary ions | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| compd | $N^a$ | *m/z* | *m/z* | *m/z* | intensity | *m/z* | intensity | *m/z* | intensity | *m/z* | intensity | *m/z* | intensity | *m/z* | *m/z* | intensity |
| **19** **20** | 6 | 513.0 | 349.1 | 367.0 | 90 | bp$^b$ | 100 | 337.0 | 5 | 319.0 | 80 | 269.1 | 5 | 173.1 | 193.0 | 35 |
| **21** **22** | 6 | 513.0 | 349.1 | 367.0 | 20 | bp | 100 | 337.0 | 95 | 319.2 | 20 | | | 163.0 | | |
| **23** **24** | 6 | 513.0 | 337.0 | 367.1 | 15 | 349.1 | 20 | bp | 100 | 319.1 | 2 | | | 173.1 | 163.1 | 27 |

$^a$ Number of LC-MS analyses. $^b$ Occurs as base peak.

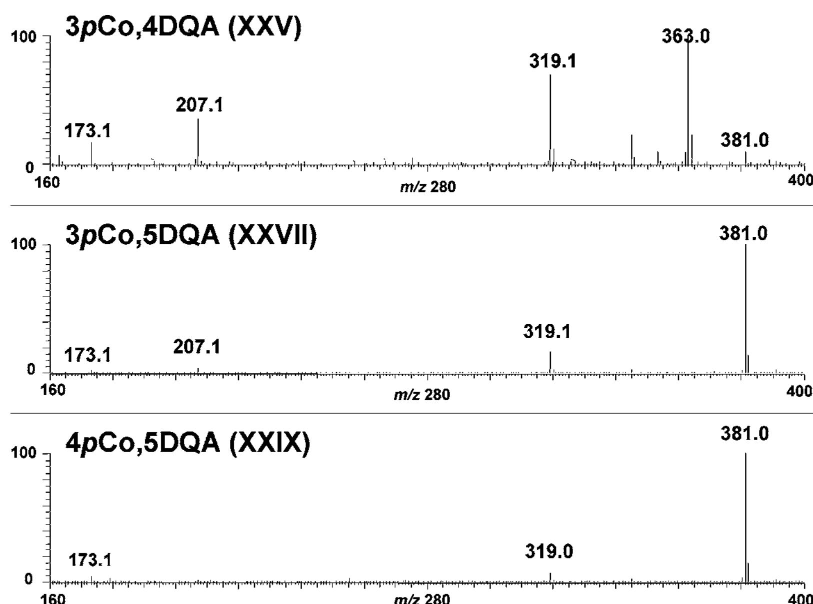



**Figure 5.** Negative ion MS$^2$ spectra of the putative *p*-coumaroyl-dimethoxycinnamoylquinic acids.

**Table 4.** Negative Ion MS$^3$ Fragmentation Data for the Putative *p*-Coumaroyl-dimethoxycinnamoylquinic Acids

| | | parent ion | MS$^2$ base peak | MS$^2$ secondary ions | | | | | | MS$^3$ base peak |
|---|---|---|---|---|---|---|---|---|---|---|
| compd | $N^a$ | *m/z* | *m/z* | *m/z* | intensity | *m/z* | intensity | *m/z* | intensity | *m/z* |
| **25** or **26** | 6 | 527.0 | 363.0 | 381.0 | 10 | bp$^b$ | 100 | 319.0 | 70 | 173.0 |
| **27** or **28** | 6 | 527.1 | 381.0 | bp | 100 | 363.1 | 2 | 319.0 | 20 | 173.0 |
| **30** | 6 | 527.1 | 381.0 | bp | 100 | | | 319.0 | 10 | 173.0 |

$^a$ Number of LC-MS analyses. $^b$ Occurs as base peak.

**Characterization of Putative *p*-Coumaroyl-dimethoxycinnamoylquinic Acids (25−30).** Three peaks were detected at *m/z* 527, but in the absence of authentic standards two signals were too weak to permit their full structural assignment. Their fragmentation is illustrated in **Figure 5** and **Table 4**. The strongest signal was obtained for the most hydrophobic *p*-coumaroyl-dimethoxycinnamoylquinic acid. This isomer loses its *p*-coumaroyl residue before its dimethoxycinnamoyl residue (MS$^2$ base peak at *m/z* 383), and this ion fragmented to *m/z* 173 at MS$^3$. By analogy with the behavior of synthetic 4-dimethoxycinnamoylquinic acid (*6*) it has been assigned as 4-dimethoxycinnamoyl-5-*p*-coumaroylquinic acid (**30**).

The first-eluting *p*-coumaroyl-dimethoxycinnamoyl quinic acid lost both cinnamoyl residues extensively, but the *p*-coumaroyl more than the dimethoxycinnamoyl. It produced a dehydrated MS$^2$ base peak at *m/z* 363 accompanied by a strong (70% of base peak) dehydrated fragment ion at *m/z* 319 and a weaker *m/z* 381 (10% of base peak) but without the other non-dehydrated *p*-coumaroylquinic acid ion at *m/z* 337. The early elution, the loss of both acyl residues with similar facility, and a ratio of dehydrated to non-dehydrated ions of 17:1 are characteristic of 3,4-*vic*-diacyl chlorogenic acids in which one of the residues is extensively methylated (*5*, *6*). The slightly greater loss of the *p*-coumaroyl residue suggests that this is located on C3 rather than C4. However, the MS$^2$ ion at *m/z* 381 yields *m/z* 207 at MS$^3$, suggesting by analogy with the reported behavior of synthetic dimethoxycinnamoylquinic acids (*6*) that this is [3-dimethoxycinnamoylquinic acid $-$ H$^+$]$^-$ rather than [4-dimethoxycinnamoylquinic acid $-$ H$^+$]$^-$. Because of this apparent conflict, it is not possible to determine whether this is 3-*p*-coumaroyl-4-dimethoxycinnamoylquinic acid (**25**) or 3-dimethoxycinnamoyl-4-*p*-coumaroylquinic acid (**26**).

On the basis of its elution between two *vic* isomers and its relative retention time (RRT), discussed below, the third *p*-coumaroyl-dimethoxycinnamoylquinic acid would appear to be a 3,5-diacyl isomer (**27** or **28**), with the relatively easy loss of the *p*-coumaroyl residue arguing in favor of **28**. However, its MS$^2$ base peak of *m/z* 381 yields an MS$^3$ base peak at *m/z* 173. In our previous LC-MS studies of chlorogenic acids a base peak at *m/z* 173 has without exception been associated with a 4-acyl substituent (*5*, *6*, *9*), suggesting that this might be 3-*p*-coumaroyl-4-dimethoxycinnamoylquinic acid (**25**). The absence

of intense dehydrated ions at *m/z* 363 and 319 argues very strongly against assignment as a 3,4-*vic* isomer, and we feel confident that this possibility can be discounted. In our investigations of synthetic dimethoxycinnamoylquinic acids (*6*) it was observed that 5-dimethoxy-cinnamoylquinic acid yielded a cinnamoyl-derived demethylated quinide-ketene MS$^2$ base peak at *m/z* 193 (rather than the expected *m/z* 207). The fragmentation pathway proposed to explain this also suggested the formation of a quinic acid derived dehydrated ion at *m/z* 173, although this was not observed in that spectrum. If for this particular *p*-coumaroyl-dimethoxycinnamoylquinic acid the formation of *m/z* 173 is favored, it would suggest that it is 3-*p*-coumaroyl-5-dimethoxycinnamoylquinic acid (**27**) rather than **28**. This tentative assignment cannot be confirmed until such time as authentic standards can be synthesized.

The ratio of dehydrated to non-dehydrated ions produced by 3,4-diacyl chlorogenic acids containing a caffeoyl residue has previously been observed to increase with increasing methylation of the second cinnamoyl residue, for example, from 0.16 for 3,4-dicaffeoylquinic acid to 0.6 for 3-feruloyl-4-caffeoylquinic acid (**8**) and to 1.8 for 3-dimethoxycinnamoyl-4-caffeoylquinic acid (*5*, *6*, *9*). We now report similar behavior for the equivalent series of *p*-coumaric acid containing diacyl chlorogenic acids because the ratio increases from 0.3 for 3-*p*-coumaroyl-4-caffeoylquinic acid (**13**) to 1.8 for 3-*p*-coumaroyl-4-feruloylquinic acid (**19**) and to 17 for one of the 3,4-*vic*-*p*-coumaroyl-dimethoxycinnamoylquinic acids (**25** or **26**). For the most extensively methylated diacyl chlorogenic acid so far studied, 3-dimethoxycinnamoyl-4-feruloylquinic acid, this ratio exceeded 100 (*6*).

**Relative Retention Time of Diacyl Chlorogenic Acids.** Our LC-MS$^n$ studies of chlorogenic acids have utilized two chromatographic packings. On the $C_{18}$ packing we have observed that for the five classes of diacyl chlorogenic acids previously studied (*5*, *6*, *9*) the RRT values of the 3,4-isomer(s), the 3,5-isomer(s), and the 4,5-isomer(s) are 1.00, 1.04 $\pm$ 0.02, and 1.12 $\pm$ 0.02, respectively. The corresponding RRT values for the four new classes of *p*-coumaric acid containing diacyl chlorogenic acids are indistinguishable at 1.00, 1.04 $\pm$ 0.01, and 1.12 $\pm$ 0.02. Although the resolution (1.07 $\pm$ 0.01) of the *p*-coumaroyl-containg 4,5-diacyl chlorogenic acids (**3**, **17**, **18**, **23**, and **30**) on the phenylhexyl packing is also identical to our previous observations (*5*, *6*, *9*) (1.08 $\pm$ 0.01), the resolution of the *p*-coumaroyl-containing 3,5-diacyl chlorogenic acids (**15**, **16**, **21**, and **27** or **28**) was poorer and incomplete in the case of 3,4-di-*p*-coumaroylquinic acid (**1**) and 3,5-di-*p*-coumaroylquinic acid (**2**).

**Missing Isomers.** Because all six theoretical isomers of the *p*-coumaroyl-caffeoylquinic acids (**13**−**18**) have been easily located in the Robusta extract, it is a little surprising that only three *p*-coumaroyl-feruloylquinic acids (**19**, **21**, and **23**) and three *p*-coumaroyl-dimethoxycinnamoyl quinic acid isomers (**25** or **26**, **27** or **28**, and **30**) have been observed. The parallel situation was observed in previous studies (*5*, *6*) reporting six caffeoyl-feruloylquinic acid isomers (**7**−**12**) but only three caffeoyl-dimethoxycinnamoylquinic acids and three feruloyl-dimethoxycinnamoylquinic acids. As discussed previously, it has so far proven to be impossible both to locate these missing isomers and to identify a rational explanation for their absence.

Although mass spectrally distinct, these *p*-coumaric acid containing diacyl chlorogenic acids were in general poorly resolved chromatographically from the many other components in the Robusta extract. With the exception of the *p*-coumaroyl-caffeoylquinic acids (**13**−**18**) they produced weak UV signals ($A_{320}$) in the range of $10^4-10^5$ $\mu$AU for a 10 $\mu$L injection, an order of magnitude weaker than the diferuloylquinic acids, caffeoyl-dimethoxycinnamoylquinic acids, and caffeoyl-feruloylquinic acids previously characterized (*5*, *6*). Estimates of the content of these newly reported chlorogenic acids were obtained as previously described (*6*) from the absorbance of individual peaks at 325 nm relative to 5-caffeoylquinic acid (**31**), assigned unit absorbance, and typically present in green Robusta coffee beans at 5% dry mass basis (dmb). Because in the UV chromatogram these *p*-coumaric acid containing chlorogenic acids are incompletely resolved, such estimates can only be approximate, but we consider it unlikely that in green coffee beans any of these novel chlorogenic acids will individually exceed ~0.02% dmb or ~0.1% dmb in total.

## ACKNOWLEDGMENT

The technical assistance of H. Roozendaal is gratefully acknowledged.

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**Received for review February 23, 2006. Revised manuscript received April 25, 2006. Accepted April 26, 2006.**

JF060536P