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Hierarchical Scheme for LC-MS^n Identification of Chlorogenic Acids

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The fragmentation behavior of 18 chlorogenic acids that are not substituted at position 1 has been investigated using LC-MS^4 applied to a methanolic coffee bean extract and commercial cider (hard cider). Using LC-MS^3, it is possible to discriminate between each of the three isomers of *p*-coumaroylquinic acid, caffeoylquinic acid, feruloylquinic acid, and dicaffeoylquinic acid, and a hierarchical key has been prepared to facilitate this process when standards are not available. MS^4 fragmentations further support these assignments, but were not essential in reaching them. The distinctive behavior of 4-acyl and 3-acyl chlorogenic acids compared with the 5-acyl chlorogenic acids is a key factor permitting these assignments. The fragmentation patterns are dependent upon the particular stereochemical relationships between the individual substituents on the quinic acid moiety. Fragmentation is facilitated by 1,2-acyl participation and proceeds through quinic acid conformers in which the relevant substituents transiently adopt a 1,3-*syn*-diaxial relationship. Selected ion monitoring at *m*/*z* 529 clearly indicated the presence in coffee of six caffeoylferuloylquinic acid isomers, whereas previously only two or three had been demonstrated. The hierarchical key permitted specific structures to be assigned to each of the six isomers. These assignments are internally consistent and consistent with the limited data previously available.

KEYWORDS: Caffeic acid; caffeoylferuloylquinic acids; caffeoylquinic acids; chlorogenic acids; cider; coffee beans; *p*-coumaric acid; *p*-coumaroylquinic acids; dicaffeoylquinic acids; ferulic acid; feruloylquinic acids; LC-MS^n; quinic acid

INTRODUCTION

Classically, chlorogenic acids (CGA) are a family of esters formed between certain *trans* cinnamic acids and (−)-quinic acid [1L-1(OH),3,4/5-tetrahydroxycyclohexanecarboxylic acid] (*1*−*3*). IUPAC (*1*) has established a preferred nomenclature for cyclitols, including quinic acid derivatives. This system will be used here, and data published using other systems have been altered to ensure consistency and clarity of discussion.

CGA are characteristic components of coffee beans and commercial coffee products, in which caffeoylquinic (CQA), *p*-coumaroylquinic (*p*CoQA), feruloylquinic (FQA), dicaffeoylquinic (diCQA), and caffeoylferuloylquinic acids (CFQA) have been reported. In coffee, esterification occurs at positions 3, 4, and 5 of the quinic acid moiety, but not at position 1 (*2*−*6*). Individual compounds are commonly referred to by a well-established (*2*, *3*, *7*, *8*) series of structure-defining abbreviations, for example, 3-CQA, 4-CQA, and 5-CQA. The full set of abbreviations and structures are illustrated in **Figure 1**. The probable existence of CFQA was first noted in 1965 (*9*). Two CFQA have been characterized by NMR (*10*, *11*). Three CFQA isomers have been demonstrated chromatographically (*12*), but it has been suggested that green robusta coffee beans would contain six (*2*, *3*). The objectives of this study are to investigate the extent to which individual CGA isomers could be distinguished by LC-MS^n and to seek convincing evidence for the presence of six CFQA isomers (**XIII**−**XVIII**) in green robusta coffee beans.

MATERIALS AND METHODS

Sample Preparation. Green robusta coffee beans were frozen overnight (−12 °C) and ground in a hammer mill to pass 0.7 mm; 500 mg was extracted (4 × 25 mL, 25 min each) with 70% v/v aqueous methanol using an HT-1043 solid−liquid continuous extraction system (Tecator, Bristol, U.K.) (*13*). The bulked extracts were treated with Carrez reagents (1 mL of reagent A plus 1 mL of reagent B) (*14*) 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 (N-Evap-111, Organomation Associates Inc., Berlin, MA), and the aqueous extracts were stored at −12 °C until required, thawed at room temperature, and used directly for LC-MS.

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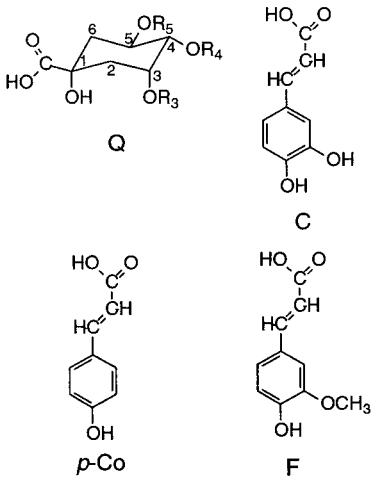


Name and abbreviation	Number	R_3	R_4	R_5
3-*O*-caffeoylquinic acid (3-CQA)	I	C	H	H
5-*O*-caffeoylquinic acid (5-CQA)	II	H	H	C
4-*O*-caffeoylquinic acid (4-CQA)	III	H	C	H
3-*O*-*p*-coumaroylquinic acid (3-*p*CoQA)	IV	*p*-Co	H	H
5-*O*-*p*-coumaroylquinic acid (5-*p*CoQA)	V	H	H	*p*-Co
4-*O*-*p*-coumaroylquinic acid (4-*p*CoQA)	VI	H	*p*-Co	H
3-*O*-feruloylquinic acid (3-FQA)	VII	F	H	H
5-*O*-feruloylquinic acid (5-FQA)	VIII	H	H	F
4-*O*-feruloylquinic acid (4-FQA)	IX	H	F	H
3,4-di-*O*-caffeoylquinic acid (3,4-diCQA)	X	C	C	H
3,5-di-*O*-caffeoylquinic acid (3,5-diCQA)	XI	C	H	C
4,5-di-*O*-caffeoylquinic acid (4,5-diCQA)	XII	H	C	C
CFQA-1 = 3-*O*-feruloyl, 4-*O*-caffeoylquinic acid[a] (3F,4CQA)	XIII	F	C	H[b]
CFQA-2 = 3-*O*-caffeoyl, 4-*O*-feruloylquinic acid[a] (3C,4FQA)	XIV	C	F	H
CFQA-3 = 3-*O*-feruloyl, 5-*O*-caffeoylquinic acid[a] (3F,5-*p*CoQA)	XV	F	H	C
CFQA-4 = 3-*O*-caffeoyl, 5-*O*-feruloylquinic acid[a] (3C,5FQA)	XVI	C	H	F
CFQA-5 = 4-*O*-feruloyl, 5-*O*-caffeoylquinic acid[a] (4F,5CQA)	XVII	H	F	C
CFQA-6 = 4-*O*-caffeoyl, 5-*O*-feruloylquinic acid[a] (4C,5FQA)	XVIII	H	C	F

Figure 1. Structure of chlorogenic acids and associated cinnamic acids (IUPAC numbering) (*1*). [a]Structure assigned during this study.

LC-MS^n. The LC equipment (ThermoFinnigan, San Jose, CA) comprised a Surveyor MS pump, an autosampler with a 20 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, turboscan, MS^n mode to obtain fragment ion *m*/*z*. MS operating conditions (negative ion) had been optimized using 5-CQA (**II**) 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.

CGA separation was achieved on a 150 × 3 mm column 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^{-1}. 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, and a return to 4% B at 105 min, followed by 5 min isocratic to re-equilibrate.

Chlorogenic acid (5-CQA) (*1*) was obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). A traditional commercial cider (hard cider) was used without further treatment as a convenient source of *p*CoQA (*8*). NMR-characterized diCQA (**X**−**XII**), 5-*p*CoQA (**V**), and 5-FQA (**VIII**) were available from previous studies (*15*). Tetramethylammonium hydroxide (30% methanolic solution) was obtained from Sigma Chemical Co. All other reagents were standard commercial items of an appropriate grade.

RESULTS AND DISCUSSION

General LC-MS Fragmentation. All data for CGA presented in this paper use the recommended IUPAC numbering system (*1*), and specimen structures are presented in **Figure 1**. When necessary, previously published data have been amended to ensure consistency and avoid ambiguity. The coffee extract

Table 1. Negative Ion MS^4 Data for Monoacylchlorogenic Acids

			MS^2							MS^3					MS^4
		MS^1 parent	base peak	secondary peaks						base peak	secondary peaks				base peak
compound	*N*	ion	*m/z*	*m/z*	intens[a]	*m/z*	intens	*m/z*	intens	*m/z*	*m/z*	intens	*m/z*	intens	*m/z*
3-CQA (**I**)	3	353.7	191.5	179.5	49			135.7	10	85.6	127.1	93	172.3	60	
4-CQA (**III**)	3	353.5	173.5	179.5	68	191.6	20	135.7	14	93.3	111.2	47			
5-CQA (**II**)	3	353.3	191.5	179.5	5	161.4	2			85.7	127.1	93	172.2	75	
3-*p*CoQA (**IV**)	6	337.7	163.5							119.4	*b*				
4-*p*CoQA (**VI**)	6	337.5	173.5	163.6	7					93.5	111.0	60			
5-*p*CoQA (**V**)	6	337.7	191.6	163.3	5					85.6	127.0	97	172.2	95	
3-FQA (**VII**)	6	367.5	193.5	191.5	3					134.0	148.8	30			106.5
4-FQA (**IX**)	3	367.4	173.5	191.8	87					93.3	111.5	44			
5-FQA (**VIII**)	3	367.6	191.6	173.6	6					85.6	127.1	100	172.2	90	

[a] Intensity. [b] Signal too weak.

Table 2. Negative Ion MS^4 Data for Diacylchlorogenic Acids

				MS^2							MS^3							MS^4				
			MS^1 parent	base peak	secondary peaks						base peak	secondary peaks						base peak	secondary peaks			
compound		*N*	ion	*m/z*	*m/z*	intens[a]	*m/z*	intens	*m/z*	intens	*m/z*	*m/z*	intens	*m/z*	intens		intens	*m/z*	*m/z*	intens	*m/z*	intens
3,4-diCQA (**X**)		3	515.7	353.4	335.6	16	173.5	18			173.5	179.5	91	191.7	53	135.6	14	93.4	111.1	70	172.9	2
3,5-diCQA (**XI**)		3	515.2	353.5							191.5	179.5	53	173.5	8	135.6	12	85.5	127.0	95	172.9	90
4,5-diCQA (**XII**)		3	515.4	353.5							173.5	179.4	80	191.6	27	135.7	12	93.3	111.4	38	172.9	15
CFQA-1 (**XIII**)	3F,4CQA	6	529.8	353.2	366.9	89	335.4	73	349.4	48	173.6	179.4	85	191.5	27	135.8	11	134.1	148.9	32	127	n.d.
CFQA-2 (**XIV**)	3C,4FQA	6	529.4	366.9	335.7	18	349.3	5			173.5	193.5	20					93.5	111.3	73	127	n.d.
CFQA-3 (**XV**)	3F,5CQA	6	529.2	367.0	335.5	9					193.3	173.4	45					134.0	149.0	48	127	n.d.
CFQA-4 (**XVI**)	3C,5FQA	6	529.3	353.2	366.8	45					191.5	179.5	56					127.2	172.4	68		
CFQA-5 (**XVII**)	4F,5CQA	6	529.3	366.9							173.5	193.5	58					93.4	111.2	57	127	n.d.
CFQA-6 (**XVIII**)	4C,5FQA	6	529.4	353.1	366.9	25					173.4	179.4	84	191.6	28	135.7	17	93.3	111.3	40	127	n.d.

[a] Intensity.

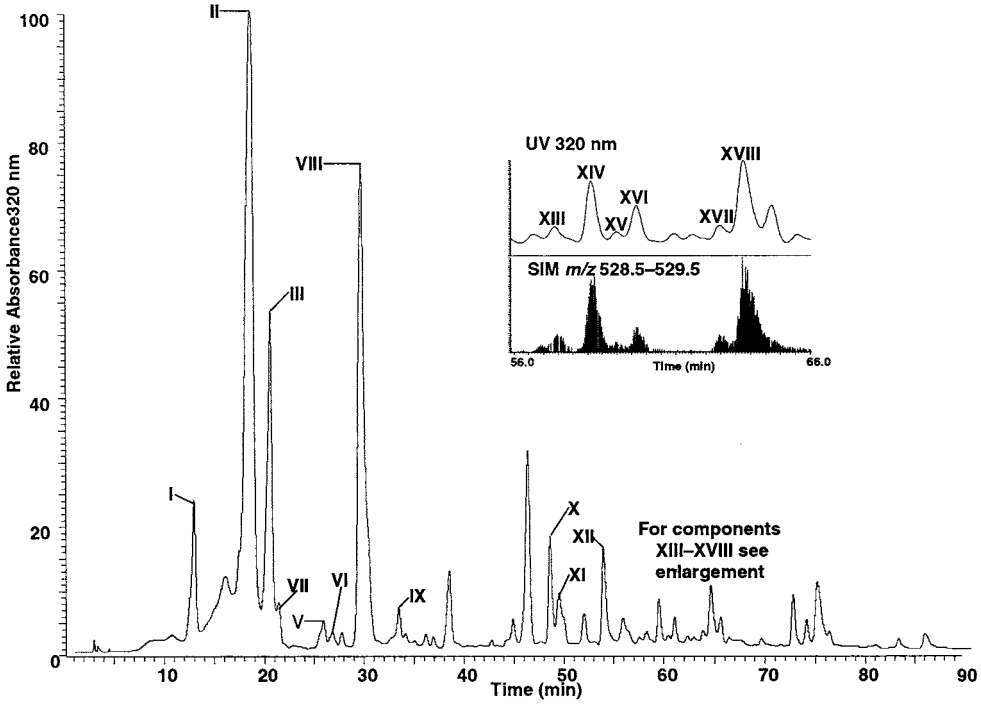


Figure 2. Specimen chromatogram (320 nm) of the coffee extract.

and cider (hard cider) sample were each examined on at least three separate occasions. Additional data were collected when the low concentration of the target molecule impaired the quality of the MS data (**Tables 1** and **2**). A specimen chromatogram (320 nm) for the coffee extract is shown in **Figure 2**. Because the content of *p*CoQA is very low in coffee and because 3-*p*-CoQA (IV) elutes fused with 5-CQA (**II**), cider (hard cider) (**Figure 3**) was used as a convenient alternative source that gives superior mass spectra for this CGA subgroup.

5-CQA (**II**) was located on the chromatogram by comparison with a commercial standard. 3-CQA (**I**) and 4-CQA (**III**) were located using standards prepared from 5-CQA (**II**) by the

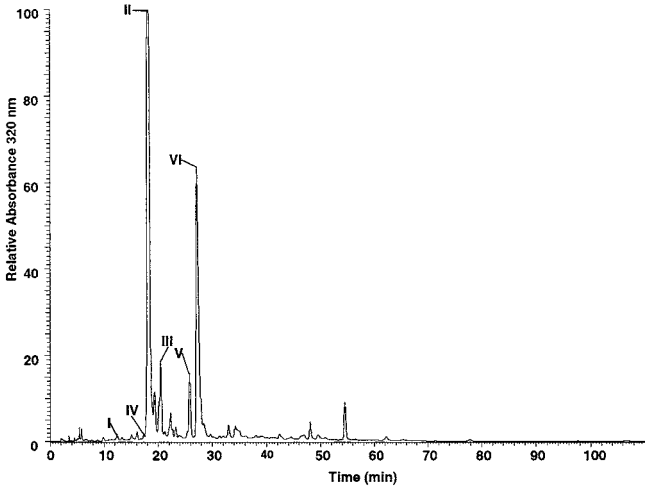


Figure 3. Specimen chromatogram (320 nm) of the cider (hard cider).

tetramethylammonium hydroxide interesterification method (*15*). 3,4-DiCQA (**X**), 3,5-diCQA (**XI**), 4,5-diCQA (**XII**), 5-*p*CoQA (**V**), and 5-FQA (**VIII**) were located by the use of NMR-characterized material isolated in previous studies (*15*). It was observed that on the phenylhexyl column packing, the monoacyl CGA eluted in the sequence 3-acyl, 5-acyl, and 4-acyl in contrast to the packings used previously in our laboratory, but which are no longer commercially available (*12*, *15*−*22*). The elution sequence of the diCQA was unchanged (3,4-di, 3,5-di, and 4,5-diCQA).

When commercial standards were not available, peak identities were assigned primarily by means of their parent ion and supported by their UV spectrum and sequence of elution/retention time relative to 5-CQA using methods validated in our laboratory (*8*, *12*, *15*). As expected from numerous previous studies (*2*, *3*, *8*), selected ion monitoring (SIM) located three CQA (**I**−**III**), three *p*CoQA (**IV**−**VI**), three FQA (**VII**−**IX**), and three diCQA (**X**−**XII**). In addition, six putative CFQA, designated CFQA-1 to CFQA-6 (**XIII**−**XVIII**), were observed for the first time. **Table 1** contains the summarized MS^n data for the monoacyl CGA and **Table 2** the equivalent data for diacyl CGA.

In general, the base peaks were consistent both within and between analyses. However, when the mass spectrum for a particular substance included two ions of similar mean intensities, within-analysis experimental error dictated that in some individual MS scans one would be more intense and for other scans the reverse would be true. This phenomenon was encountered primarily when the signal intensity was lower, that is, with quantitatively minor components and/or higher order spectra. For example, the monoacyl CGA MS^3 ions at *m*/*z* ∼85.6 (Q_5) and at *m*/*z* ∼127.0 (Q_7) are essentially co-equal in some spectra. However, in this particular case, the lower mass ion has been assigned consistently as the base peak because in the spectra of several compounds this was clearly the case. Fragment ions with intensities of <10% of the base peak have been reported only when they are needed for comparison. Selected diagnostic mass spectra are shown (**Figures 5**−**9**) to support the arguments advanced.

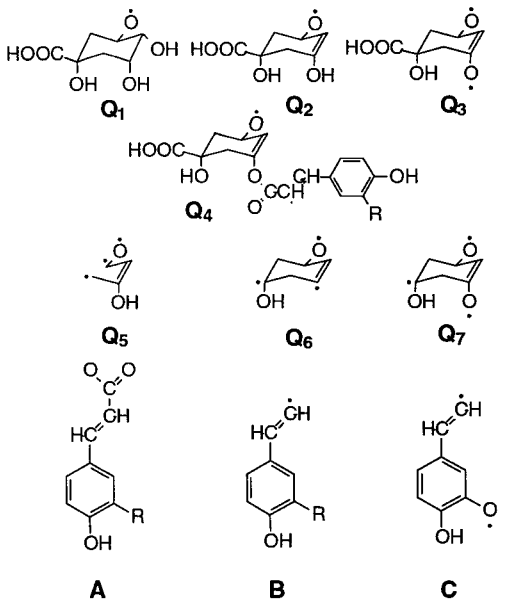
All monoacyl CGA gave the expected parent ion [monoacyl CGA − H^+]$^-$. The fragment structures are presented in **Figure 4**. In the case of 3-*p*CoQA (**IV**) and 3-FQA (**VII**) the MS^2 and MS^3 base peak ions were derived from the cinnamic acid moiety. For 3-*p*CoQA (**IV**), these ions are a [cinnamoyl − H^+]$^-$ ion (A_3) and its decarboxylation product [cinnamoyl − CO_2 − H^+]$^-$ (B_3), respectively. 3-FQA (**VII**) also yields a [cinnamoyl − H^+]$^-$ ion (A_2), but the MS^3 base peak is the "demethylated" ion (C) at *m*/*z* ∼134.0. For the remaining seven monoacyl CGA the base peak ions were derived from the quinic acid moiety. Two distinct fragmentation pathways occur. One pathway gives [quinic acid − H^+]$^-$ (Q_1) at MS^2 and a fragment (Q_5) at *m*/*z* ∼85.6 in MS^3; the other gives [quinic acid − H_2O − H^+]$^-$ (Q_2) and an unassigned MS^3 fragment at *m*/*z* ∼93.3 (**Figures 5** and **6**). The MS^1 and MS^2 data are consistent with previous publications (*8*, *23*); MS^3 data have not been reported previously.

The diacyl CGA behaved similarly, giving the equivalent parent ion [diacyl CGA − H^+]$^-$. All nine compounds examined lost either caffeic acid or ferulic acid, yielding a [diacyl CGA − cinnamoyl − H^+]$^-$ ion as the MS^2 base peak. These ions are identical to the parent ions obtained from CQA and FQA, and it is clear that in general ions produced from diacyl CGA at MS$^{(n+1)}$ are identical to those produced from monoacyl CGA at MS^n—for example, compare **Figures 5** and **7**. CFQA-1 (**XIII**) and CFQA-3 (**XV**) closely resemble 3-FQA (**VII**), both yielding C, the demethylated (base peak), and the decarboxylated ions (B_1, B_2) at MS^4. CFQA-4 (**XVI**) has an MS^4 base peak (Q_7) at *m*/*z* ∼127.2. For the remaining six diacyl CGA the MS^3 base peak is one or the other of the quinic acid-derived ions that form the MS^2 base peaks of the monoacyl CGA. The base peaks reported for diCQA are consistent with previous publications (*8*, *23*), but the fragment ions differ from those reported by Fang et al. (*23*) It must be noted that some of the mass assignments as printed in that paper appear to be in error. For example, [M − C_1 − H_2O]$^-$ is given as *m*/*z* 353 when M = 516 and C_1 = 179 and has apparently been confused/transposed with [M − C_1]$^-$ that is assigned as *m*/*z* 335. The term "chlorogenic acids" also is used confusingly when apparently "caffeic acid" is meant. Because there may also be less obvious errors, a more detailed comparison of their data and those reported here has not been attempted.

Key objectives of this investigation were to ascertain whether individual mono- or diacyl CGA could be identified unequivocally by their mass spectra and to seek to demonstrate the six CFQA that have been predicted as occurring in coffee beans.

Identification of Isomeric Monoacyl CGA. In zoom scan mode, the molecular masses predicted for the nine monoacyl CGA (**I**−**IX**) were consistently within 0.02 amu of the calculated accurate masses. In turboscan mode the mass predicted for 5-CQA (**II**) from the parent ion was 0.2 amu greater than the calculated accurate mass (353.10). The corresponding predictions for the remaining eight monoacyl CGA (**I**, **III**−**IX**)were higher by not more than 0.6 amu, and this level of imprecision is assumed for the fragment *m*/*z* values. It is clear from the data summarized in **Table 1** that the parent ion clearly identifies the CGA subclass to which each monoacyl CGA belongs.

More importantly, it is easy to distinguish a 4-acyl CGA by its "dehydrated" MS^2 base peak Q_2 at *m*/*z* ∼173.5 (**Figure 5**), supported by strong MS^3 ions at *m*/*z* ∼93.3 (unassigned) and Q_6 at *m*/*z* ∼111.2 (**Figure 6**). In contrast, all three 5-acyl CGA (**II**, **V**, and **VIII**) and 3-CQA (**I**) produce an MS^2 base peak at *m*/*z* ∼191.5 (Q_1), supported by strong MS^3 ions at *m*/*z* ∼85.6 (Q_5), ∼127.0 (Q_7), and ∼172.0 (Q_3). The distinctive MS^2 behavior of 4-CQA (**III**) has been reported previously (*23*), but the fragmentation could not be investigated further with the equipment available, and its structure−diagnostic value was not fully discussed. 3-*p*CoQA (**IV**) and 3-FQA (**VII**) are readily distinguished by their cinnamic acid-derived MS^2 base peaks (A_3 at *m*/*z* ∼163.5 and A_2 at *m*/*z* ∼193.5, respectively). 3-CQA (**I**) gives the same base peak as 5-CQA (**II**) but can be



Fragment	R	Cinnamic Acid	Accurate Mass
Q_1			191.06
Q_2			173.04
Q_3			172.04
Q_4	OH	Caffeic	335.08
	OCH_3	Ferulic	349.08
Q_5			85.03
Q_6			111.04
Q_7			127.04
A_1	OH	Caffeic	179.04
A_2	OCH_3	Ferulic	193.04
A_3	H	*p*-Coumaric	163.04
B_1	OH	Caffeic	135.04
B_2	OCH_3	Ferulic	149.04
B_3	H	*p*-Coumaric	119.04
C		Ferulic	134.04

Figure 4. Structure of quinic acid-derived and cinnamic acid-derived fragments.

distinguished from 5-CQA (**II**) by a comparatively intense caffeic acid-derived ion A_1 at *m*/*z* ~179.5 (mean intensity of 49% compared with 5%) that is reminiscent of A_3 produced from 3-*p*CoQA (**IV**) and A_2 produced from 3-FQA (**VII**). These data for 3-CQA (**I**) and 5-CQA (**II**) are identical to those reported by Fang et al. (*23*).

It is thus possible to discriminate between each of the nine monoacyl CGA (**I**−**IX**) on the basis of MS^1 and MS^2 spectra. The MS^3 spectra provide confirmation of these assignments, but are not essential in making them. The potential diagnostic value of this distinctive MS behavior has not previously been recognized so far as we are aware (*8*). A hierarchical key to facilitate its exploitation is presented in **Figure 10**.

Identification of Isomeric DiCQA. The molecular masses predicted from the diCQA parent ions differed from the calculated accurate masses by not more than 0.03 amu in zoom scan mode and by not more than 0.5 amu in turboscan mode.

The *vic* diCQA [i.e., 3,4-diCQA (**X**) and 4,5-diCQA (**XII**)] give Q_2 as the MS^3 base peak at *m*/*z* ~173.5 (**Figure 7**), supported by strong MS^4 ions at *m*/*z* ~93.3 (unassigned) and Q_6 at *m*/*z* ~111.2 (**Table 2**), as previously observed for 4-CQA (**III**), 4-*p*CoQA (**VI**), and 4-FQA (**IX**). These ions were not detectable in the spectrum of 3,5-diCQA (**XI**) (**Figure 7**), which gives Q_1 as an MS^3 base peak at *m*/*z* ~191.5, supported by strong MS^3 ions at *m*/*z* ~85.6 (Q_5), *m*/*z* ~127.0 (Q_7), and *m*/*z* ~172.0 (Q_3), as previously observed for 3-CQA (**I**) and 5-CQA (**II**). 3,5-DiCQA (**XI**) can thus be distinguished unequivocally from the *vic* diCQA (**X** and **XII**), and the ion at Q_2 at *m*/*z* ~173.5 in either an MS^2 or an MS^3 spectrum is clearly diagnostic for substitution at position 4. There remains a need to determine which of the two *vic* diCQA has a substituent at position 3 and which at position 5.

The two *vic* diCQA isomers (**X** and **XII**) differ (**Figure 7**) with regard to the intensity of Q_4 the MS^2 "dehydrated" ion $[CQA - H_2O - H^+]^-$. In 3,4-diCQA (**X**) Q_4 is more intense (16% of base peak). In contrast, in 4,5-diCQA (XII) Q_4 is barely detectable (<5% of base peak). Similarly, the 3,4-isomer (**X**) (**Figure 7**; **Table 2**) produces Q_1 in MS^3 and Q_6 in MS^4 at approximately double the intensities of the 4,5-isomer (**XII**).

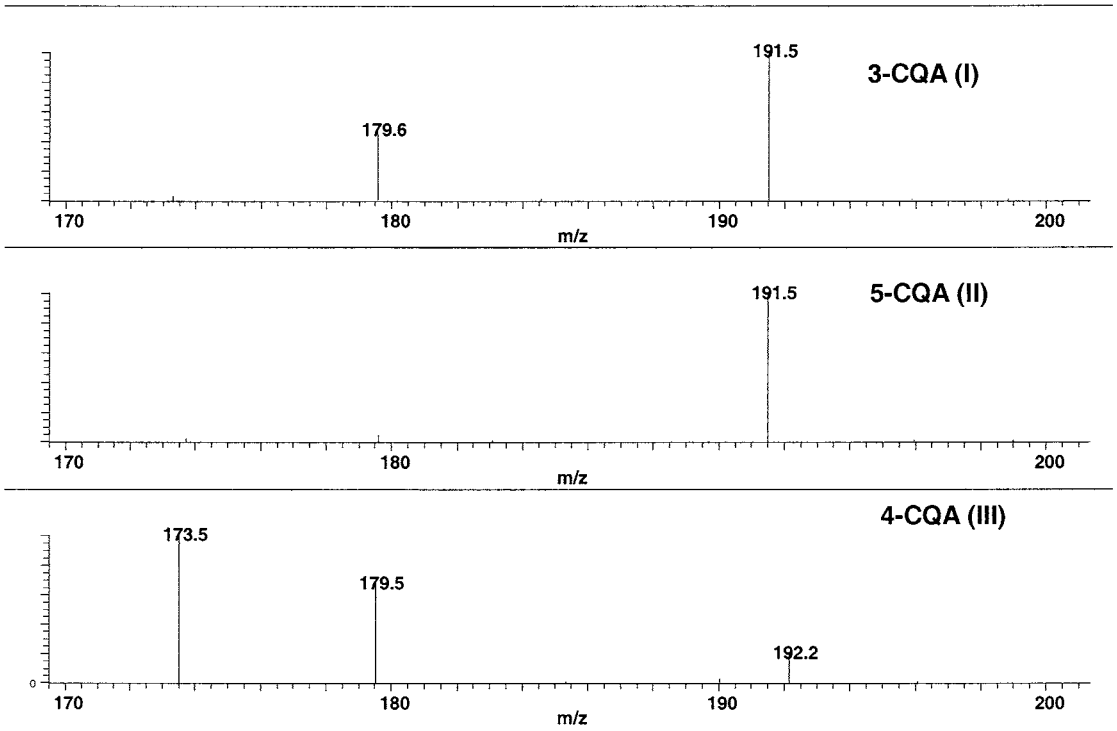


Figure 5. MS^2 spectra for isomeric caffeoylquinic acids.

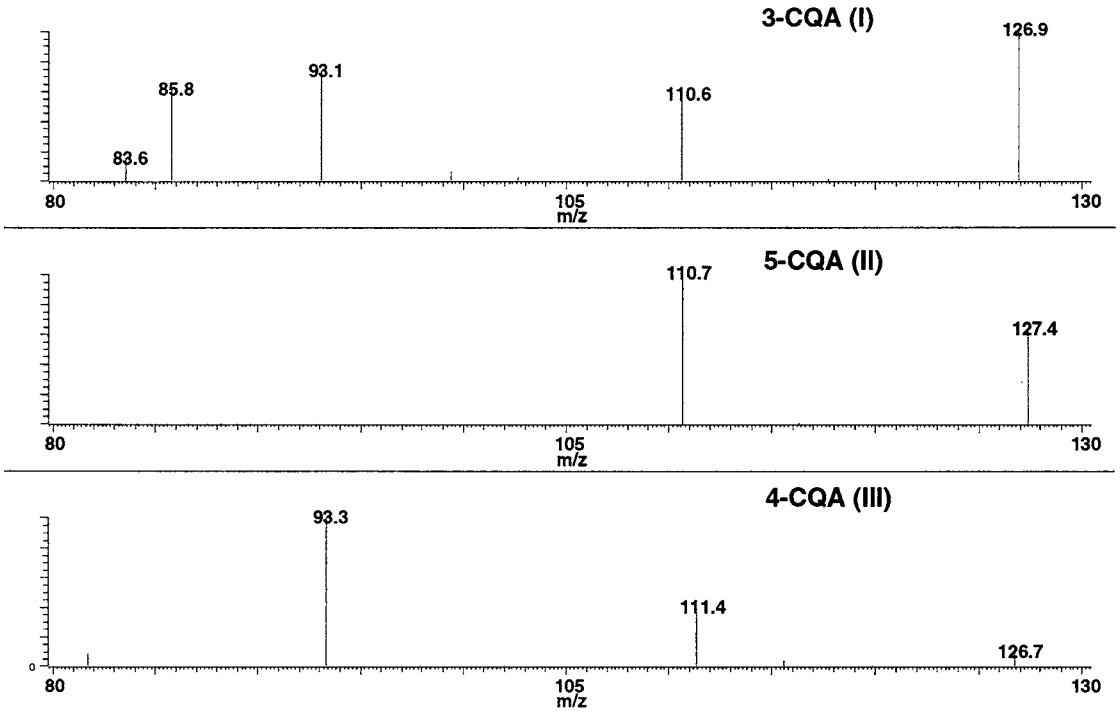


Figure 6. MS^3 spectra for isomeric caffeoylquinic acids.

Because this behavior was highly consistent over six separate determinations, it is sufficiently robust to discriminate between these two isomers.

As noted above, the MS^2 base peaks for the diCQA are identical to the parent ions for the CQA. The subsequent degradation of these ions will therefore be identical regardless of whether they have been derived from CQA or diCQA. Thus, by comparing the diCQA MS^3 data (**Figure 7**; **Table 2**) with the CQA MS^2 data (**Figure 5**; **Table 1**) it is possible to define the precise structure of the ions responsible for the diCQA MS^2 base peaks. With this information it becomes possible to specify which of the caffeoyl moieties is removed from the diCQA during MS^1 and which during MS^2.

Because the ion Q_2 at m/z ~173.5 is characteristic for an isomer substituted at position 4, the MS^2 base peak for the *vic* diCQA (**X** and **XII**) must be $[4\text{-CQA} - H^+]^-$ rather than $[3\text{-CQA} - H^+]^-$ or $[5\text{-CQA} - H^+]^-$. It follows, therefore, that 3,4-diCQA (**X**) initially must lose the caffeoyl moiety at position 3, whereas 4,5-diCQA (**XII**) initially must lose that at position 5.

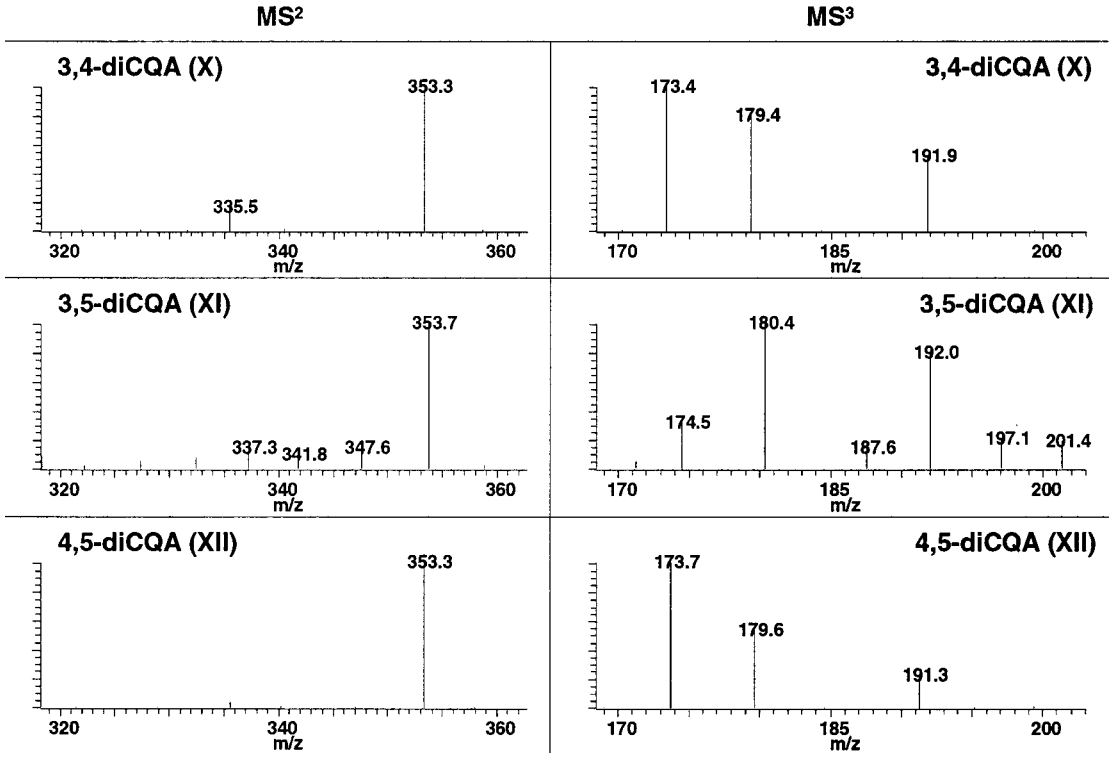


Figure 7. MS^2 and MS^3 spectra for isomeric dicaffeoylquinic acids.

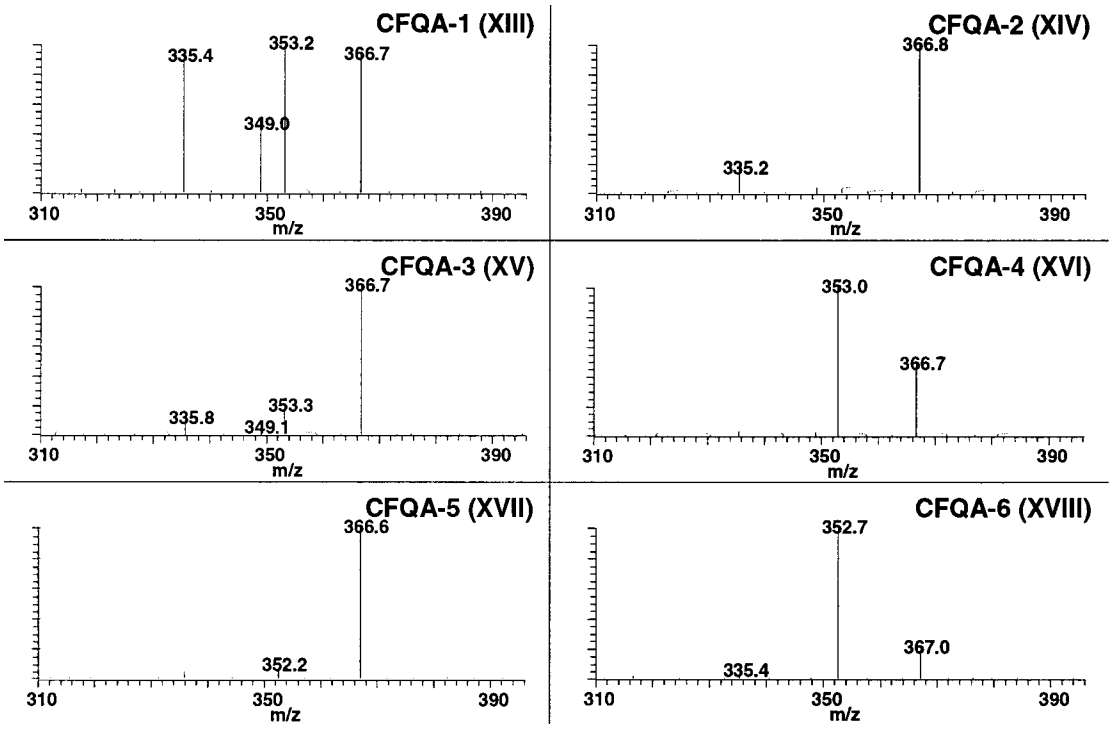


Figure 8. MS^2 spectra for isomeric caffeoylferuloyl quinic acids.

Fragmentation of the MS^2 base peak for 3,5-diCQA (**XI**) yields (**Figure 7**) a comparatively intense [caffeoyl − H^+]$^-$ ion (A_1 = 50% of base peak). This is consistent with [3-CQA − H^+]$^-$ being the MS^2 base peak rather than [5-CQA − H^+]$^-$ where only a weak [caffeoyl − H^+]$^-$ ion (A_1 ~5% of base peak) would be expected (**Table 1**; **Figure 5**). These observations lead to the conclusion that the acylating residue at position 4 is the most difficult to remove, whereas that at position 5 is the easiest. This proposal is supported also by the observation (**Figure 7**; **Table 2**) that *vic* diCQA (**X** and **XII**) do not give strong "dehydrated" ions (Q_4) at *m/z* ~335.5 that would otherwise be expected. These observations have been incorporated in the hierarchical key (**Figure 10**), and this has been used to interpret the spectra for the putative CFQA isomers (**XIII**−**XVIII**).

Characterization of Putative CFQA Isomers. Although previously only three CFQA isomers have been unequivocally characterized in any one coffee sample (*12*), in theory, one would expect six CFQA isomers (**XIII**−**XVIII**) to be present (*12*). SIM at *m/z* 529.0 ± 0.5 detected six peaks (**Figure 8**)

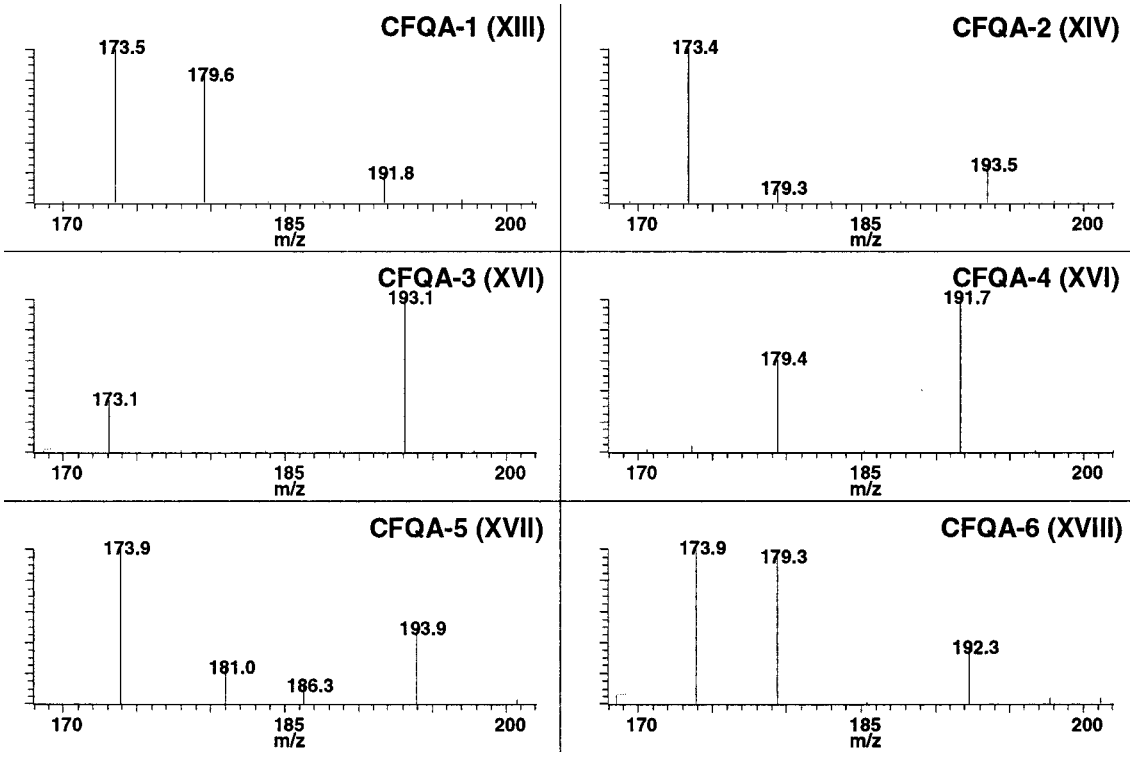


Figure 9. MS^3 spectra of the isomeric caffeoylferuloylquinic acids.

with 325 nm λ_{max} that eluted between 57 and 65 min (**Figure 2**). These six compounds elute as three pairs with, in each case, the faster eluting component being present at a much smaller concentration than the slower eluting. The CFQA are minor components that, relative to unit concentration of 5-CQA (the major CGA in coffee), range from ≈0.004 unit (**XV**) to ≈0.08 unit (**XVIII**) assuming similar propensities to ionize.

In turboscan mode parent ions fall in the range *m*/*z* 529.2−529.9 compared with a calculated exact mass of 530.14. In zoom scan mode the predicted masses were within 0.1 amu of the calculated accurate masses. All six compounds show MS^2 base peaks (**Figure 8**) of either *m*/*z* 353.1−353.2 ([CQA − H$^+$]$^-$) or *m*/*z* 366.9−367.0 ([FQA − H$^+$]$^-$) and at MS^3 (**Figure 9**) significant yields of A_1 ([caffeoyl − H$^+$]$^-$) or A_2 ([feruloyl − H$^+$]$^-$) consistent with these compounds being CFQA isomers.

The absence of the MS^3 base peak Q_2 at *m*/*z* ∼173.5 (**Figure 9**) clearly defines **XV** and **XVI** as lacking substitution at position 4. These isomers are, therefore, 3F,5CQA and 3C,5FQA. Similarly, the detection of Q_2 as the MS^3 base peak clearly defines the four *vic* isomers as **XIII**, **XIV**, **XVII**, and **XVIII**.

The two forms of Q_4 at *m*/*z* 335.4−353.7 ([CQA − H_2O − H$^+$]$^-$) and *m*/*z* 349.3−349.4 ([FQA − H_2O − H$^+$]$^-$) are found in the MS^2 spectra (**Figure 8**) of **XIII** and **XIV** with intensities never less than 20% of the MS^2 base peaks. **XVII** and **XVIII** yield the same "dehydrated" ions with intensities never exceeding 6% of base peak. Therefore, by analogy with the behavior of the *vic* diCQA (**Figure 7**), it seems reasonable to conclude that **XIII** and **XIV** are the 3,4-substituted isomers and that **XVII** and **XVIII** are the 4,5-substituted isomers. Such assignments are also consistent with the known greater hydrophobicity of 4,5-substituted CGA compared with 3,4-substituted isomers (*12*, *15*). It now remains to define which cinnamic acid is at which position.

Because **XVIII** yields an MS^2 base peak at *m*/*z* ∼353.1 (**Figure 8**), this compound loses its feruloyl residue before its caffeoyl residue. Because during MS^3 the base peak (**Figure 9**) at *m*/*z* ∼173.5 (Q_2) is accompanied by an intense A_1 (70% of base peak) at *m*/*z* ∼179.4 ([caffeoyl − H$^+$]$^-$), the MS^2 base peak must be [4-CQA − H$^+$]$^-$ rather than [4-FQA − H$^+$]$^-$. Accordingly, **XVIII** is 4C,5FQA.

It follows that if this assignment is correct, then **XVII** must be 4F,5CQA. A strong MS^3 ion (**Figure 9**) at *m*/*z* ∼193.5 (40% of the *m*/*z* 173.5 base peak) and the release of the caffeoyl residue at C5 before the feruloyl residue at C4 are consistent with these assignments. This order of elution is consistent also with previous studies (*10*, *11*) in which 4F,5CQA (**XVII**) and 4C,5FQA (**XVIII**) had been isolated and characterized by NMR (but note that the original reports used non-IUPAC numbering).

XV yields an MS^2 base peak (**Figure 8**) at *m*/*z* ∼367.0 indicating the loss of its caffeoyl residue before its feruloyl residue, suggesting that this compound is 3F,5CQA. This assignment is also supported by the observation of A_2 as the MS^3 base peak at *m*/*z* ∼193.3, a feature also of the MS^2 spectrum of 3-FQA (**VII**) and no other FQA isomer. In contrast, **XV** yields an MS^2 base peak at *m*/*z* ∼353.2 indicating the loss of its feruloyl residue before its caffeoyl residue, implying consistently that this compound is 3C,5FQA (**XVI**). This isomer has a distinctive MS^4 spectrum (base peak Q_7 at *m*/*z* ∼127.2 and strong secondary ion Q_4 at *m*/*z* ∼172.4), which are seen clearly also in the MS^3 spectra of 3-CQA (**I**), 5-CQA (**II**), and 5-FQA (**VIII**) (**Table 1**).

XIV yields an MS^2 base peak at *m*/*z* ∼366.9 indicating the loss of the caffeoyl residue prior to the feruloyl residue. This implies that the feruloyl residue is at the more stable location, which indicates that this compound is 3C,4FQA (**XIV**). If this assignment is correct, then **XIII** must be 3F,4CQA. However, **XIII** has distinctive behavior (**Figure 8**) that requires further comment. **XIII** gives an MS^2 base peak with a mean value (N = 6) of *m*/*z* 353.2 that is compatible with this assignment. However, it must be noted that it gives also a very strong MS^2 ion at *m*/*z* ∼367.0 (mean intensity of 89% of base peak). In approximately half of the individual scans this ion is more

1. Parent ion *m/z* 337.5. ***p*-Coumaroylquinic acids. *Go to 2***
   Parent ion *m/z* 353.5. **Caffeoy;quinic acids. *Go to 3***
   Parent ion *m/z* 367.5. **Feruloylquinic acids. *Go to 4***
   Parent ion *m/z* 515.5. **Dicaffeoylquinic acids. *Go to 5***
   Parent ion *m/z* 529.5. **Caffeoylferuloylquinic acids. *Go to 6***

2. MS^2 base peak *m/z* 163.5. **3-*p*-Coumaroylquinic acid (IV)**
   MS^2 base peak *m/z* 173.5. **4-*p*-Coumaroylquinic acid (VI)**
   MS^2 base peak *m/z* 191.5. **5-*p*-Coumaroylquinic acid (V)**

3 MS^2 base peak *m/z* 191.5, and relatively intense (*ca* 50% base peak) secondary ion at *m/z* 179.5. **3-Caffeoy;quinic acid (I)**
   MS^2 base peak *m/z* 173.5. **4-Caffeoy;quinic acid (III)**
   MS^2 base peak *m/z* 191.5, and weak or undetectable (<5% base peak) secondary ion at *m/z* 179.5. **5-Caffeoy;quinic acid (II)**

4. MS^2 base peak *m/z* 193.5. **3-Feruloylquinic acid (VII)**
   MS^2 base peak *m/z* 173.5. **4-Feruloylquinic acid (IX)**
   MS^2 base peak *m/z* 191.5. **5-Feruloylquinic acid (VIII)**

5. MS^2 base peak *m/z* 353.5, MS^3 base peak *m/z* 173.5, and comparatively intense (*ca* 20% of base peak) secondary ion at *m/z* 335.5. **3,4-Dicaffeoylquinic acid (X)**
   MS^2 base peak *m/z* 353.5 and MS^3 base peak *m/z* 191.5. **3,5-Dicaffeoylquinic acid (XI)**
   MS^2 base peak *m/z* 353.5, MS^3 base peak *m/z* 173.5, and comparatively intense (*ca* 20% of base peak) secondary ion at *m/z* 335.5. **4,5-Dicaffeoylquinic acid (XII)**

6. MS^3 base peak *m/z* 173.5. ***Vic*-Caffeoylferuloylquinic acids. *Go to 7***
   MS^3 base peak **not** *m/z* 173.5. **3,5-Caffeoylferuloylquinic acids *Go to 10***

7. MS^2 secondary ions at *m/z* 335.5 or *m/z* 349.5 with intensities not less than *ca* 40% of base peak. **3,4-Caffeoylferuloylquinic acids. *Go to 8***
   MS^2 secondary ions at *m/z* 335.5 or *m/z* 349.5 with intensities not more than *ca* 20% of base peak. **4,5-Caffeoylferuloylquinic acids. *Go to 9***

8. MS^2 base peak at *m/z* 367.0 and MS^3 secondary ion at *m/z* 193.5. **3-Caffeoyl-4-feruloylquinic acid (XIV)**
   MS^2 base peak at *m/z* 353.5 **or** *m/z* 367.0 with *m/z* 353.5 of near identical intensity, and MS^3 secondary ion at *m/z* 179.5. **3-Feruloyl-4-caffeoylquinic acid (XIII)**

9. MS^2 base peak at *m/z* 367.0 and an intense (>50% of base peak) MS^3 secondary ion at *m/z* 193.5. **4-Feruloyl-5-caffeoylquinic acid (XVII)**
   MS^2 base peak at *m/z* 353.5 and an intense (>50% of base peak) MS^3 secondary ion at *m/z* 179.5. **4-Caffeoyl-5-feruloylquinic acid (XVIII)**

10. MS^2 base peak at *m/z* 367.0 and MS^3 base peak at *m/z* 193.5. **3-Feruloyl-5-caffeoylquinic acid (XV)**
    MS^2 base peak at *m/z* 353.5 and an intense (>50% of base peak) MS^3 secondary ion at *m/z* 179.5. **3-Caffeoyl-5-feruloylquinic acid (XVI)**

Figure 10. Hierarchical key for identification by LC-MS^n of mono- and diacylchlorogenic acids not substituted at position 1.

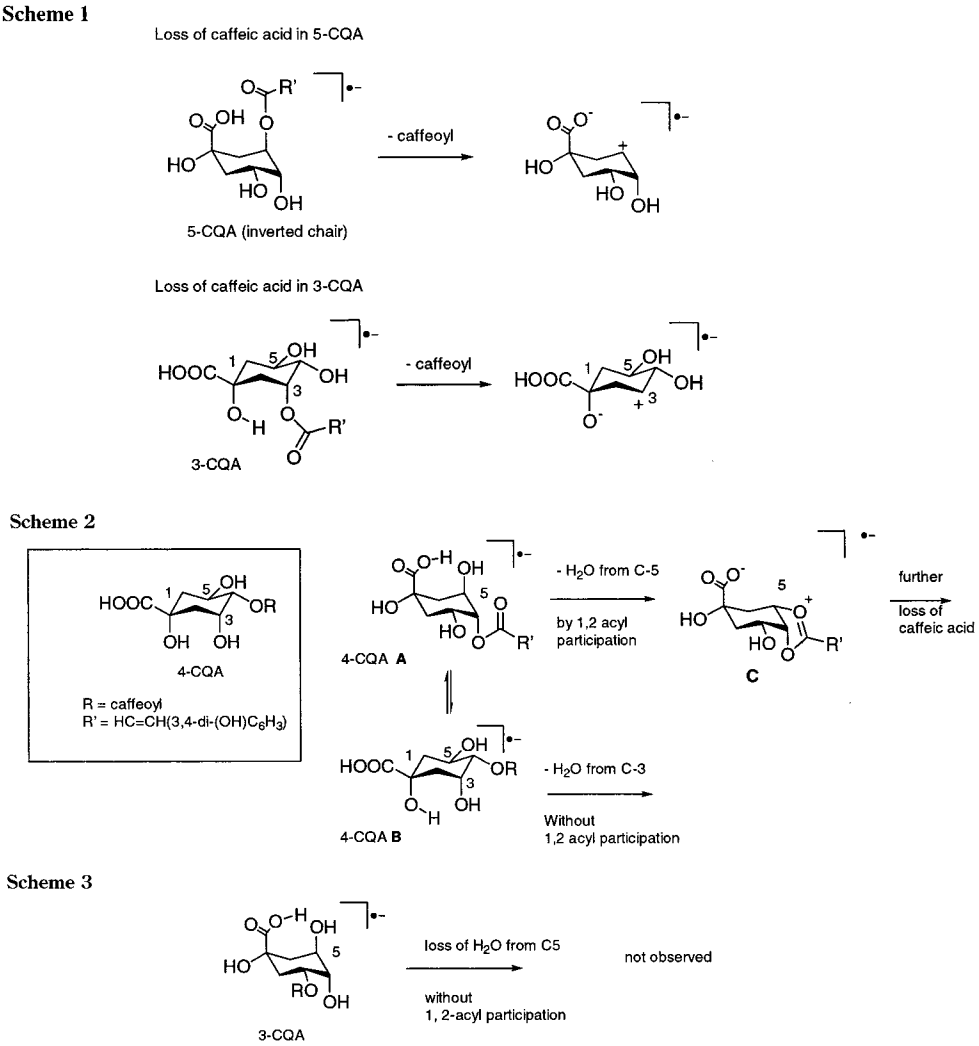


Figure 11. Schemes of fragmentation.

intense than the ion at *m*/*z* ~353.2 and on these occasions is returned as the base peak. The distinctive MS^4 ions at *m*/*z* ~134.1 (C) and *m*/*z* ~148.9 (B_2) seen previously in the MS^3 spectrum of 3-FQA (**VII**) also suggest that **XIII** is indeed 3F,4CQA.

Such behavior is not seen in any other CFQA isomer, but it is consistent with the behavior of 3-FQA (**VII**) and 3F,5CQA (**XV**) discussed above. Such behavior, if it were to occur in the corresponding diCQA, could not be detected because both acylating residues have the same mass. However, closer examination of the MS^3 spectra for 3,4-diCQA (**X**) reveals that in ~50% of the scans A_1 at *m*/*z* ~179.5 is more intense than the Q_2 at *m*/*z* ~173.5. This implies that such behavior is characteristic of acylation at C3 whichever cinnamic acid is involved and further strengthens the assignment of **XIII** as 3F,-4CQA.

Mechanisms of Fragmentation. The comparatively easy loss of the 5-acyl moiety, for example, from 5-CQA (**II**), can be rationalized by an abstraction of the proton from the C1 carboxyl while in an inverted chair conformation where the C1 carboxyl and the C5 caffeoyl have a 1,3-*syn*-diaxial arrangement (**Figure 11**, **Scheme 1**). The caffeoyl substituent in 3-CQA (**I**) has relative to the C1 hydroxyl a similar 1,3-*syn*-diaxial arrangement and can be lost by the same mechanism (**Figure 11**, **Scheme 1**). We expect the C5 hydroxyl to be lost more readily than the C1 hydroxyl due to a stronger hydroxyl-associated C−O bond α to COOH. The high p*K* of the hydroxyl (p*K* > 15) relative to the carboxyl (p*K* ≈ 3.5) explains why 3,5-diCQA (**XI**) loses the residue at C5 prior to the residue at C3. For loss of a substituent from C4 no suitable conformation can be found that would allow transfer of a proton required for the elimination step. In radical cation chemistry it has been argued that loss of water in a substituted cyclohexanol system occurs via abstraction of a C4 hydrogen in a boat conformation (*24*, *25*). In the absence of a deuterated substrate we cannot exclude this stereochemical pathway for fragmentation. However, we believe that in radical anion chemistry any elimination pathway should lead to a more stable oxygen-centered anion rather than the carbon-centered anion that would be obtained as a result of such a *syn*-1,4 elimination. It should be noted, however, that although the rationale we have presented does not require such a *syn*-1,4 elimination pathway, all of our arguments would remain consistent and in complete agreement with the experimental observations if it were to occur.

It follows, therefore, that loss of water from the chlorogenic acids cannot involve the hydroxyls at C4 and C1, but could involve those at C3 or C5. From the foregoing, it is postulated that 4-CQA (**III**), for example, can lose water from either of two conformers (A or B) as illustrated in **Figure 11** and **Scheme 2**. Although in the absence of the deuterated analogues it is not possible to establish which conformer is involved, we suggest that conformer A is preferred because it allows 1,2-acyl participation by the *trans*-vicinal acyl moiety on C4, thus facilitating the formation of a bicyclic oxonium radical anion

C by loss of OH from C5 and subsequent loss of the caffeoyl at C4. 1,2-Acyl participation is not only a powerful tool in synthetic chemistry, but common in mass spectrometric fragmentation (*26*, *27*). In contrast, the loss of OH from C3 of conformer B would have to occur without 1,2-acyl participation and should be less favored. Further evidence for the importance of the 1,2-acyl participation hypothesis is given below.

Consistent with this proposal is the observation (**Figure 5**) that for 3-CQA (**I**) the ion Q_2 at *m*/*z* ~173.5 is only ~5% of the MS^2 base peak intensity; that is, water is not eliminated significantly during the fragmentation of this isomer in which the acyl residue is not *trans* relative to the vicinal hydroxyl and 1,2-acyl participation is thus not possible (**Figure 11**, **Scheme 3**). If 1,2-acyl participation were not involved in the fragmentation process, then 3-CQA (**I**) also should fragment via dehydration. In 5-CQA (**II**) the 5-acyl group is *trans* to the OH at C4. However, as argued before, loss of the C4 hydroxyl by 1,2-acyl participation is not observed because it would require abstraction of a *syn* proton that in the reactive conformation is unavailable in this particular isomer.

The increased intensity of the dehydrated peak in the MS^2 spectrum of 3,4-diCQA (**X**) as compared with 4,5-diCQA (**XII**) (**Figure 7**) further supports the above argument that loss of OH takes place from C5 preferentially by 1,2-acyl participation. Furthermore, loss of the caffeoyl group from C5 in 4,5-diCQA (**XII**), where 1,2-acyl participation can occur, is more facile than loss of caffeoyl from C3 in 3,4-diCQA (**X**), where 1,2-acyl participation is impossible.

The data obtained for the fragmentation of the diCQA indicate clearly that a caffeoyl residue is most easily removed from C5, somewhat less readily removed from C3, and not easily removed from C4. We have presented arguments based on the relative acidities of COOH and OH that perfectly explain these observations, but 1,2-acyl participation can be used as an alternative rationale. This increased stability of the C4 caffeoyl compared with the alternative 3- and 5-positions in 3,4-diCQA (**X**) and 4,5-diCQA (**XII**), respectively, where 1,2-acyl participation facilitates loss of 5-OH [in 3,4-diCQA (**X**)] or loss of 5-caffeoyl [in 4,5-diCQA (**XII**)], provides in summary a consistent rationale for all observed fragmentations.

However, comparisons of the fragmentation behavior of 3C,-4FQA (**XIV**) with 3F,4CQA (**XIII**) and of 3-CQA (**I**) with 3-FQA (**VII**) and 3-pCoQA (**IV**) suggest that for C3 the ease with which an acyl residue is removed depends also upon its identity. The fragmentation process has to be initiated by the capture of an electron by the aromatic side chain to produce a radical anion. If the caffeic ester is able to capture an electron more readily than the ferulic or *p*-coumaric ester, then the stereochemcial relationships between the substituents might become less relevant than for the diCQA and CQA. For a caffeoyl residue, electron capture will give a radical anion that subsequently loses an H radical or proton. The *para* hydroxyl of a *vic* dihydroxyphenol such as caffeic acid has a bond dissociation enthalpy (BDE) of ~72 kcal mol^{-1}, lower than for isolated phenolic hydroxyls (*28*), and the anion/radical once formed is further stabilized by H-bonding to the adjacent hydroxyl (*29*, *30*). The BDE for the *para* hydroxyl of a feruloyl or *p*-coumaroyl moiety is some 14 kcal mol^{-1} higher and the anion/radical cannot hydrogen bond and is thus less stable. This stabilization available to the caffeoyl residue is sufficient, at least at C3, to account for its distinctive behavior compared with feruloyl or *p*-coumaroyl esters. That this effect is not apparent with 4-acyl-CGA suggests that the additional energy required to remove a C4 caffeoyl moiety compared with a C3 caffeoyl moiety is somewhat greater than 14 kcal mol^{-1}.

Hierarchical Key to the Identification by LC-MS^n of Mono- and Diacyl CGA in Coffee. In conclusion, green robusta coffee beans contain six isomers of caffeoylferuloylquinic acid. Of greater importance, it is clear from the data in **Tables 1** and **2**, and the discussion above, that the 18 coffee chlorogenic acids examined (**I**−**XVIII**) give mass spectra that are amenable to logical interpretation, thus permitting their unequivocal discrimination. Such LC-MS^n procedures are thus potentially of much wider application because CGA are widespread in plant material and commercial standards are restricted to one or two individual compounds (*8*). The hierarchical key (**Figure 10**) is proposed to facilitate this process. All masses are approximate (±0.3 amu). It must be noted however, that the key as presented is not intended to accommodate the 1-substituted CGA (as found in some Asteraceae) and has not been evaluated on CGA that contain other than the "normal" isomer of quinic acid (*8*, *31*, *32*).

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