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ARTICLE

Profile and Characterization of the Chlorogenic Acids in Green Robusta Coffee Beans by LC-MS^n: Identification of Seven New Classes of Compounds

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LC-MS^n (n = 2−4) has been used to detect and characterize in green Robusta coffee beans 15 quantitatively minor sinapic acid and trimethoxycinnamoylquinic acid-containing chlorogenic acids, all reported for the first time from this source, with 13 of them not previously reported in nature. These comprise 3-sinapoylquinic acid, 4-sinapoylquinic acid, and 5-sinapoylquinic acid (M_r 398); 3-sinapoyl-5-caffeoylquinic acid, 3-sinapoyl-4-caffeoylquinic acid, and 4-sinapoyl-3-caffeoylquinic acid (M_r 560); 3-(3,5-dihydroxy-4-methoxy)cinnamoyl-4-feruloylquinic acid (M_r 560); 3-sinapoyl-5-feruloylquinic acid, 3-feruloyl-4-sinapoylquinic acid, and 4-sinapoyl-5-feruloylquinic acid (M_r 574); 4-trimethoxycinnamoyl-5-caffeoylquinic acid, 3-trimethoxycinnamoyl-5-caffeoylquinic acid (M_r 574); and 5-feruloyl-3-trimethoxycinnamoylquinic acid, 3-trimethoxycinnamoyl-4-feruloylquinic acid, and 4-trimethoxycinnamoyl-5-feruloylquinic acid (M_r 588). Furthermore, a series of structures including nine new triacyl quinic acids have been assigned on the basis of LC-MS^n patterns of fragmentation, relative hydrophobicity, and analogy of fragmentation patterns if compared to feruloyl, caffeoyl, and dimethoxycinnamoyl quinic acids. Sixty-nine chlorogenic acids have now been characterized in green Robusta coffee beans.

KEYWORDS: Chlorogenic acids; coffee; sinapoylquinic acids; sinapoyl-caffeoylquinic acids; sinapoyl-feruloylquinic acids; trimethoxycinnamoylquinic acids; caffeoyl-trimethoxycinnamoylquinic acids; feruloyl-trimethoxycinnamoylquinic acids; dimethoxy-feruloyl-caffeoylquinic acid; triacyl quinic 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 acid (*1*−*3*). Representative structures are shown in **Figure 1**. In the IUPAC system (−)-quinic acid is defined as 1 L-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 45 chlorogenic acids that are not acylated at C1 of the quinic acid moiety. In previous work we have profiled the chlorogenic acids in green Arabica coffee beans and identified a total of 45 different chlorogenic acids (*5*−*8*).

The aim of this study was to profile green Robusta coffee beans with respect to their chlorogenic acid profile. Coffee trees are usually divided botanically into two categories, *Coffea arabica* (Arabica coffee) and *Coffea canephora* (usually referred to as Robusta coffee). Supposedly high-quality coffee blends consist typically of 100% Arabica coffee beans. Lower quality, cheaper blends may have some proportion of Robusta beans, or they may consist entirely of Robusta. Arabica beans produce allegedly a superior taste in the cup, being more flavorful and complex than their Robusta counterparts. Robusta beans in contrast tend to produce a more bitter brew, with a musty flavor and less body (*9*). Obviously this difference in sensory properties could be related to the individual phytochemical profile of the two coffee varieties. Because chlorogenic acids are the most common secondary metabolites found in the green coffee bean and are therefore precursors for the formation of further compounds with sensory properties in the coffee roasting process, a closer inspection of their chlorogenic acid profile deserves attention to allow a direct comparison of compounds present in either coffee variety. Hofmann and coworkers (*9*), for example, have shown that chlorogenic acids and their derivatives contribute considerably to the sensory properties of the coffee brew.

The cultivation of Robusta coffee if compared to Arabica has several advantages because their trees produce their first crops within about 2−3 years of being planted. Arabica trees, in comparison, require about 4−5 years to yield fruit. Farmers have hence a good motive to grow the faster growing variety and take advantage of upswings in the price of coffee. Additionally, the Robusta coffee tree can grow under a larger variety of climatic conditions than can the Arabica because it is in general more tolerant to cold climate and tolerates a wider range of altitudes.

Recently, LC-MS^n has been used to characterize cinnamoyl-amino acid conjugates (*10*) and to discriminate between individual isomers of monoacyl and diacyl chlorogenic acids (*5*−*8*).

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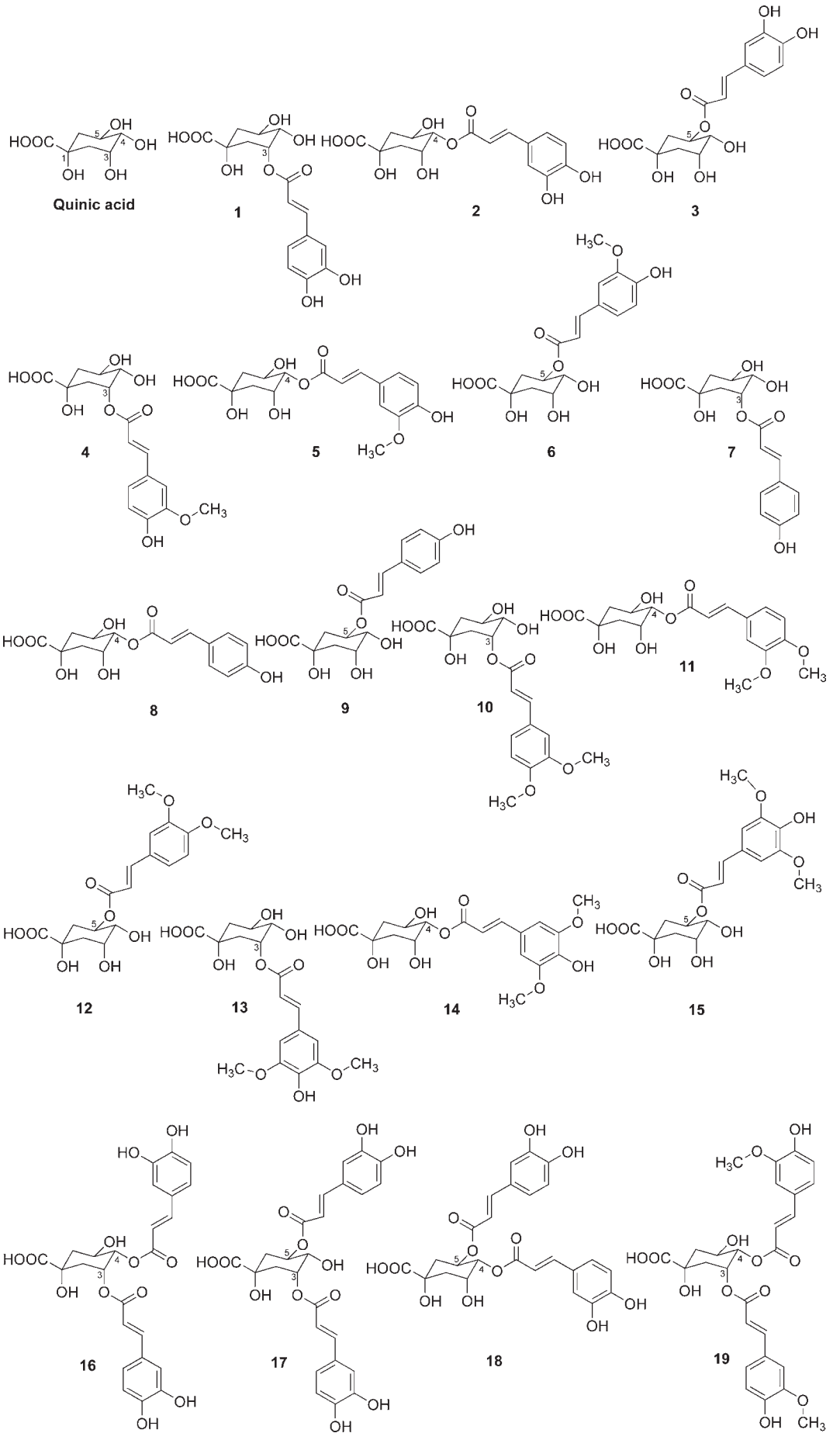
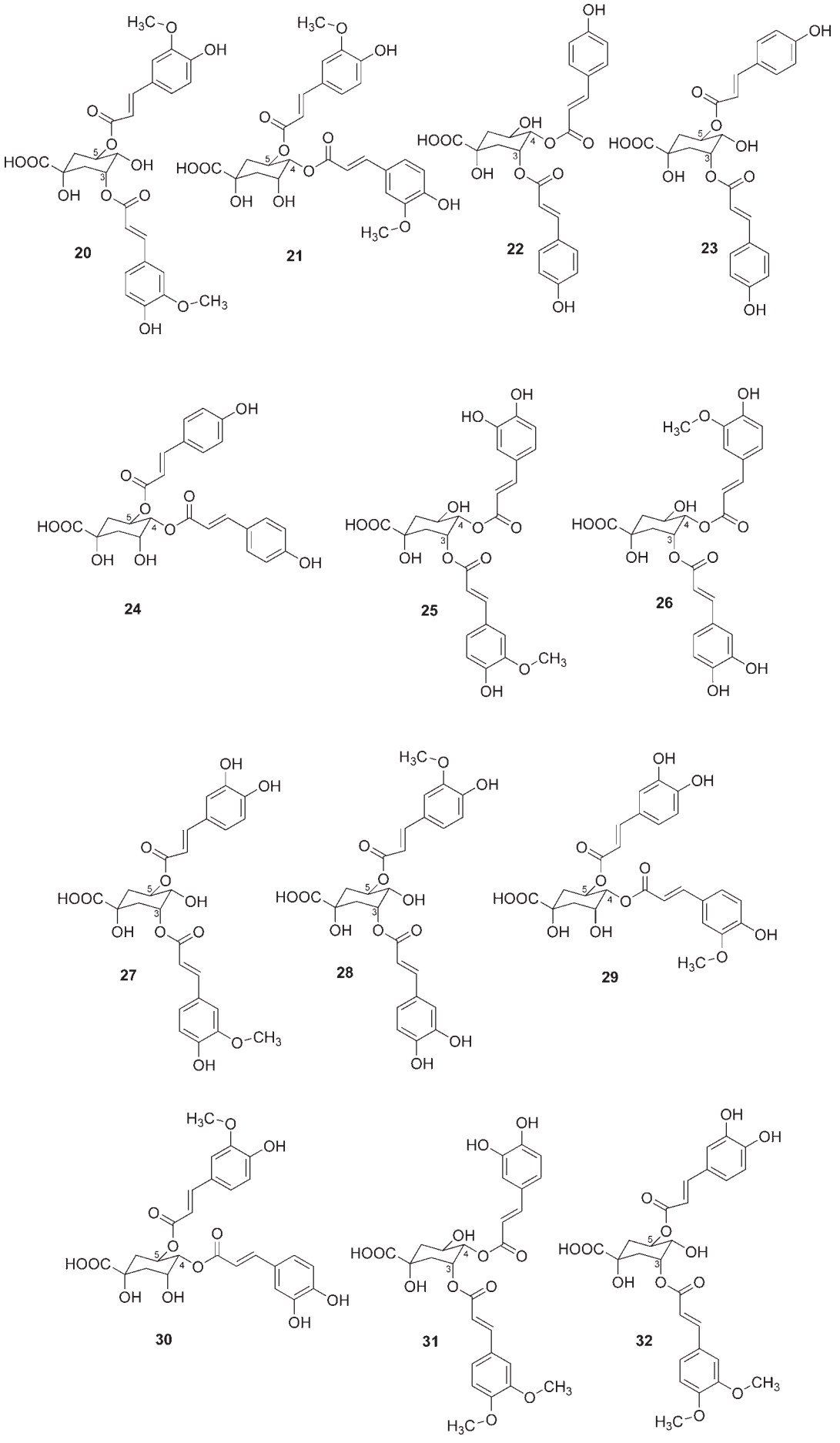


Figure 1. Continued



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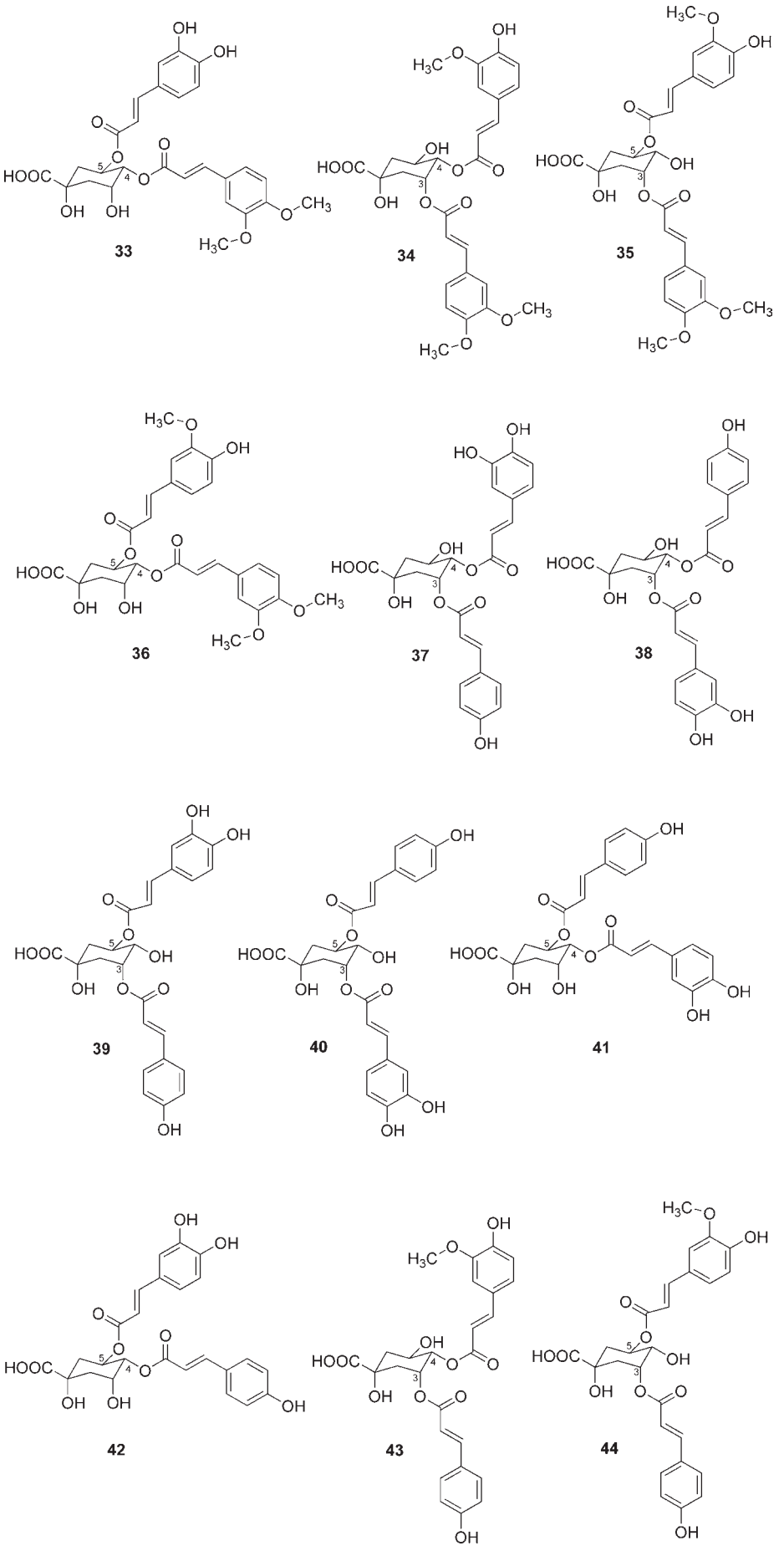

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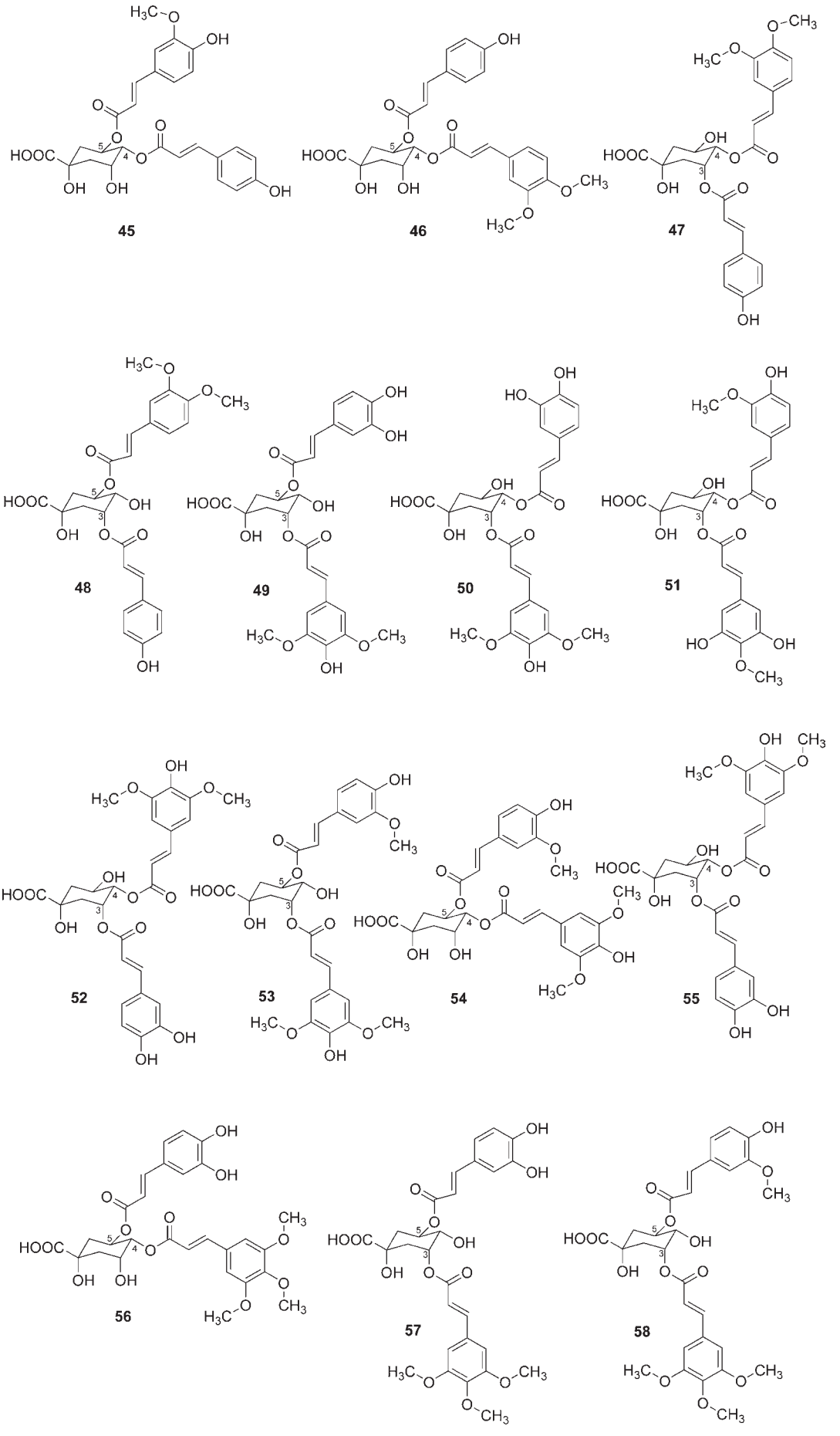

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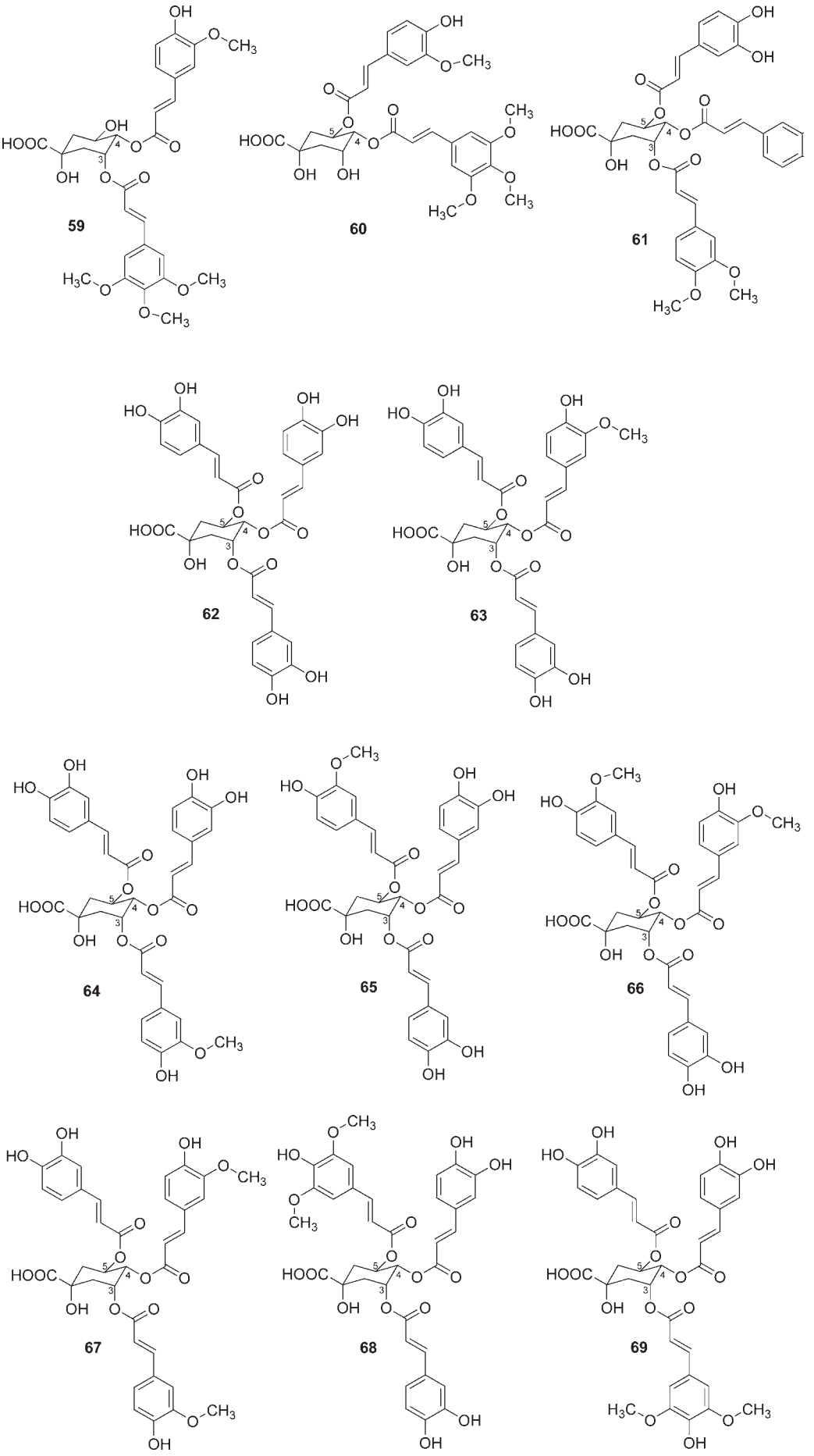


Figure 1. Continued

No.	Name	Abbreviation	R^3	R^4	R^5
1	3-*O*-caffeoylquinic acid	3-CQA	C	H	H
2	4-*O*-caffeoylquinic acid	4-CQA	H	C	H
3	5-*O*-caffeoylquinic acid	5-CQA	H	H	C
4	3-*O*-feruloylquinic acid	3-FQA	F	H	H
5	4-*O*-feruloylquinic acid	4-FQA	H	F	H
6	5-*O*-feruloylquinic acid	5-FQA)	H	H	F
7	3-*O*-*p*-coumaroylquinic acid	3-*p*CoQA	*p*Co	H	H
8	4-*O*-*p*-coumaroylquinic acid	4-*p*CoQA	H	*p*Co	H
9	5-*O*-*p*-coumaroylquinic acid	5-*p*CoQA	H	H	*p*Co
10	3-*O*-dimethoxycinnamoylquinic acid	3-DQA	D	H	H
11	4-*O*-dimethoxycinnamoylquinic acid	4-DQA	H	D	H
12	5-*O*-dimethoxycinnamoylquinic acid	5-DQA	H	H	D
13	3-*O*-sinapoylquinic acid[b]	3-SiQA	Si	H	H
14	4-*O*-sinapoylquinic acid[a]	4-SiQA	H	Si	H
15	5-*O*-sinapoylquinic acid[b]	5-SiQA	H	H	Si
16	3,4-di-*O*-caffeoylquinic acid	3,4-diCQA	C	C	H
17	3,5-di-*O*-caffeoylquinic acid	3,5-diCQA	C	H	C
18	4,5-di-*O*-caffeoylquinic acid	4,5-diCQA	H	C	C
19	3,4-di-*O*-feruloylquinic acid	3,4-diFQA	F	F	H
20	3,5-di-*O*-feruloylquinic acid	3,5-diFQA	F	H	F
21	4,5-di-*O*-feruloylquinic acid	4,5-diFQA	H	F	F
22	3,4-di-*O*-*p*-coumaroylquinic acid	3,4-di*p*CoQA	*p*Co	*p*Co	H
23	3,5-di-*O*-*p*-coumaroylquinic acid	3,5-di*p*CoQA	*p*Co	H	*p*Co
24	4,5-di-*O*-*p*-coumaroylquinic acid	4,5-di*p*CoQA	H	*p*Co	*p*Co
25	3-*O*-feruloyl-4-*O*-caffeoylquinic acid	3F-4CQA	F	C	H
26	3-*O*-caffeoyl-4-*O*-feruloylquinic acid	3C-4FQA	C	F	H
27	3-*O*-feruloyl-5-*O*-caffeoylquinic acid	3F-5CQA	F	H	C
28	3-*O*-caffeoyl-5-*O*-feruloylquinic acid	3C-5FQA	C	H	F
29	4-*O*-feruloyl-5-*O*-caffeoylquinic acid	4F-5CQA	H	F	C
30	4-*O*-caffeoyl-5-*O*-feruloylquinic acid	4C-5FQA	H	C	F
31	3-*O*-dimethoxycinnamoyl-4-*O*-caffeoylquinic acid	3D-4CQA	D	C	H
32	3-*O*-dimethoxycinnamoyl-5-*O*-caffeoylquinic acid	3D-5CQA	D	H	C
33	4-*O*-dimethoxycinnamoyl-5-*O*-caffeoylquinic acid	4D-5CQA	H	D	C
34	3-*O*-dimethoxycinnamoyl-4-*O*-feruloylquinic acid	3D-4FQA	D	F	H
35	3-*O*-dimethoxycinnamoyl-5-*O*-feruloylquinic acid	3D-5FQA	D	F	H
36	4-*O*-dimethoxycinnamoyl-5-*O*-feruloylquinic acid	4D-5FQA	H	D	F
37	3-*O*-p-coumaroyl-4-*O*-caffeoylquinic acid	3*p*Co-4CQA	*p*Co	C	H
38	3-*O*-caffeoyl-4-*O*-*p*-coumaroylquinic acid	3C-4*p*CoQA	C	*p*Co	H
39	3-*O*-*p*-coumaroyl-5-*O*-caffeoylquinic acid	3*p*Co-5CQA	*p*Co	H	C
40	3-*O*-caffeoyl-5-*O*-*p*-coumaroylquinic acid	3C-5*p*CoQA	C	H	*p*Co
41	4-*O*-caffeoyl-5-*O*-*p*-coumaroylquinic acid	4C-5*p*CoQA	H	C	*p*Co
42	4-*O*-*p*-coumaroyl-5-*O*-caffeoylquinic acid	4*p*Co-5CQA	H	*p*Co	C
43	3-*O*-*p*-coumaroyl-4-*O*-feruloylquinic acid	3*p*Co-4FQA	*p*Co	F	H
44	3-*O*-*p*-coumaroyl-5-*O*-feruloylquinic acid	3*p*Co-5FQA	*p*Co	H	F
45	4-*O*-*p*-coumaroyl-5-*O*-feruloylquinic acid	4*p*Co-5FQA	H	*p*Co	F
46	4-*O*-dimethoxycinnamoyl-5-*O*-*p*-coumaroylquinic acid	4D-5*p*CoQA	H	D	*p*Co
47	3-*O*-*p*-coumaroyl-4-*O*-dimethoxycinnamoylquinic acid	3*p*Co-4DQA	*p*Co	D	H
48	3-*O*-*p*-coumaroyl-5-*O*-dimethoxycinnamoylquinic acid	3*p*Co-5DQA	*p*Co	H	D
49	3-*O*-sinapoyl-5-*O*-caffeoylquinic acid[a]	3Si-5CQA	Si	H	C
50	3-*O*-sinapoyl-4-*O*-caffeoylquinic acid[a]	3Si-4CQA	Si	C	H
51	3-*O*-(3,5-dihydroxy-4-methoxy)cinnamoyl-4-*O*-feruloylquinic acid[a]	3DM-4FQA	DM	F	H
52	4-*O*-sinapoyl-3-*O*-caffeoylquinic acid[a]	4Si-3CQA	C	Si	H
53	3-*O*-sinapoyl-5-*O*-feruloylquinic acid[a]	3Si-5FQA	Si	H	F
54	3-*O*-feruloyl-4-*O*-sinapoylquinic acid[a]	4Si-5FQA	H	Si	F
55	4-*O*-sinapoyl-3-*O*-feruloylquinic acid[a]	4Si-3FQA	F	Si	H
56	4-*O*-trimethoxycinnamoyl-5-*O*-caffeoylquinic acid[b]	4T-5CQA	H	T	C
57	3-*O*-trimethoxycinnamoyl-5-*O*-caffeoylquinic acid[b]	3T-5CQA	T	H	C
58	3-*O*-trimethoxycinnamoyl-5-*O*-feruloylquinic acid[b]	3T-5FQA	T	H	F
59	3-*O*-trimethoxycinnamoyl-4-*O*-feruloylquinic acid[b]	3T-4FQA	T	F	H
60	4-*O*-trimethoxycinnamoyl-5-*O*-feruloylquinic acid[b]	4T-5FQA	H	T	F

Figure 1. Continued

No.	Name	Abbreviation	R^3	R^4	R^5
61	3-*O*-dimethoxycinnamoyl-4-*O*-feruloyl-5-*O*-caffeoylquinic acid[b]	3D-4F-5-CQA	D	F	C
62	3,4,5-tri-*O*-caffeoylquinic acid	3,4,5-triCQA	C	C	C
63	3,5-di-*O*-caffeoyl-4-*O*-feruloylquinic acid	3,5-diC-4FQA	C	F	C
64	3-*O*-feruloyl-4,5-di-*O*-caffeoylquinic acid	3F-4,5-diCQA	F	F	C
65	3,4-di-*O*-caffeoyl-5-*O*-feruloylquinic acid	3,4-diC-5FQA	C	C	F
66	3-*O*-caffeoyl-4,5-di-*O*-feruloylquinic acid	3C-4,5-diFQA	C	F	F
67	3,4-di-*O*-feruloyl-5-*O*-caffeoylquinic acid	3,4-diF-5CQA	F	F	C
68	3,4-di-*O*-caffeoyl-5-*O*-sinapoylquinic acid	3,4-diC-5SiQA	C	C	S
69	3-*O*-sinapoyl-4,5-di-*O*-caffeoylquinic acid	3Si-4,5-diCQA	S	C	C

C = caffeoyl; D = dimethoxycinnamoyl; F = feruloyl; *p*Co = *p*-coumaroyl; Si = sinapoyl; H = hydrogen; DM= 3,5-dihydroxy-4-methoxycinnamoyl; T= trimethoxycinnamoyl; a = first time reported in nature; b = first time from this source.

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

The MS fragmentation patterns in tandem MS spectra, UV spectrum, retention time, and relative hydrophobicity have been utilized to develop structure-diagnostic hierarchical keys for the identification of chlorogenic acids. In this study, we applied these methods to the qualitative profiling of chlorogenic acids in green Robusta coffee bean extract.

MATERIALS AND METHODS

All of the chemicals (analytical grade) were purchased from Sigma-Aldrich (Bremen, Germany). Green Robusta coffee beans (Cameroon, India, Indonesia, Tanzania, Togo, and Uganda Robusta) were purchased from supermarkets in Bremen, Germany.

Methanolic Extract of Coffee Beans. Six different samples of green Robusta coffee (Cameroon, India, Indonesia, Tanzania, Togo, and Uganda Robusta) beans (5 g of each) were freeze-dried overnight at −20 °C and ground to fine powder; methanolic extracts were prepared by Soxhlet extraction using aqueous methanol (70%) for 5 h. The extract was treated with Carrez reagents (1 mL of reagent A plus 1 mL of reagent B) (*11*) to precipitate colloidal material and filtered through a Whatman no. 1 filter paper. The methanol and water were removed in vacuo, and the residue was stored at −20 °C until required, thawed at room temperature, dissolved in methanol (60 mg/10 mL), filtered through a membrane filter, and used for LC-MS.

LC-MS^n. The LC equipment (Agilent 1100 series, Karlsruhe, Germany) comprised a binary pump, an autosampler with a 100 μL loop, and a DAD detector with a light-pipe flow cell (recording at 320 and 254 nm and scanning from 200 to 600 nm). This was interfaced with an ion-trap mass spectrometer fitted with an ESI source (Bruker Daltonics HCT Ultra, Bremen, Germany) operating in full scan, auto MS^n mode to obtain fragment ion m/z. As necessary, MS^2, MS^3, and MS^4 fragment-targeted experiments were performed to focus only on compounds producing a parent ion at m/z 397, 559, 573, 587, 677, 691, 705, or 719. Tandem mass spectra were acquired in auto-MS^n mode (smart fragmentation) using a ramping of the collision energy. Maximum fragmentation amplitude was set to 1 V, starting at 30% and ending at 200%. MS operating conditions (negative mode) had been optimized using 5-caffeoylquinic acid with a capillary temperature of 365 °C, a dry gas flow rate of 10 L/min, and a nebulizer pressure of 10 psi. High-resolution LC-MS was carried out using the same HPLC equipped with a MicrOTOF Focus mass spectrometer (Bruker Daltonics, Bremen, Germany) fitted with an ESI source, and internal calibration was achieved with 10 mL of 0.1 M sodium formate solution injected through a six-port valve prior to each chromatographic run. Calibration was carried out using the enhanced quadratic mode.

HPLC. Separation was achieved on a 150 × 3 mm i.d. column containing diphenyl 5 μm, with a 5 mm × 3 mm i.d. guard column (Varian, Darmstadt, Germany). Solvent A was water/formic acid (1000:0.005 v/v) and solvent B was methanol. Solvents were delivered at a total flow rate of 500 μL/min. The gradient profile was from 10 to 70% B linearly in 60 min followed by 10 min isocratic and a return to 10% B at 90 and 10 min isocratic to re-equilibrate.

Synthesis of Mixture of Regioisomers of Sinapoylquinic Acid (13−15 and 70). To a solution of quinic acid (100 mg, 0.52 mmol) and DMAP (8 mg, 0.06 mmol) in CH_2Cl_2 (10 mL) were added pyridine (4 mL) and 4-acetylsinapic acid chloride (147 mg, 0.52 mmol) at room temperature. The reaction mixture was stirred for 12 h and acidified with 1 M HCl (pH ≈3). The layers were separated, and the aqueous phase was re-extracted with CH_2Cl_2 (3 × 20 mL). The combined organic layers were dried over Na_2SO_4 and filtered, and the solvents were removed in vacuo. The resulting crude esters were dissolved in a mixture of 20 mL of trifluoroacetic acid and water (7:3) at room temperature and stirred for 30 min. The solvents were removed in vacuo, and the resulting reddish product was analyzed by HPLC-MS.

Synthesis of Mixture of Regioisomers of (3,4,5-Trimethoxycinnamoyl)quinic Acid (71−74). 3,4,5-Trimethoxycinnamic acid (500 mg, 2.1 mmol) was suspended in toluene (15 mL) containing 3 drops of DMF, and oxalyl chloride (480 mg, 3.78 mmol) was added at 0 °C. The suspension was stirred for 10 h at room temperature, and a clear yellowish solution was formed. Toluene and unreacted oxalyl chloride were removed in vacuo. The yellow residue was washed with petroleum ether and dried in vacuo to give 3,4,5-trimethoxycinnamic acid chloride (511 mg, 1.99 mmol, 95%). To a solution of quinic acid (100 mg, 0.52 mmol) and DMAP (8 mg, 0.06 mmol) in CH_2Cl_2 (10 mL) were added pyridine (4 mL) and 3,4,5-trimethoxycinnamic acid chloride (134 mg, 0.52 mmol) at room temperature. The reaction mixture was stirred for 12 h at room temperature and acidified with 1 M HCl (pH ≈3). The layers were separated, and the aqueous phase was re-extracted with CH_2Cl_2 (3 × 20 mL). The combined organic layers were dried over Na_2SO_4 and filtered, and the solvents were removed in vacuo. The resulting esters were analyzed by HPLC.

RESULTS AND DISCUSSION

Preliminary Assessment of Data. All data for chlorogenic acids presented in this paper 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. In this study a selection of six different samples of green Robusta coffee beans were analyzed covering a selection of growing areas and countries of origin. Chlorogenic acids were isolated as described previously (*8*) using a Soxhlet extraction followed by protein precipitation and removal.

In a first set of experiments the Robusta coffee extracts were analyzed using a slightly modified HPLC method from the one previously reported, in which the stationary phase phenylhexyl packing was exchanged for a diphenyl packing and in the mobile phase acetonitrile was replaced with methanol. Using these modified conditions chromatograms were produced using a high-resolution TOF mass detector. The Robusta coffee extract gave a typical chromatogram, in which the 37 chlorogenic acids previously reported in green Arabica beans were readily located according to their m/z ratio (*5*). All 37 chlorogenic acids shown in **Table 1** could be identified according to their high-resolution mass data in the negative ion mode and using tandem LC-MS.

Table 1. High-Resolution Mass (MS-TOF) Data of Chlorogenic Acids and Their Parent Ions (M − H)

no.	name	abbrev	mol formula	theor *m/z* (M − H)	exptl *m/z* (M − H)	error (ppm)
1	3-*O*-caffeoylquinic acid	3-CQA	$C_{16}H_{18}O_9$	353.0878	353.0881	−0.7
2	4-*O*-caffeoylquinic acid	4-CQA	$C_{16}H_{18}O_9$	353.0878	353.0884	−1.6
3	5-*O*-caffeoylquinic acid	5-CQA	$C_{16}H_{18}O_9$	353.0878	353.0892	−3.9
4	3-*O*-feruloylquinic acid	3-FQA	$C_{17}H_{20}O_9$	367.0929	367.1047	−3.4
5	4-*O*-feruloylquinic acid	4-FQA	$C_{17}H_{20}O_9$	367.0929	367.1038	−0.8
6	5-*O*-feruloylquinic acid	5-FQA)	$C_{17}H_{20}O_9$	367.0929	367.1045	−2.9
7	3-*O*-*p*-coumaroylquinic acid	3-*p*CoQA	$C_{16}H_{18}O_8$	337.0929	337.0931	−0.5
8	4-*O*-*p*-coumaroylquinic acid	4-*p*CoQA	$C_{16}H_{18}O_8$	337.0929	337.0921	2.4
9	5-*O*-*p*-coumaroylquinic acid	5-*p*CoQA	$C_{16}H_{18}O_8$	337.0929	337.0921	2.4
10	3-*O*-dimethoxycinnamoylquinic acid	3-DQA	$C_{18}H_{22}O_9$	381.1191	381.1202	−2.8
11	4-*O*-dimethoxycinnamoylquinic acid	4-DQA	$C_{18}H_{22}O_9$	381.1191	381.1191	−2.5
12	5-*O*-dimethoxycinnamoylquinic acid	5-DQA	$C_{18}H_{22}O_9$	381.1191	381.1202	−2.8
13	3-*O*-sinapoylquinic acid	3-SiQA	$C_{18}H_{22}O_{10}$	397.1140	397.1125	3.8
14	4-*O*-sinapoylquinic acid	4-SiQA	$C_{18}H_{22}O_{10}$	397.1140	397.1150	−2.5
15	5-*O*-sinapoylquinic acid	5-SiQA	$C_{18}H_{22}O_{10}$	397.1140	397.1140	−4.9
16	3,4-di-*O*-caffeoylquinic acid	3,4-diCQA	$C_{25}H_{24}O_{12}$	515.1195	515.1190	1.0
17	3,5-di-*O*-caffeoylquinic acid	3,5-diCQA	$C_{25}H_{24}O_{12}$	515.1195	515.1172	4.5
18	4,5-di-*O*-caffeoylquinic acid	4,5-diCQA	$C_{25}H_{24}O_{12}$	515.1195	515.1170	4.9
19	3,4-di-*O*-feruloylquinic acid	3,4-diFQA	$C_{27}H_{28}O_{12}$	543.1508	543.1512	−0.8
20	3,5-di-*O*-feruloylquinic acid	3,5-diFQA	$C_{27}H_{28}O_{12}$	543.1508	543.1514	−1.1
21	4,5-di-*O*-feruloylquinic acid	4,5-diFQA	$C_{27}H_{28}O_{12}$	543.1508	543.1539	−3.4
25	3-*O*-feruloyl-4-*O*-caffeoylquinic acid	3F-4CQA	$C_{26}H_{26}O_{12}$	529.1351	529.1343	1.7
26	3-*O*-caffeoyl-4-*O*-feruloylquinic acid	3C-4FQA	$C_{26}H_{26}O_{12}$	529.1351	529.1351	−0.1
27	3-*O*-feruloyl-5-*O*-caffeoylquinic acid	3F-5CQA	$C_{26}H_{26}O_{12}$	529.1351	529.1373	−4.0
28	3-*O*-caffeoyl-5-*O*-feruloylquinic acid	3C-5FQA	$C_{26}H_{26}O_{12}$	529.1351	529.1367	−3.0
29	4-*O*-feruloyl-5-*O*-caffeoylquinic acid	4F-5CQA	$C_{26}H_{26}O_{12}$	529.1351	529.1351	0.1
30	4-*O*-caffeoyl-5-*O*-feruloylquinic acid	4C-5FQA	$C_{26}H_{26}O_{12}$	529.1351	529.1349	0.5
31	3-*O*-dimethoxycinnamoyl-4-*O*-caffeoylquinic acid	3D-4CQA	$C_{27}H_{28}O_{12}$	543.1508	543.1488	3.6
32	3-*O*-dimethoxycinnamoyl-5-*O*-caffeoylquinic acid	3D-5CQA	$C_{27}H_{28}O_{12}$	543.1508	543.1491	3.1
33	4-*O*-dimethoxycinnamoyl-5-*O*-caffeoylquinic acid	4D-5CQA	$C_{27}H_{28}O_{12}$	543.1508	543.1526	−3.4
34	3-*O*-dimethoxycinnamoyl-4-*O*-feruloylquinic acid	3D-4FQA	$C_{27}H_{28}O_{12}$	543.1508	543.1508	−4.1
35	3-*O*-dimethoxycinnamoyl-5-*O*-feruloylquinic acid	3D-5FQA	$C_{27}H_{28}O_{12}$	543.1508	543.1515	−1.4
36	4-*O*-dimethoxycinnamoyl-5-*O*-feruloylquinic acid	4D-5FQA	$C_{27}H_{28}O_{12}$	543.1508	543.1525	−3.1
37	3-*O*-p-coumaroyl-4-*O*-caffeoylquinic acid	3*p*Co-4CQA	$C_{25}H_{24}O_{11}$	499.1246	499.1227	3.7
38	3-*O*-caffeoyl-4-*O*-*p*-coumaroylquinic acid	3C-4*p*CoQA	$C_{25}H_{24}O_{11}$	499.1246	499.1247	−0.2
39	3-*O*-*p*-coumaroyl-5-*O*-caffeoylquinic acid	3*p*Co-5CQA	$C_{25}H_{24}O_{11}$	499.1246	499.1248	−0.5
40	3-*O*-caffeoyl-5-*O*-*p*-coumaroylquinic acid	3C-5*p*CoQA	$C_{25}H_{24}O_{11}$	499.1246	499.1247	−0.2
41	4-*O*-caffeoyl-5-*O*-*p*-coumaroylquinic acid	4C-5*p*CoQA	$C_{25}H_{24}O_{11}$	499.1246	499.1246	−4.9
42	4-*O*-*p*-coumaroyl-5-*O*-caffeoylquinic acid	4*p*Co-5CQA	$C_{25}H_{24}O_{11}$	499.1246	499.1249	−0.6
43	3-*O*-*p*-coumaroyl-4-*O*-feruloylquinic acid	3*p*Co-4FQA	$C_{26}H_{26}O_{11}$	513.1402	513.1389	2.6
44	3-*O*-*p*-coumaroyl-5-*O*-feruloylquinic acid	3*p*Co-5FQA	$C_{26}H_{26}O_{11}$	513.1402	513.1141	−2.9
45	4-*O*-*p*-coumaroyl-5-*O*-feruloylquinic acid	4*p*Co-5FQA	$C_{26}H_{26}O_{11}$	513.1402	513.1406	−0.7
49	3-*O*-sinapoyl-5-*O*-caffeoylquinic acid	3Si-5CQA	$C_{27}H_{28}O_{13}$	559.1457	559.1481	−4.2
50	3-*O*-sinapoyl-4-*O*-caffeoylquinic acid	3Si-4CQA	$C_{27}H_{28}O_{13}$	559.1457	559.1472	−2.6
51	3-*O*-(3,5-dihydroxy-4-methoxy)cinnamoyl-4-*O*-feruloylquinic acid	3DM-4FQA	$C_{27}H_{28}O_{13}$	559.1457	559.1458	−0.2
52	4-*O*-sinapoyl-3-*O*-caffeoylquinic acid	4Si-3CQA	$C_{27}H_{28}O_{13}$	559.1457	559.1457	0.9
53	3-*O*-sinapoyl-5-*O*-feruloylquinic acid	3Si-5FQA	$C_{28}H_{30}O_{13}$	573.1614	573.1641	−4.7
54	4-*O*-sinapoyl-5-*O*-feruloylquinic acid	4Si-5FQA	$C_{28}H_{30}O_{13}$	573.1614	573.1599	−2.5
55	4-*O*-sinapoyl-3-*O*-feruloylquinic acid	4Si-3FQA	$C_{28}H_{30}O_{13}$	573.1614	573.1634	−3.5
56	4-*O*-trimethoxycinnamoyl-5-*O*-caffeoylquinic acid	4T-5CQA	$C_{28}H_{30}O_{13}$	573.1614	573.1611	0.4
57	3-*O*-trimethoxycinnamoyl-5-*O*-caffeoylquinic acid	3T-5CQA	$C_{28}H_{30}O_{13}$	573.1614	573.1623	−1.7
58	3-*O*-trimethoxycinnamoyl-5-*O*-feruloylquinic acid	3T-5FQA	$C_{29}H_{32}O_{13}$	587.1770	587.1748	3.8
59	3-*O*-trimethoxycinnamoyl-4-*O*-feruloylquinic acid	3T-4FQA	$C_{29}H_{32}O_{13}$	587.1770	587.1766	0.7
60	4-*O*-trimethoxycinnamoyl-5-*O*-feruloylquinic acid	4T-5FQA	$C_{29}H_{32}O_{13}$	587.1770	587.1764	1.0
61	3-*O*-dimethoxycinnamoyl-4-*O*-feruloyl-5-*O*-caffeoylquinic acid	3D-4F-5CQA	$C_{37}H_{36}O_{15}$	719.1981	719.2001	−2.7
62	3,4,5-tri-*O*-caffeoylquinic acid	3,4,5-triCQA	$C_{34}H_{29}O_{15}$	677.1512	677.1522	−3.5
63	3,5-di-*O*-caffeoyl-4-*O*-feruloylquinic acid	3,5-diC-4FQA	$C_{35}H_{31}O_{15}$	691.1668	691.1647	3.1
64	3-*O*-feruloyl-4,5-di-*O*-caffeoylquinic acid	3F-4,5-diCQA	$C_{35}H_{31}O_{15}$	691.1668	691.1711	−6.2[a]
65	3,4-di-*O*-caffeoyl-5-*O*-feruloylquinic acid	3,4-diC-5FQA	$C_{35}H_{31}O_{15}$	691.1668	691.1647	3.1
66	3-*O*-caffeoyl-4,5-di-*O*-feruloylquinic acid	3C-4,5-diFQA	$C_{36}H_{33}O_{15}$	705.1825	705.1851	−3.8
67	3,4-di-*O*-feruloyl-5-*O*-caffeoylquinic acid	3,4-diF-5CQA	$C_{36}H_{33}O_{15}$	705.1825	705.1833	−1.1
68	3,4-di-*O*-caffeoyl-5-*O*-sinapoylquinic acid	3,4-diC-5SiQA	$C_{36}H_{33}O_{16}$	721.1774	721.1795	−2.9
69	3-*O*-sinapoyl-4,5-di-*O*-caffeoylquinic acid	3Si-4,5-diCQA	$C_{36}H_{33}O_{16}$	721.1774	721.1766	1.1

[a] In 10 chromatographic runs it was the lowest error and reason for this higher value might be lower concentration of the compound in extract. All MS^n data were in agreement on the presence of compound **64**.

The mass error was typically under 5 ppm, confirming the molecular formulas of the chlorogenic acids from Robusta coffee previously assigned (*5*−*8*, *12*). All 37 previously reported chlorogenic acids could be readily identified in all five samples of green

Table 2. Negative Ion MS^2 and MS^3 Fragmentation Data for Monoacyl Sinapoylquinic Acids (**13**−**15** and **70**) and Trimethoxycinnamoylquinic Acids (**71**−**74**)

		MS^1	MS^2					MS^3						
			base peak	secondary peak				base peak	secondary peak					
no.	compd	parent ion	*m/z*	*m/z*	int	*m/z*	int	*m/z*	*m/z*	int	*m/z*	int	*m/z*	int
13	3-SiQA	397.0	222.9	164.0	13	149.0	10	163.9	178.9	15	148.9	15		
14	4-SiQA	397.0	172.9	222.9	12			93.3	111.1	62	71.6	58	154.9	22
15	5-SiQA	397.0	190.9	222.9	5			127.0	172.9	70	93.3	60	85.4	59
70	1-SiQA	397.0	222.9	172.9	21	190.7	7	207.9	178.9	32	163.9	31		
71	1-TQA	411.1	172.9	143.0	20	236.9	10	143.0	155.0	50	111.2	60	93.3	43
72	3-TQA	411.0	236.9					133.0	221.9	50	192.9	40	103.3	23
73	5-TQA	411.0	172.9					93.0	155.0	20	111.1	45	61.8	5
74	4-TQA	411.1	172.9	236.9	21			93.0	155.0	15	111.1	49	71.6	13

Robusta beans analyzed with some noticeable differences in intensities. A detailed discussion about these differences is outside the scope of this paper and requires a full principal component analysis (see the Supporting Information for a listing of the CGAs found in the Robusta beans).

In a second round of experiments using the same chromatographic conditions, tandem MS spectra were recorded in the negative ion mode using an ion trap MS detector. Selected ion monitoring and analysis of the individual tandem MS spectra again revealed and confirmed the presence of those 37 chlorogenic acids in all six green Robusta coffee bean samples.

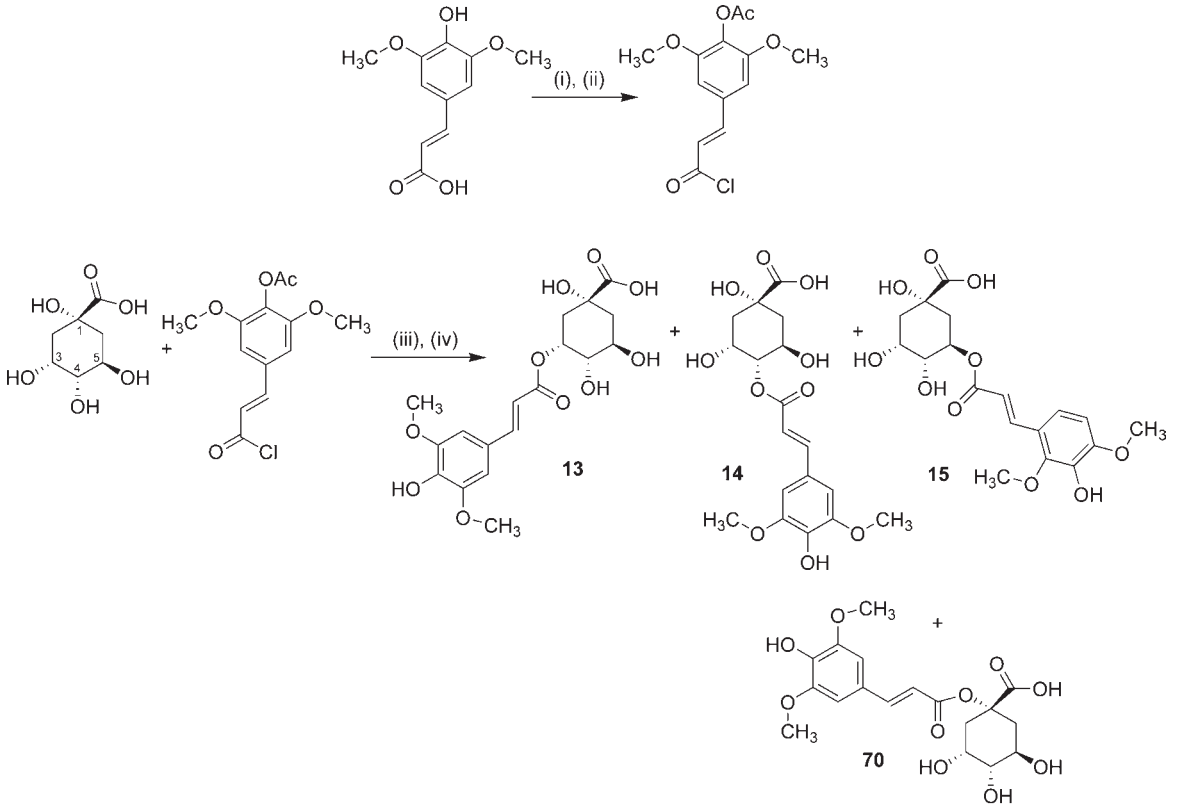
In general, new CGAs can be identified in an All MS^n EIC (extracted ion chromatogram) by their unique fragments at *m/z* 173 and 191. Selected ion monitoring at *m/z* 397, 559, 573, 588, and 719 immediately located 16 chromatographic peaks eluting between 23 and 60 min, each with a UV spectrum typical of chlorogenic acids (λ_{max} 320 nm). However, because the quinic acid moiety is not acylated at C1 in coffee beans and produces only three caffeoylquinic acids (**1**−**3**), three feruloylquinic acids (**4**−**6**), and three *p*-coumaroylquinic acid (**7**−**9**), only three sinapoylquinic acids (**13**−**15**) and three trimethoxycinnamoylquinic acids (**72**−**74**) were expected. During this study we did not observe any monoacyl trimethoxycinnamoylquinic acid isomers. Three peaks produced MS^2 fragment ions at either *m/z* 173 or 191, 12 peaks produced MS^3 fragment ions at *m/z* 173 or 191, and a further peak produced MS^4 fragment ions at *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, 219, or 233 confirmed that none of these substance were derivative of alkyl quinate. Five of the 16 peaks produced MS^2 ions at *m/z* 397 ([sinapoylquinic acid − H^+]$^-$) and 379 ([sinapoylquinic acid − H_2O − H^+]$^-$) and MS^3 ions at *m/z* 223 ([sinapic acid − H^+]$^-$) analogous to those produced by dicaffeoylquinic acids, caffeoyl-feruloylquinic acids, dimethoxycinnamoyl-feruloylquinic acid, and *p*-coumaroyl-feruloylquinic acids (*5*, *7*, *8*, *12*).

The other two peaks produced MS^2 ions at *m/z* 353 and 349 and MS^3 ions at *m/z* 173 and 193. The MS^2 ion at *m/z* 353 and the MS^3 ion at *m/z* 173 are characteristic (*5*) of caffeic acid derived diacyl chlorogenic acids and can be assigned as [caffeoylquinic acid − H^+]$^-$, and the MS^2 ion at *m/z* 349 and MS^3 ion at *m/z* 193 are characteristic of ferulic acid derived diacyl chlorogenic acids and can be assigned as [sinapoylquinic acid − H_2O − CH_2O − H^+]$^-$. The remaining ions were tentatively assigned to a similar series of fragments 30 amu larger than the ferulic acid related fragments (46 amu larger than the caffeic acid related fragments), strongly suggesting that they might have one more methyl ether group at position 5 of the cinnamic acid residue, that is, 5-*O*-methyl ether of ferulic acid (sinapic acid). Those compounds of M_r 574 produced MS^2 ions at *m/z* 379 and compounds of M_r 560 produced MS^2 ions at *m/z* 349 were tentatively assigned as feruloyl-sinapoylquinic acids and caffeoyl-sinapoylquinic acids, respectively.

Another five peaks produced MS^2 ions at *m/z* 411 ([trimethoxycinnamoylquinic acid − H^+]$^-$) and MS^3 ions at *m/z* 237 ([trimethoxycinnamic acid − H^+]$^-$), analogous to those produced by caffeoyl-feruloylquinic acids, dimethoxycinnamic acids, and *p*-coumaroyl-feruloylquinic acids (*5*, *7*, *8*, *12*). The other two peaks produced MS^2 ion at *m/z* 349 and MS^3 ion at *m/z* 193. The MS^2 ion at *m/z* 349 and the MS^3 ion at *m/z* 193 are characteristic (*5*) of ferulic acid derived diacyl chlorogenic acids and can be assigned as [feruloylquinic acid − H_2O − H^+]$^-$. The remaining ions were tentatively assigned to similar series of fragments 30 amu larger than the dimethoxycinnamic acid, strongly suggesting that they might have one more methyl ether group at position 5 of the cinnamic acid, that is, 5-*O*-methyl ether of 3,4-dimethoxycinnamic acid (trimethoxycinnamic acid). Those compounds of M_r 573 produced MS^2 ions at *m/z* 393, 353, and 335 and compounds of M_r 587 produced MS^2 ion at *m/z* 349 were tentatively assigned as caffeoyl-trimethoxycinnamoylquinic acids and feruloyl-trimethoxycinnamic acids, respectively. It is conceivable that a feruloyl-sinapoylquinic acid, a caffeoyl-sinapoylquinic acid, and a feruloyl-trimethoxycinnamic acid might produce ions at *m/z* 379 or 349, and there can be no doubt that all three series of chlorogenic acids were present.

Characterization of Putative Sinapoylquinic Acids (M_r 398). The Robusta extract contained three minor components with molecular ions at *m/z* 397 that eluted between 23 and 30 min. All three peaks had UV spectra typical of chlorogenic acids. MS^2 data are presented in **Table 2**.

Because the monoacyl chlorogenic acids examined on diphenyl packing elute in the sequence 3-acyl, 5-acyl, and 4-acyl, the first sinapoylquinic acid was tentatively assigned as the 3-isomer (**13**). The MS^2 base peak at *m/z* 223 ([sinapoylquinic acid − quinic acid − H^+]$^-$), which subsequently decarboxylates and demethylates at MS^3 (concentration was below the limits of detection but compared with the MS^4 of diacyl CGAs), is analogous behavior to 3-feruloylquinic acid (**4**) and 3-dimethoxycinnamoylquinic acid (**10**) (*5*, *7*, *8*). The most strongly retained of the sinapoylquinic acid isomers has a fragmentation characteristic of a 4-acyl chlorogenic acid (MS^2 and MS^3 base peaks at *m/z* 173 and 93, respectively, which were not in the range of the limit of detection) and can be tentatively assigned as 4-sinapoylquinic acid (**14**). The third isomer has fragmentation characteristic of a 5-acyl chlorogenic acid (MS^2 at *m/z* 191) and the retention time is between those of 3- and 4-acylated sinapoylquinic acid, which strongly suggest 5-sinapoylquinic acid (**15**). Further evidence for the assignment of monoacyl sinapoylquinic acids (**13**−**15**) came from an independent synthesis of a mixture of all four possible regioisomers of sinapoylquinic acid (**Figure 2**). A direct comparison of the chromatograms of the synthetic mixture and coffee bean extracts allows an ambiguous confirmation of the presence of sinapoylquinic acids (**13**−**15**) by comparison of retention times, UV−vis data, and tandem mass spectra (**Figure 3** and **Table 2**).



Reagents and conditions : (i) Pyridine, Ac_2O; (ii)Toluene, DMF, $(COCl)_2$; (iii) DMAP, CH_2Cl_2, pyridine, rt, 8h; (iv) TFA, H_2O (7:3), 30 min.

Figure 2. Synthesis of mixture of regioisomers of sinapoylquinic acid (**13**−**15** and **70**).

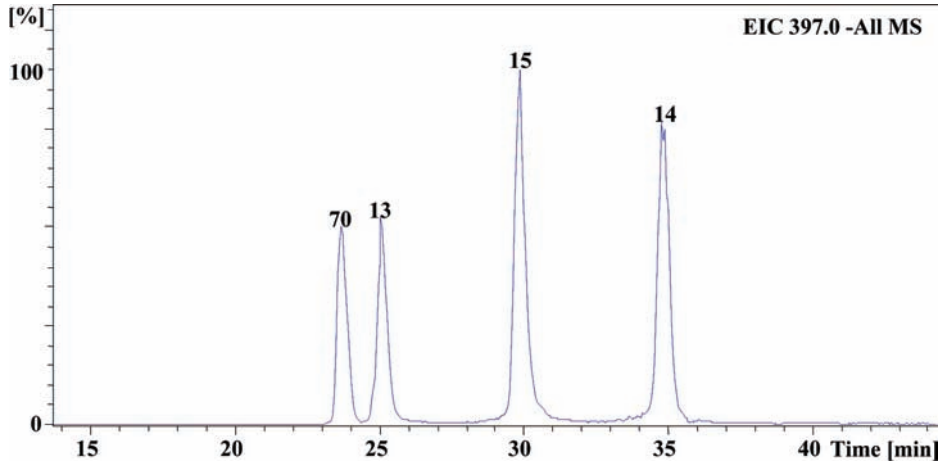


Figure 3. Extracted ion chromatogram (EIC) at *m/z* 397 (compounds 13−15 and 70) in negative ion mode.

Characterization of Putative Caffeoyl-sinapoylquinic Acids (M_r 560). Of the four peaks that yielded molecular ions at *m/z* 559, two produced an MS^2 base peak at *m/z* 397 with an MS^3 base peak at *m/z* 223, suggestive of caffeoyl-sinapoylquinic acids. The third peak showed an MS^2 base peak at *m/z* 349 followed by an MS^3 base peak at *m/z* 192.8, and the fourth peak produced an MS^2 base peak at *m/z* 353. Theoretically, six caffeoyl-sinapoylquinic acid isomers would have been expected, as seen previously (*5*) for the caffeoyl-feruloylquinic acids. **Table 3** compares the fragmentation patterns of the three putative caffeoyl-sinapoylquinic acids with data previously obtained for the six caffeoyl-feruloylquinic acids (*5*). 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 at C5 was the most easily eliminated, whereas that at C4 was the most stable and that at C3 was 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

Table 3. Negative Ion MS^2, MS^3, and MS^4 Fragmentation Data for the Dimethoxycinnamoyl-caffeoylquinic Acids, Dimethoxycinnamoyl-feruloylquinic Acid, Caffeoyl-sinapoylquinic Acids, Feruloyl-sinapoylquinic Acids, Trimethoxycinnamoyl-caffeoylquinic Acids, and Trimethoxycinnamoyl-feruloylquinic Acids

		MS^1	MS^2							MS^3							MS^4				
			base peak	secondary peak						base peak	secondary peak						base peak	secondary peak			
no.	compd	parent ion	*m/z*	*m/z*	int	*m/z*	int	*m/z*	int	*m/z*	*m/z*	int	*m/z*	int	*m/z*	int	*m/z*	*m/z*	int	*m/z*	int
31	3D-4CQA	543.0	381.1	335.1	92	363.1	4	298.7	12	207.1	173.3	10					149.1				
32	3D-5CQA	543.0	381.1	335.1	2					207.1	173.3	25					149.0	162.7	80	133.1	22
33	4D-5CQA	543.0	381.0	335.1	2					173.1	207.0	55	191.0	32	135.1	9	93.2	155.1	20	111.1	35
34	3D-4FQA	557.0	349.1			363.1	75			172.9	178.9	76	190.9	9	135.0	19	93.1			111.0	20
35	3D-5FQA	557.0	381.2	349.2	75					175.0	193.0	80	269.0	55	313.0	25	160.1				
36	4D-5FQA	557.0	381.2	349.2	30					207.1	173.3	47					93.1	155.1	10	111.1	40
49	3Si-5CQA	559.1	397.1	335.2	5	375.0	20			222.8							163.7	148.9	20		
50	3Si-4CQA	559.1	353.1	334.9	35	378.9	28	397.0	60												
51	3C-4SiQA	559.1	349.0	333.0	16	490.7	20	521.0	42	192.8	172.6	45	149.0	60	154.6	50	93.2	111.1	40		
52	4Si-5CQA	559.1	397.1							172.9	222.8	99	149.0	5			98.9	83.2	36		
53	3Si-5FQA	573.1	397.0	349.1	40					222.8							163.8	148.7	36		
54	3F-4SiQA	573.1	379.0	349.1	60	367.0	18	397.0	40	222.8	204.8	90	166.8	38	283.8	60					
55	4Si-5FQA	573.1	397.1	349.1	8	379.0	5			172.7	222.8	60					93.2	155.0	12	111.0	10
56	4T-5CQA	573.1	411.1	236.9	10	434.8	55	502.7		172.8	236.8	70									
57	3T-5CQA	573.1	411.0	236.9	30	379.0	5	535.1	8	236.8	172.8	20					132.8	221.8	45		
58	3T-5FQA	587.1	411.0	348.9	65	236.9	20	172.9	10	236.9	172.9	10					133.1				
59	3T-4FQA	587.2	348.9	411.1	45	393.0	55	236.9	65	192.9	268.9	20	172.9	42							
60	4T-5FQA	587.2	411.0	348.9	30	236.9	17	172.9	12	93.0	111.0	62									

(*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*, *12*, *13*) it should be expected that the first caffeoyl-sinapoylquinic acid to elute would be one of the 3,4-caffeoyl-sinapoylquinic acid isomers. However, we observed earliest elution of 3-sinapoyl-5-caffeoylquinic acid, which loses its caffeoyl residue before its sinapoyl residue. It has been shown that a caffeoyl residue at C5 is easily eliminated (*5*). Such an elimination would produce [3-sinapoylquinic acid $- H^+]^-$ as the MS^2 base peak, and as a argued above for **49**, the MS^3 base peak at *m/z* 223 is consistent with such an assignment, suggesting that the first eluting caffeoyl-sinapoylquinic acid is 3-sinapoyl-5-caffeoylquinic acid (**49**). Its fragmentation pattern more closely resembles 3-feruloyl-5-caffeoylquinic acid (**27**) than 3-caffeoyl-5-feruloylquinic acid (**28**).

The next eluting caffeoyl-sinapoylquinic acid loses its two cinnamoyl residues with nearly equal facility (slightly more favoring the sinapoyl residue) and produces strong dehydrated ions at *m/z* 335 and 379, thus closely resembling 3-feruloyl-4-caffeoylquinic acid (**25**) (**Table 3**) (*5*). Its MS^3 spectrum was not detected and tentatively assigned as 3-sinapoyl-4-caffeoylquinic acid (**50**).

The fragmentation of the third eluting isomer (**51**) produced an MS^2 base peak at *m/z* 349, an MS^3 base peak at *m/z* 193, and an MS^4 base peak at *m/z* 134, which we consider fingerprint ions of feruloylquinic acids. An MS^3 secondary peak at *m/z* 173 (80% of base peak), which is consistent with a 4-substituted chlorogenic acid, was observed (*8*). The MS^2 and MS^3 spectra indicate the presence of a feruloyl substituent at C4 rather than a caffeoyl or sinapoyl substituent (*8*). Two secondary peaks at *m/z* 269 and 305 are characteristic for a diacyl CGA containing a feruloyl substituent at C4, that is, 3,4-acylated CGA (*8*). The MS^2 base peak at *m/z* 349 ([diacyl CGA $- H_2O$ − cinnamoyl $- H^+]^-$) is due to the loss of H_2O (18 amu) and one cinnamoyl residue (192 amu). This identifies the second acyl group as a methoxy-dihydroxy cinnamic acid residue. There are two possible regiochemistries to consider, 4-methoxy **A** or 3-methoxy **B**. In the absence of further evidence in favor of either structure **A** or **B**, structure **B** was considered more likely because it would represent a logical biosynthetic precursor for 3Si-4FQA, and **51** was tentatively assigned as 4-feruloyl-3-(3,4-dihydroxy-3-methoxy)cinnamoylquinic acid. To the best of our knowledge this hydroxycinnamoyl substituent is reported here for the first time in nature.

The most hydrophobic isomer has the characteristic fragmentation 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 sinapoyl residue [MS^2 base peak at *m/z* 397 and a strong MS^3 secondary ion (99% of base peak) at *m/z* 223]. The MS^2 base peak must be either [4-sinapoylquinic acid $- H^+]^-$ or [5-sinapoylquinic acid $- H^+]^-$, and the subsequent fragmentation to the dehydrated ion *m/z* 173 indicates [4-sinapoylquinic acid $- H^+]^-$ and is thus tentatively assigned as 3-caffeoyl-4-sinapoylquinic acid (**52**). The absence of the MS^2 ions at *m/z* 299 and 255, suggesting that **52** does not have a caffeoyl residue at C4, is consistent. Its fragmentation pattern more closely resembles 3-caffeoyl-4-feruloylquinic acid (**26**) than 3-feruloyl-4-caffeoylquinic acid (**25**) (**Table 3**).

Characterization of Putative Feruloyl-sinapoylquinic Acids (M_r 574). The LC-MS data for the feruloyl-sinapoylquinic acids (**53**−**55**) are summarized in **Table 3**. The fragmentation pattern of the slowest eluting isomer (**55**) is identical to that of caffeoyl-sinapoylquinic acid **52** 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 tentatively assigned as 4-sinapoyl-3-feruloylquinic acid (**55**).

The preceding feruloyl-sinapoylquinic acid **54** resembles the analogous feruloyl-dimethoxycinnamoylquinic acid **34** (**Table 3**) in that both produce a dehydrated MS^2 base peak. On the basis of the above argument, feruloyl-sinapoylquinic acid was tentatively assigned as 4-sinapoyl-5-feruloylquinic acid (**54**).

The fragmentation of the most rapidly eluting feruloyl-sinapoylquinic acid (**53**) resembles the analogous caffeoyl-sinapoylquinic acid (**49**) in that both lose their caffeoyl or feruloyl residue more preferentially than their sinapoyl residue. Fragmentation of the MS^2 base peak (*m/z* 397) produces an MS^3 base peak at *m/z* 223 (**Table 3**), which is consistent with 3,5-caffeoyl-sinapoylquinic acid. Accordingly, it is assigned as 3-sinapoyl-5-feruloylquinic acid (**53**).

From our previous studies it has been proven that MS^n spectra of monoacyl CGAs are similar to MS^{n+1} spectra of corresponding

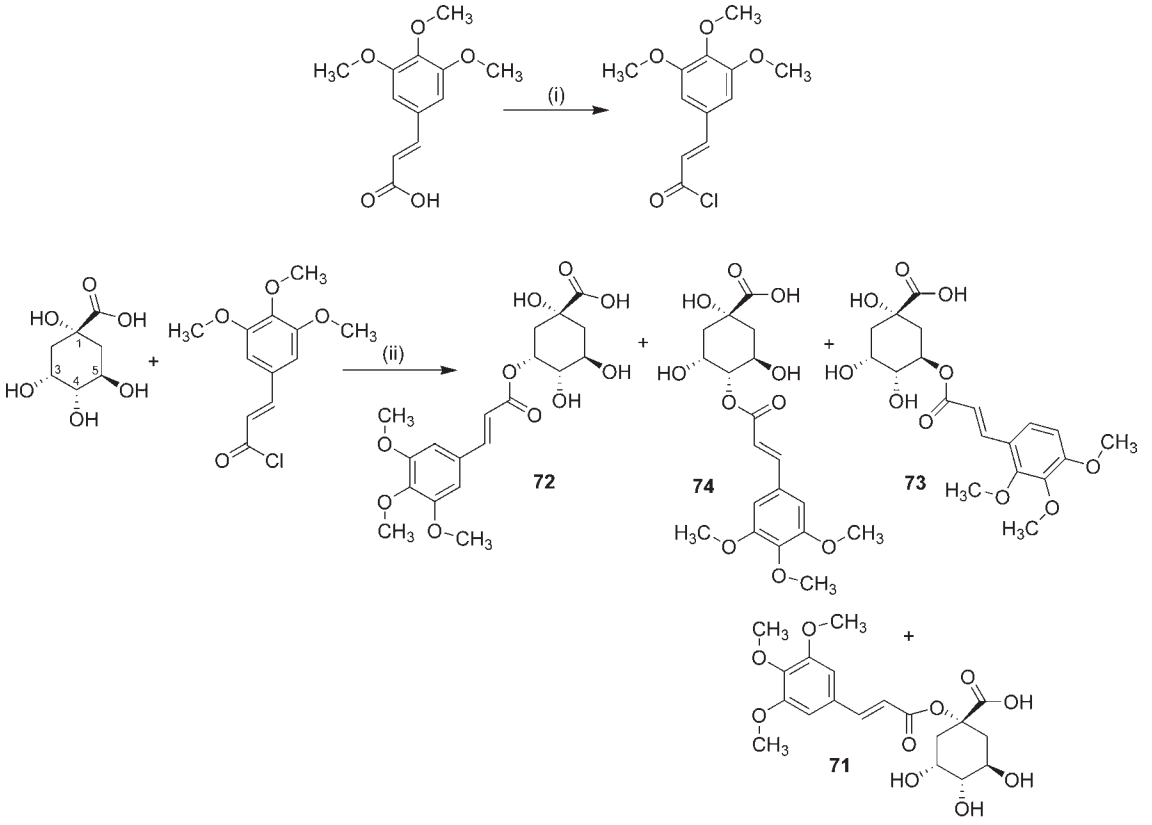


Figure 4. Synthesis of regioisomers of trimethoxycinnamoylquinic acid (**71**−**74**).

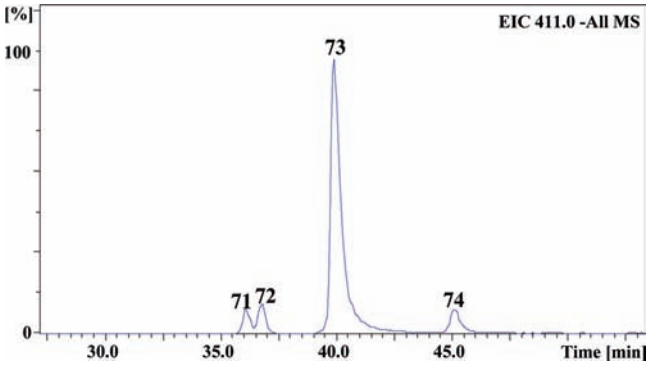


Figure 5. Extracted ion chromatogram (EIC) of synthetic trimethoxycinnamoylquinic acids at *m*/*z* 411 (negative ion mode).

diacyl CGAs. For the further identification and characterization of trimethoxycinnamic acid containing diacyl CGAs, a nonselective synthesis of a mixture of all four possible regioisomers of monoacyl trimethoxycinnamoylquinic acid (**63**−**66**) has been carried out (**Figure 4**). In the first step of synthesis, trimethoxycinnamic acid was converted into its acid chloride by using oxalyl chloride and DMF to give the acid chloride. This was subsequently reacted with quinic acid in the presence of pyridine and DMAP to yield a mixture of four regioisomers of trimethoxycinnamoylquinic acid (**63**−**66**). A fragmentation pattern and LC behavior of all four regioisomers of trimethoxycinnamoylquinic acid is discussed below.

LC-MS^n Characterization of Trimethoxycinnamoylquinic Acid Regioisomers (71−74). The monoacyl trimethoxycinnamic acids examined on diphenyl packing elute in the sequence 1-acyl, 3-acyl, 5-acyl, and 4-acyl trimethoxycinnamic acid (**Figure 5**). The second eluting isomer **72** produced an MS^2 base peak at *m*/*z* 237 ([trimethoxycinnamic acid − H^+]$^-$), which subsequently decarboxylates and demethylates at MS^3 (*m*/*z* 133) (**Figure 6** and **Table 2**) and is analogous behavior to 3-feruloylquinic acid (**4**) and 3-dimethoxycinnamic acid (**10**) (*5*, *7*, *8*). All three other isomers produced MS^2 base peaks at *m*/*z* 173 ([quinic acid − H_2O − H^+]$^-$), and MS^2 secondary peaks at *m*/*z* 237 ([trimethoxycinnamic acid − H^+]$^-$) (which is absent in 5-trimethoxycinnamoylquinic acid **73**). 4- (**74**) and 5-trimethoxycinnamic acid produced MS^3 base peaks at *m*/*z* 93, and 1-trimethoxycinnamoylquinic acid **71** produced MS^3 base peak at *m*/*z* 143.

Characterization of Putative Caffeoyl-trimethoxycinnamoylquinic Acids (M_r 574). Two peaks were detected at *m*/*z* 573, and their LC-MS data are summarized in **Table 3**. The first eluting isomer **56** produced an MS^2 base peak at *m*/*z* 411 by the loss of a caffeoyl residue ([trimethoxycinnamoylquinic acid − H^+]$^-$) and MS^3 base

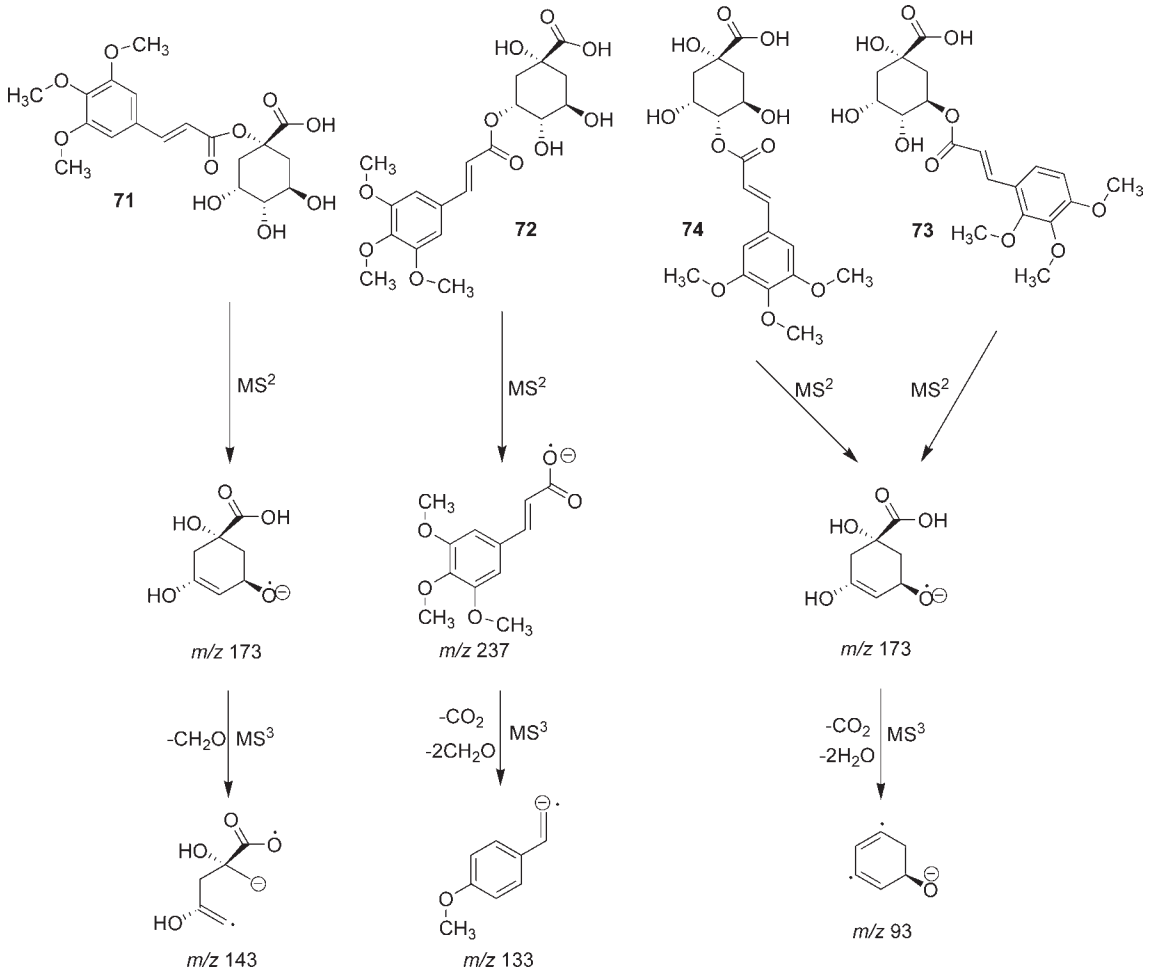


Figure 6. Fragmentation mechanism of trimethoxycinnamoylquinic acids (**71**−**74**).

peak at *m*/*z* 172.8 and MS^2 secondary peak at *m*/*z* 236.9 ([trimethoxycinnamic acid − H^+]$^-$). MS^2 and MS^3 data of **56** are similar to MS^2 data of 4-trimethoxycinnamoylquinic acid **74**, which confirms the presence of a trimethoxycinnamoyl residue at C-4 of the quinic acid moiety. The absence of an MS^2 secondary peak at *m*/*z* 353 ([caffeoylquinic acid − H^+]$^-$) or 335 ([caffeoylquinic acid − H_2O − H^+]$^-$) confirms the caffeoyl residue at C-5, and isomer **56** was tentatively assigned as 5-caffeoyl-4-trimethoxycinnamoylquinic acid. The next eluting isomer, **57**, produced an MS^2 base peak at *m*/*z* 411 by the loss of a caffeoyl residue ([trimethoxycinnamoylquinic acid − H^+]$^-$), an MS^3 base peak at *m*/*z* 236.8, and an MS^4 base peak at *m*/*z* 132.8. MS^3 and MS^4 spectra of **57** are similar to MS^2 and MS^3 spectra of 3-trimethoxycinnamoylquinic acid (**72**), which confirms the presence of a trimethoxycinnamoyl residue at C3. The caffeoyl residue should be at C5 because if it is at C4, then the MS^3 base peak will be at *m*/*z* 173 instead of 236.8. On the basis of the above arguments **57** was tentatively assigned as 3-trimethoxy-5-caffeoylquinic acid.

Characterization of Putative Feruloyl-trimethoxycinnamoylquinic Acids (M_r 588). The LC-MS data for the feruloyl-trimethoxycinnamoylquinic acids (**58**−**60**) are summarized in **Table 3**. The fragmentation of the slowest eluting isomer (**60**) is identical to that of feruloyl-sinapoylquinic acid **55** if allowance is made for the weak ferulic acid-derived ion at *m*/*z* 349 being replaced by the more intense peak, suggesting that logically it can be tentatively assigned as 4-trimethoxycinnamoyl-5-feruloylquinic acid.

The preceding feruloyl-trimethoxycinnamoylquinic acid **59** resembles the analogous feruloyl-sinapoylquinic acid **54** (**Table 3**) in that both produce a dehydrated MS^2 base peak. On the basis of the above argument, feruloyl-trimethoxycinnamoylquinic acid was tentatively assigned as 3-trimethoxycinnamoyl-4-feruloylquinic acid (**59**).

The fragmentation of the most rapidly eluting feruloyl-trimethoxycinnamoylquinic acid **58** resembles the analogous feruloyl-sinapoylquinic acid **53** in that both lose feruloyl residue more preferentially than sinapoyl or trimethoxycinnamoyl residue. Fragmentation of the MS^2 base peak (*m*/*z* 411) produces an MS^3 base peak at *m*/*z* 237 (**Table 3**), which is consistent with 3-sinapyl-5-feruloylquinic acid. Accordingly, it is assigned as 3-trimethoxycinnamoyl-5-feruloylquinic acid.

Characterization of Putative Triacylquinic Acids. Next to the monoacyl and diacyl quinic acid derivatives a series of triacyl quinic acids derivatives were identified by their characteristic quinic acid fragments at *m*/*z* 191 and 173 in the MS^4 tandem mass spectra. According to our hierarchical scheme for assignment of chlorogenic acid regiochemistry (*5*−*8*), the following assumptions were made to assign the regiochemistry of the triacyl quinic acid derivatives.

(1) All of the CGAs reported so far are not acylated at C-1 of the quinic acid moiety, which suggests the absence of C-1 acylation, so we consider only the presence of 3-,4-, and 5-acyl CGA.

(2) Loss of the acyl moiety occurs most readily from C-5. Hence, the fragments and neutral losses in MS^2 define the nature of the substituent at C-5.

(3) Loss of the acyl moiety occurs more reluctantly from C-3. Hence, the fragments and neutral losses in MS^3 define the nature of the substituent at C-3.

Table 4. Negative Ion MS^2, MS^3, and MS^4 Fragmentation Data for the Triacylquinic Acids **61**−**69**

		MS^1	MS^2					MS^3							MS^4				
			base peak	secondary peak				base peak	secondary peak						base peak	secondary peak			
no.	compd	parent ion	*m/z*	*m/z*	int	*m/z*	int	*m/z*	*m/z*	int	*m/z*	int	*m/z*	int	*m/z*	*m/z*	int	*m/z*	int
61	3D-4F-5CQA	719.3	557.1			349.1	5	349.0	381.0	25	304.9	5	268.8	10	174.7	192.9	85	149.0	25
62	3,4,5-triCQA	677.1	515.1	497.0	5	353.1	25	353.0	335.0	10	254.8	6	298.9	6	172.7	178.9	80	190.8	50
63	3,5diC-4FQA	691.1	529.1	335.0	28	367.0	19	367.0	334.9	15	172.7	55			172.7	192.7	40		
64	3F-4,5-diCQA	691.2	529.0	515.1	30			352.9	367.1	55	254.8	5			172.7	178.8	80	190.8	22
65	3,4-diC-5FQA	691.2	515.1	529.1	90	497.0	10	352.9	366.9	50	334.9	8	254.7	8	172.7	178.7	80	190.8	50
66	3C-4,5-diFQA	705.2	529.1	543.0	72	511.0	23	367.0	353.0	74	335.0	37			172.6	192.7	16		
67	3,4-diF-5CQA	705.2	543.0	529.0	50			349.0	367.0	42	381.0	20	172.7	26	192.7	268.7	53	312.9	82
68	3,4-diC-5SiQA	721.2	515.1	559.1	59			352.9	335.0	25	298.8	5	255.0	2	172.6	178.6	80	191.0	50
69	3Si-4,5-diCQA	721.2	559.1	515.1	23	298.9	8	353.0	335.0	10	172.7	26			172.7	178.6	35	190.6	70

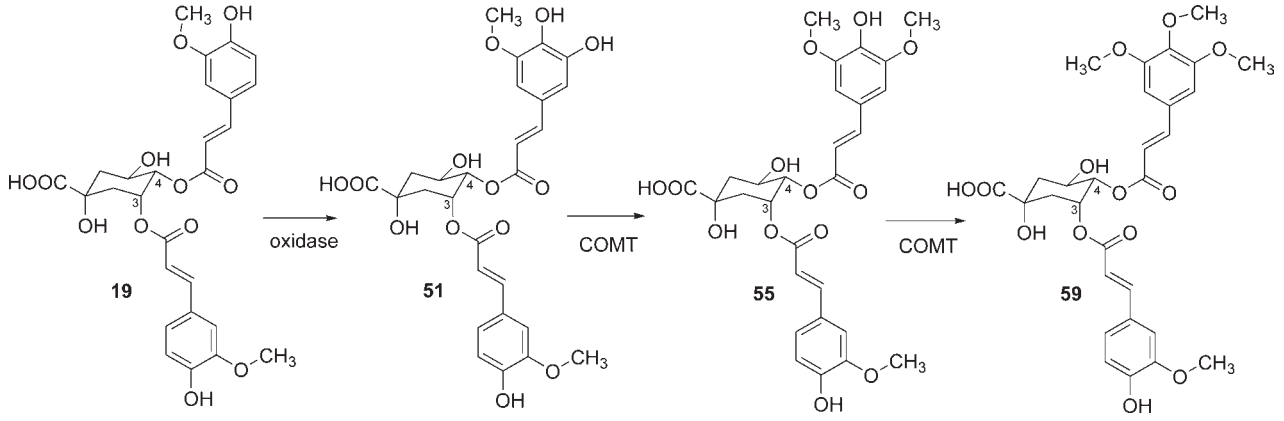


Figure 7. Proposed scheme for the biosynthesis of trimethoxycinnamoylquinic acids (COMT = catechol-*O*-methyl transferase).

(4) Loss of the acyl moiety occurs most reluctantly accompanied by loss of water from C-4. Hence, the fragments and neutral losses in MS^4 define the nature of the substituent at C-4.

Following identification of a total of nine triacyl quinic acids by all MS^n searches, the molecular formulas were confirmed (**Table 1**) according to their high-resolution mass data in the negative ion mode. The mass error was typically under 5 ppm, confirming the molecular formulas of the triacyl quinic acids. Selected ion monitoring at *m*/*z* 677, 691, 705, 719, and 721 immediately located nine chromatographic peaks eluting between 40 and 60 min, each with a UV spectrum typical of chlorogenic acids (λ_{max} 320 nm). Structures have been assigned on the basis of LC-MS^n patterns of fragmentation, and MS^n data are given in **Table 4**. This detailed analysis of MS^n data allowed the identification of 3-dimethoxycinnamoyl-4-feruloyl-5-caffeoylquinic acid (**61**; M_r 720); 3,4,5-tricaffeoylquinic acid (**62**; M_r 678); 3,5-dicaffeoyl-4-feruloylquinic acid (**63**); 3-feruloyl-4,5-dicaffeoylquinic acid (**64**), 3,4-dicaffeoyl-5-feruloylquinic acid (**65**; M_r 692); 3-caffeoyl-4,5-diferuloylquinic acid (**66**), 3,4-diferuloyl-5-caffeoylquinic acid (**67**; M_r 706); 3,4-dicaffeoyl-5-sinapoylquinic acid (**68**); and 3-sinapoyl-4,5-dicaffeoylquinic acid (**69**; M_r 722). Compounds **61** and **63**−**69** have been reported here for the first time in nature and for the first time from this source.

Missing Isomers. Because all six theoretically possible regioisomers of caffeoyl-feruloylquinic acids (**25**−**30**) are easily located in the Robusta coffee extract (*5*), it is a little surprising that only three caffeoyl-sinapoyl (**49**, **50**, and **52**), three feruloyl-sinapoylquinic acids (**53**−**55**), two caffeoyl-trimethoxycinnamoylquinic acids (**56** and **57**), and three feruloyl-trimethoxycinnamoylquinic acids (**58**−**60**) have been observed. This could reflect the failure of the coffee plant to synthesize all six isomers. However, all three sinapoylquinic acids are produced, which could be elaborated into the full set of diacyl derivatives. It is possible that these other isomers are present but below the limits of detection in our method. If that is the case, then the most concentrated of the "missing" caffeoyl-sinapoylquinic acids, feruloyl-sinapoylquinic acids, caffeoyl-trimethoxycinnamoylquinic acids, and feruloyl-trimethoxycinnamoylquinic acids must be present at concentrations considerably below that of the weakest isomer found, because analysis of deliberately (10 times) concentrated extracts did not allow their detection. A third possibility is that they are present at low concentration and not resolved chromatographically.

Intermediates in Biosynthesis of Trimethoxycinnamoyl Quinic Acid Derivatives. From the current study an interesting detail becomes apparent. For one full series of compounds (3-feruloyl 4-acyl quinic acids) a full series of possible biochemical intermediates have been observed, hence allowing the proposal of a biosynthetic route for the biosynthesis of trimethoxycinnamoyl quinic acids. This proposed route is based on the structures of four intermediates observed and shown in **Figure 7**. We propose that starting from 3,4-diferuloyl quinic acid (**19**) an oxidase enzyme inserts an oxygen into position 5 of the 4-feruloyl substituent, yielding intermediate **51**. Methylation presumably by catechol-*O*-methyl transferase (COMT) yields 3-feruloyl-4-sinapoyl quinic acid (**55**). A final COMT-mediated methylation furnishes 3-ferulyol-4-trimethoxycinnamoyl quinic acid (**59**).

Conclusion. All sinapoylquinic acid and trimethoxycinnamoylquinic acid derivatives could be located in all 6 Robusta samples, whereas they were absent in 10 Arabica samples investigated. As a preliminary conclusion, we therefore suggest that sinapoyl quinic acid derivatives can act as phytochemical markers that enable the distinction between Arabica and Robusta coffee varieties.

Interestingly, differences in such minor components have not yet been established in other works studying the phytochemical profile of coffee varieties, mainly by multivariant statistical techniques (*14*, *15*). Detailed knowledge of these differences might help to distinguish adulterations of ground or roasted coffee products in the future. Whether these classes of secondary metabolites contribute to the inferior sensory properties of Robusta coffee must be the topic of further investigations. As in other examples reported in the literature, the chlorogenic acid profile of individual plant species or variety can serve as a phytochemical profile of the species investigated (*16*−*18*).

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Supporting Information Available: Additional figures and table. This material is available free of charge via the Internet at http://pubs.acs.org.

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