JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2005,** *53,* 3821−3832 **3821**

Discriminating between the Six Isomers of Dicaffeoylquinic Acid by LC-MS^n

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The fragmentation behavior of all six dicaffeoylquinic acids (diCQA) has been investigated using LC-MS^4. It is possible to discriminate between each of the isomers including those for which commercial standards are not available. For diCQA, the ease of removal of the caffeoyl residue during fragmentation is 1 ≈ 5 > 3 > 4. The distinctive fragmentation observed for the little-studied 1,4-dicaffeoylquinic acid involves elimination of the C1 caffeoyl residue, repeated dehydrations leading to the aromatization of the quinic acid moiety, and its decarboxylation. It is suggested that this process is initiated by the C1 carboxyl protonating the C5 hydroxyl in the inverted chair conformer, followed by its protonating the C1 caffeoyl residue in the favored chair conformation. The fragmentation of 1-caffeoylquinic acid is indistinguishable from that of the commercially available 5-caffeoylquinic acid, but these two isomers can be distinguished easily by their facile chromatographic resolution on reversed phase packings. The hierarchical key previously developed for characterizing chlorogenic acids has been extended to accommodate 1-caffeoylquinic acid and the 1-acyl dicaffeoylquinic acids.

KEYWORDS: Asteraceae; caffeoylquinic acids; chlorogenic acids; coffee; *Cynara*; cynarin; dicaffeoylquinic acids; LC-MS^n

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-tetrahydroxycyclohexane carboxylic acid] (*1*−*3*). Specimen structures are shown in **Figure 1**. CGA are widely distributed in plants (*2*, *3*); however, few commercial standards are available (*4*), and precise identification of individual CGA in complex mixtures is not easy. An LC-MS^n method developed for this purpose can discriminate between the isomers of caffeoylquinic acid (CQA), *p*-coumaroylquinic acid (*p*CoQA), feruloylquinic acid (FQA), dicaffeoylquinic acid (diCQA), and caffeoyl-feruloylquinic acid (CFQA) that occur in green robusta coffee beans (*5*). Key steps in this scheme are as follows:

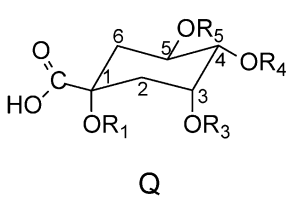
1. MS^n for a monoacyl CGA corresponds to MS^{n+1} for a related diacyl CGA.

2. Individual CGA subgroups can be distinguished by their molecular ion [CGA − H$^+$]$^-$.

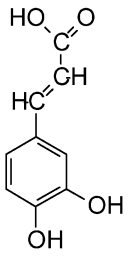
3. 4-Acyl CGA can be distinguished by an MS^2 or MS^3 base peak at *m*/*z* 173.

4. 5-Acyl CGA can be distinguished by an MS^2 or MS^3 base peak at *m*/*z* 191 accompanied by a weak (<5% of base peak) [cinnamate − H$^+$]$^-$ fragment.

5. 3-Acyl CGA can be distinguished by an MS^2 or MS^3 [cinnamate − H$^+$]$^-$ base peak (FQA, *p*CoQA, and CFQA) or by a combination of an MS^2 or MS^3 base peak at ∼*m*/*z* 191 accompanied by an intense (>50% base peak) [cinnamate − H$^+$]$^-$ fragment (CQA and diCQA).



Q



C

Name and abbreviation	Number	R_1	R_3	R_4	R_5
1-*O*-caffeoylquinic acid (1-CQA)	I	C	H	H	H
3-*O*-caffeoylquinic acid (3-CQA)	II	H	C	H	H
5-*O*-caffeoylquinic acid (5-CQA)	III	H	H	H	C
4-*O*-caffeoylquinic acid (4-CQA)	IV	H	H	C	H
1,3-di-*O*-caffeoylquinic acid (1,3-diCQA)	V	C	C	H	H
1,4-di-*O*-caffeoylquinic acid (1,4-diCQA)	VI	C	H	C	H
1,5-di-*O*-caffeoylquinic acid (1,5-diCQA)	VII	C	H	H	C
3,4-di-*O*-caffeoylquinic acid (3,4-diCQA)	VIII	H	C	C	H
3,5-di-*O*-caffeoylquinic acid (3,5-diCQA)	IX	H	C	H	C
4,5-di-*O*-caffeoylquinic acid (4,5-diCQA)	X	H	H	C	C

Q = quinic acid; C = caffeic acid

Figure 1. Structure of caffeoylquinic and dicaffeoylquinic acids (IUPAC numbering) (*1*).

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10.1021/jf050046h CCC: $30.25
Published on Web 04/14/2005

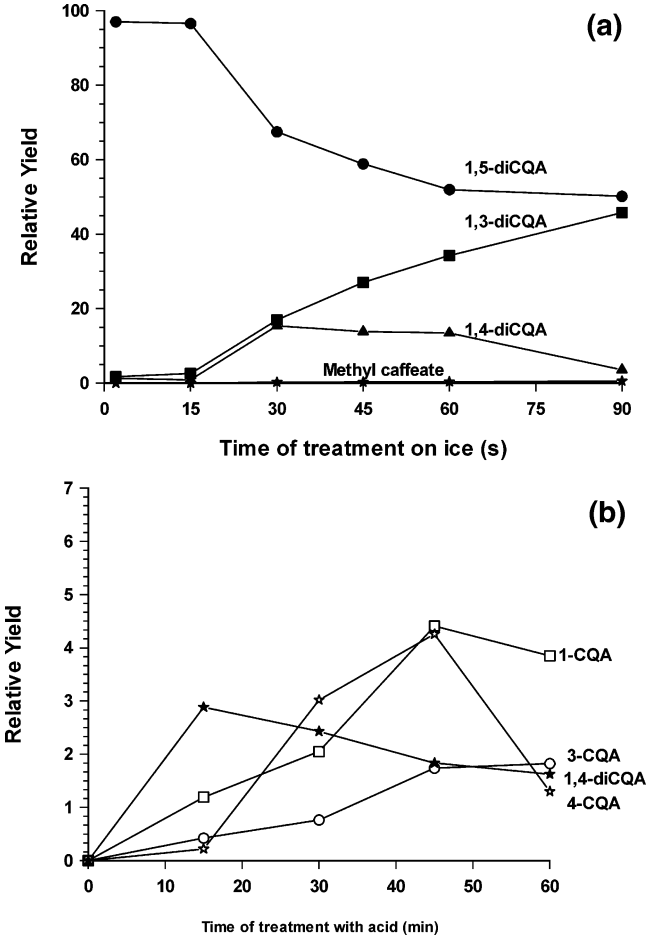


Figure 2. Generation of 1,4-diCQA and 1-CQA: (**a**) relative yield of 1,3-diCQA, 1,4-diCQA, and methyl caffeate produced by treating 1,5-diCQA on ice with TMAH for various times; (**b**) relative yield of 1-CQA, 3-CQA, 4-CQA, and 1,4-diCQA produced by treating 1,3-diCQA with 2 M HCl at 100 °C.

Many plants, including coffee, produce CGA in which esterification occurs at positions 3, 4, and 5 of the quinic acid moiety, but not at position 1. However, 1-acyl CGA are found in some Asteraceae, for example, artichoke, burdock, and arnica (*6*−*10*), and it is desirable that this procedure be evaluated also for 1-acyl CGA. This manuscript presents the results of this study.

MATERIALS AND METHODS

CQA and DiCQA. A methanolic extract of green arabica coffee beans was used as a convenient source of 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA (**VIII**−**X**) as previously described (*5*). The coffee extract was treated with Carrez reagents (1 mL of reagent A plus 1 mL of reagent B) (*11*) 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 extract stored at −12 °C until required, thawed at room temperature, centrifuged (1360*g*, 10 min), and used directly for LC-MS.

Cynarin (1,3-diCQA) (**V**) and 1,5-diCQA (**VII**) were obtained from LGC Promochem (Hatfield, U.K.). Stock solutions (∼1 mg/mL) were prepared in 10% methanol and diluted 100 times. 1,4-DiCQA (**VI**) was prepared by interesterification with tetramethylammonium hydroxide (TMAH) as previously described (*5*, *12*, *13*) using a 60 s treatment of 1,5-diCQA (**VII**) (∼10 μg/mL) on ice. The 1-acyl diCQA working solutions were centrifuged (1360*g*, 10 min) and used directly for LC-MS.

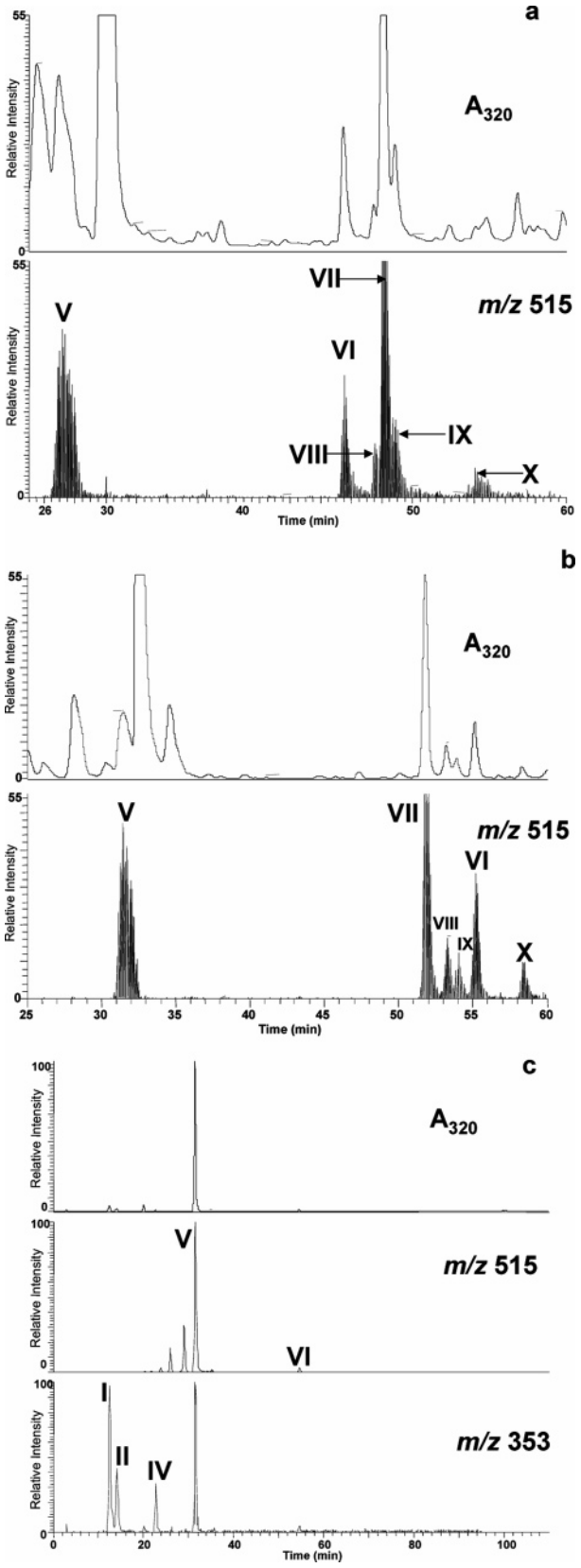


Figure 3. Specimen LC-MS chromatograms: (**a**) UV chromatogram (320 nm) and selected ion chromatogram (*m*/*z* 515) of the arabica coffee extract spiked with TMAH-treated 1,5-diCQA (**VII**) on C_{18} packing; (**b**) UV chromatogram (320 nm) and selected ion chromatogram (*m*/*z* 515) of the arabica coffee extract spiked with TMAH-treated 1,5-diCQA (**VII**) on phenylhexyl packing; (**c**) UV chromatogram (320 nm) and selected ion chromatograms (*m*/*z* 353 and 515) of acid-treated 1,3-diCQA (**V**) on phenylhexyl packing.

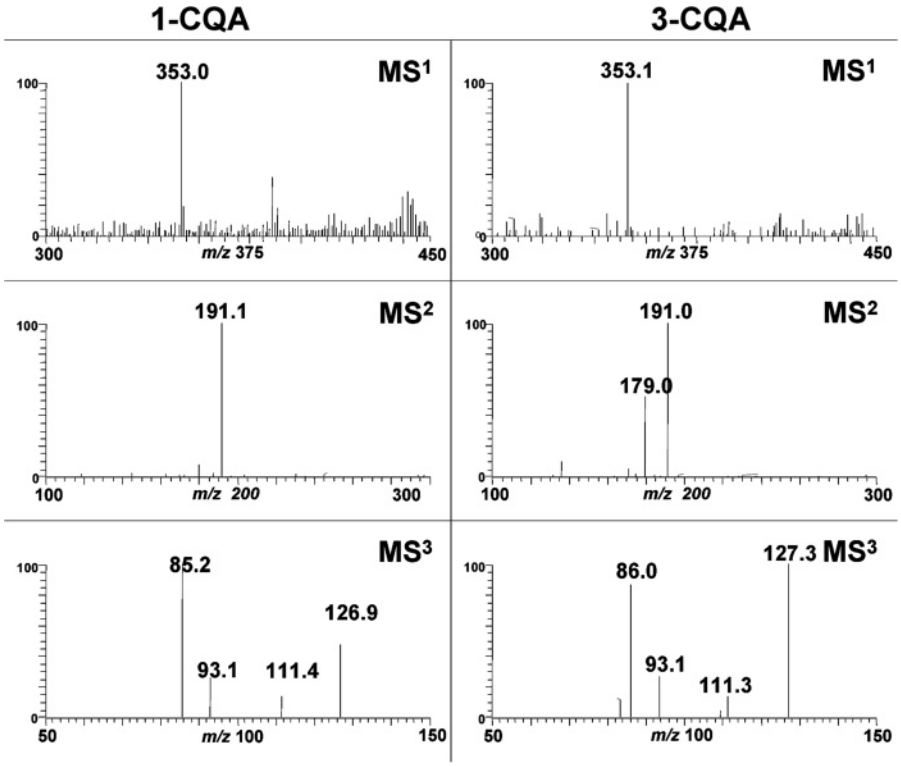


Figure 4. Negative ion MS^n spectra for 1-CQA and 3-CQA.

Table 1. Negative Ion MS^3 Fragmentation Data for Caffeoylquinic Acids

		parent	MS^2 base	MS^2 ions						MS^3 base	MS^3 ions							
compd	*N*	ion *m*/*z*	peak *m*/*z*	*m*/*z*	int[a]	*m*/*z*	int	*m*/*z*	int	peak *m*/*z*	*m*/*z*	int	*m*/*z*	int	*m*/*z*	int	*m*/*z*	int
1-CQA	3	353.0	191.1	179.3	3	b.p.[b]	100			85.1	110.9	35	127.3	65	172.3	60	93.4	40
3-CQA	3	353.1	191.0	179.2	55	b.p.	100	135.1	10	85.2	111.4	40	127.2	70	172.3	65	93.5	40
4-CQA	3	353.3	173.0	179.0	70	191.1	20	135.1	15	93.1	111.1	65	126.9	5			b.p.	100
5-CQA	3	352.9	191.1	179.1	3	b.p.	100	135.1	3	85.0	110.7	40	127.3	30	172.3	45	93.1	50

[a] Int, intensity. [b] b.p., occurs as base peak.

Table 2. Negative Ion MS^4 Fragmentation Data for Dicaffeoylquinic Acids

		parent	MS^2 base	MS^2 ions														
compd	*N*	ion *m*/*z*	peak *m*/*z*	*m*/*z*	int[a]	*m*/*z*	int	*m*/*z*	int	*m*/*z*	int	*m*/*z*	int	*m*/*z*	int	*m*/*z*	int	*m*/*z*	int
1,3-diCQA	3	515.1	353.1	335.1	35									191.1	10	179.0	30		
1,4-diCQA	3	515.1	353.0	335.1	25	317.1	35	299.1	50	255.0	22	203.1	55			179.0	7	173.1	15
1,5-diCQA	3	515.0	353.1	335.0	7							203.1	3	191.1	20				
3,4-diCQA	3	515.1	353.0	335.1	16			299.1	5	255.1	5	203.1	7			179.1	15	173.0	18
3,5-diCQA	3	515.0	353.1									203.0	10	191.2	20				
4,5-diCQA	3	515.1	353.1			317.0	7	299.0	12	255.2	8	203.1	15			179.1	5	173.1	12

		MS^3 base	MS^3 ions							MS^4 base	MS^4 ions							
compd	*N*	peak *m*/*z*	*m*/*z*	int	*m*/*z*	int	*m*/*z*	int	*m*/*z*	int	peak *m*/*z*	*m*/*z*	int	*m*/*z*	int	*m*/*z*	int	*m*/*z*	int
1,3-diCQA	3	191.1	179.1	60	b.p.[b]	100			135.2	10	85.1	127.2	78	172.3	60	110.9	35	93.1	60
1,4-diCQA	3	173.2	179.1	70	191.1	27			135.1	20	93.0	127.1	5			111.1	45	b.p.	100
1,5-diCQA	3	191.0	179.0	7	b.p.	100					85.1	127.0	60	172.1	75	110.8	55	93.0	55
3,4-diCQA	3	173.1	179.1	91	191.1	53			135.1	14	93.1	127.1	3	172.0	2	111.1	50	b.p.	100
3,5-diCQA	3	191.1	179.2	53	b.p.	100	173.1	5	135.1	12	85.2	127.0	95	172.0	90	110.9	60	93.1	80
4,5-diCQA	3	173.1	179.2	80	191.2	27			135.1	12	93.1	127.0	3	172.0	15	111.4	38	b.p.	100

[a] Int, intensity. [b] b.p., occurs as base peak.

1-CQA (**I**) was prepared from 1,3-diCQA (**V**) by partial acid hydrolysis. Stock solution (10 μL) was added to 2 M HCl (1 mL) in a screw-cap test tube and heated in a boiling water bath for 2 h. The products were cooled and centrifuged (1360*g*, 10 min) and used directly for LC-MS.

LC-MS^n. The LC equipment (ThermoFinnigan, San Jose, CA) comprised a Surveyor MS pump, an autosampler with a 50 μL loop, and a PDA detector with a light-pipe flow cell (recording at 320, 280, and 254 nm and scanning from 200 to 600 nm). This was interfaced with an LCQ Deca XP Plus mass spectrometer fitted with an ESI source (ThermoFinnigan) and operating in zoom scan mode for the accurate determination of parent ion *m*/*z* and in data-dependent, MS^n full scan mode to obtain fragment ion *m*/*z*. Unless specified otherwise, the parent ion or base peak ion was selected for further fragmentation. MS

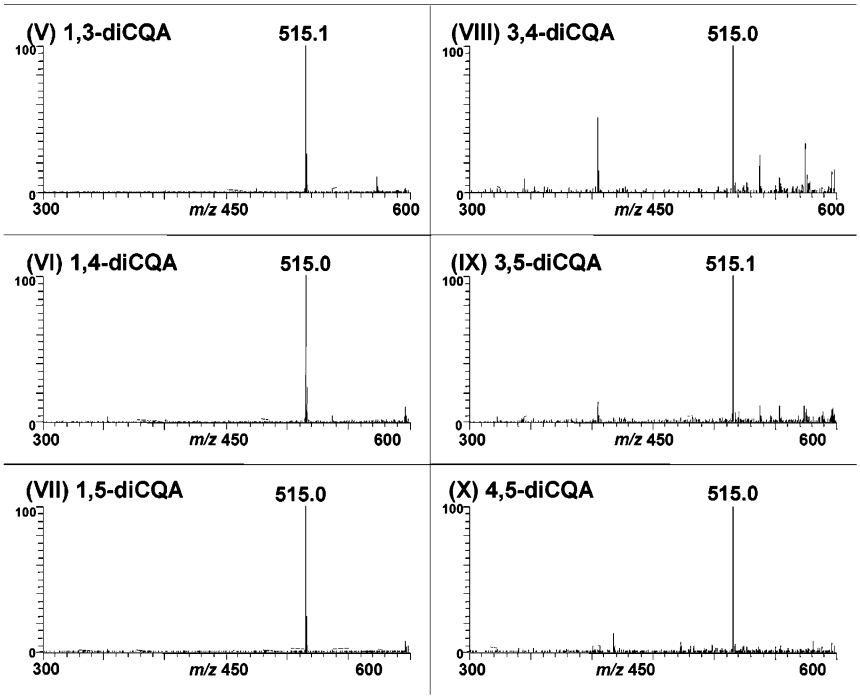


Figure 5. Negative ion MS^1 spectra for isomeric dicaffeoylquinic acids.

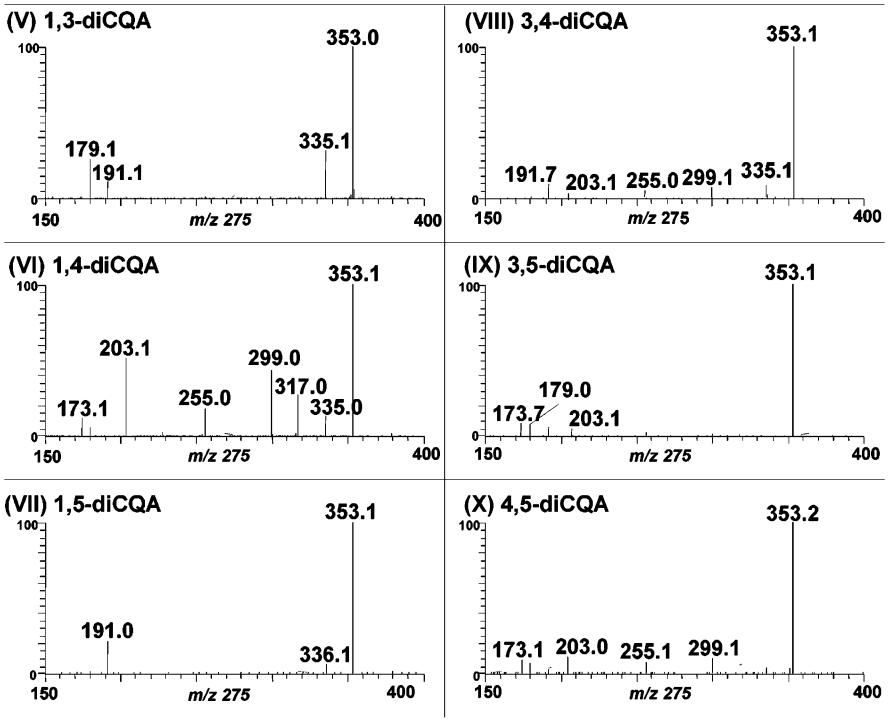


Figure 6. Negative ion MS^2 spectra for isomeric dicaffeoylquinic acids.

operating conditions (negative ion) had been optimized using 5-CQA (**III**) 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 separations were achieved on 150 × 3 mm columns containing either Luna 5 μm phenylhexyl packing or Kromasil C_{18} 5 μm (Phenonemex, Macclesfield, U.K.). For routine separations 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, a return to 4% B at 105 min, and 5 min isocratic to re-equilibrate. In experiments designed to investigate ionization mechanisms and rationalize fragmentation pathways, analyses were performed in which acetic acid was omitted from the HPLC solvents.

RESULTS AND DISCUSSION

Chromatographic Data. 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 gave a typical chromatogram, and 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA (**VIII**−**X**) were located by their parent ion at *m*/*z* 515 and distinguished by their patterns

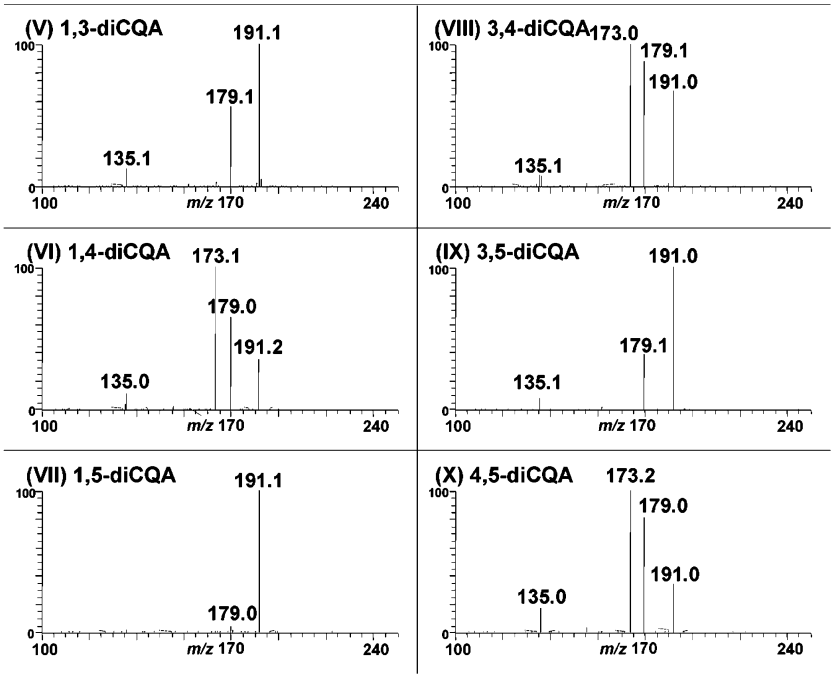


Figure 7. Negative ion MS^3 spectra for isomeric dicaffeoylquinic acids.

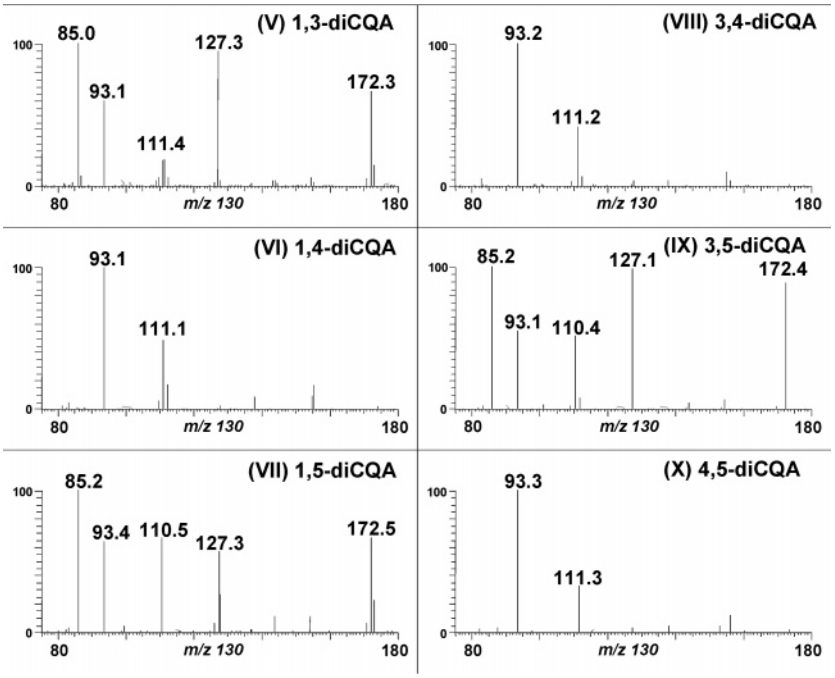


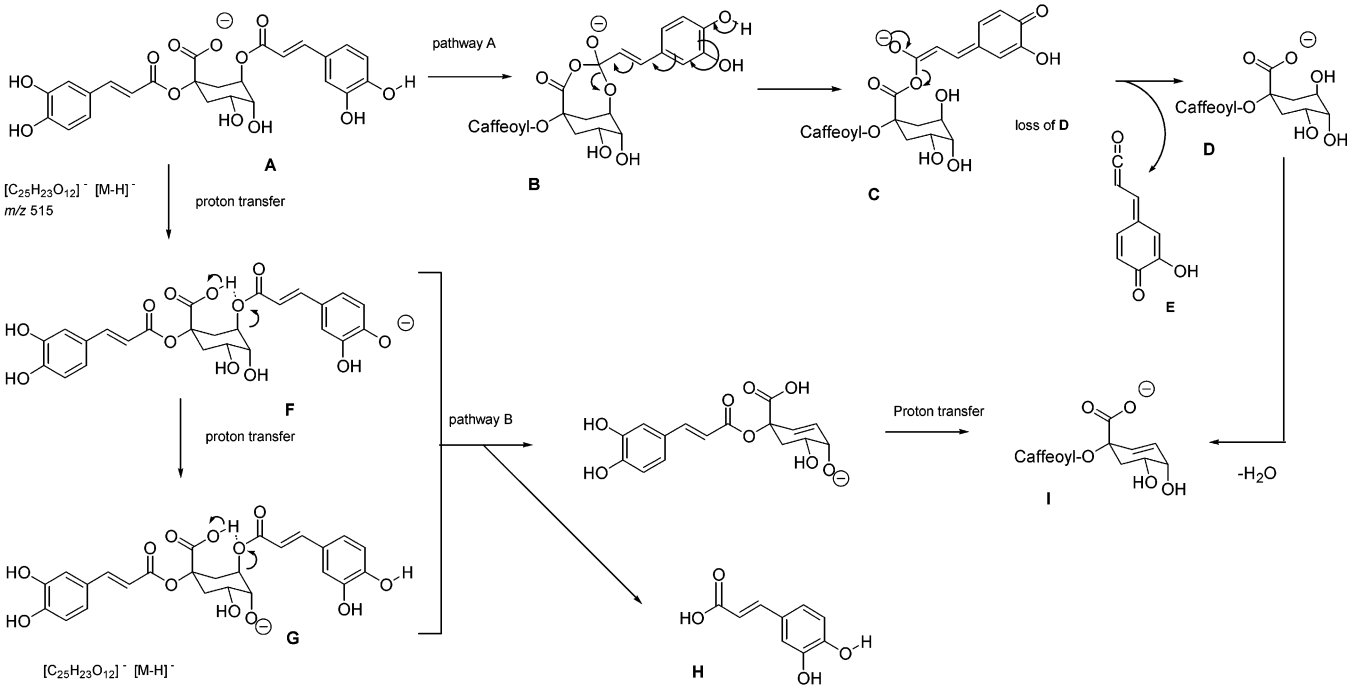
Figure 8. Negative ion MS^4 spectra for isomeric dicaffeoylquinic acids.

of fragmentation as previously reported (*5*). These compounds eluted from the C_{18} packing between 47 and 55 min and from the phenylhexyl packing between 53 and 58 min. As previously reported (*14*) 1,3-diCQA (**V**) eluted much earlier (~27 min for C_{18} and ~31 min for phenylhexyl), whereas 1,5-diCQA (**VII**) eluted at ~48 min (C_{18}) and ~52 min (phenylhexyl).

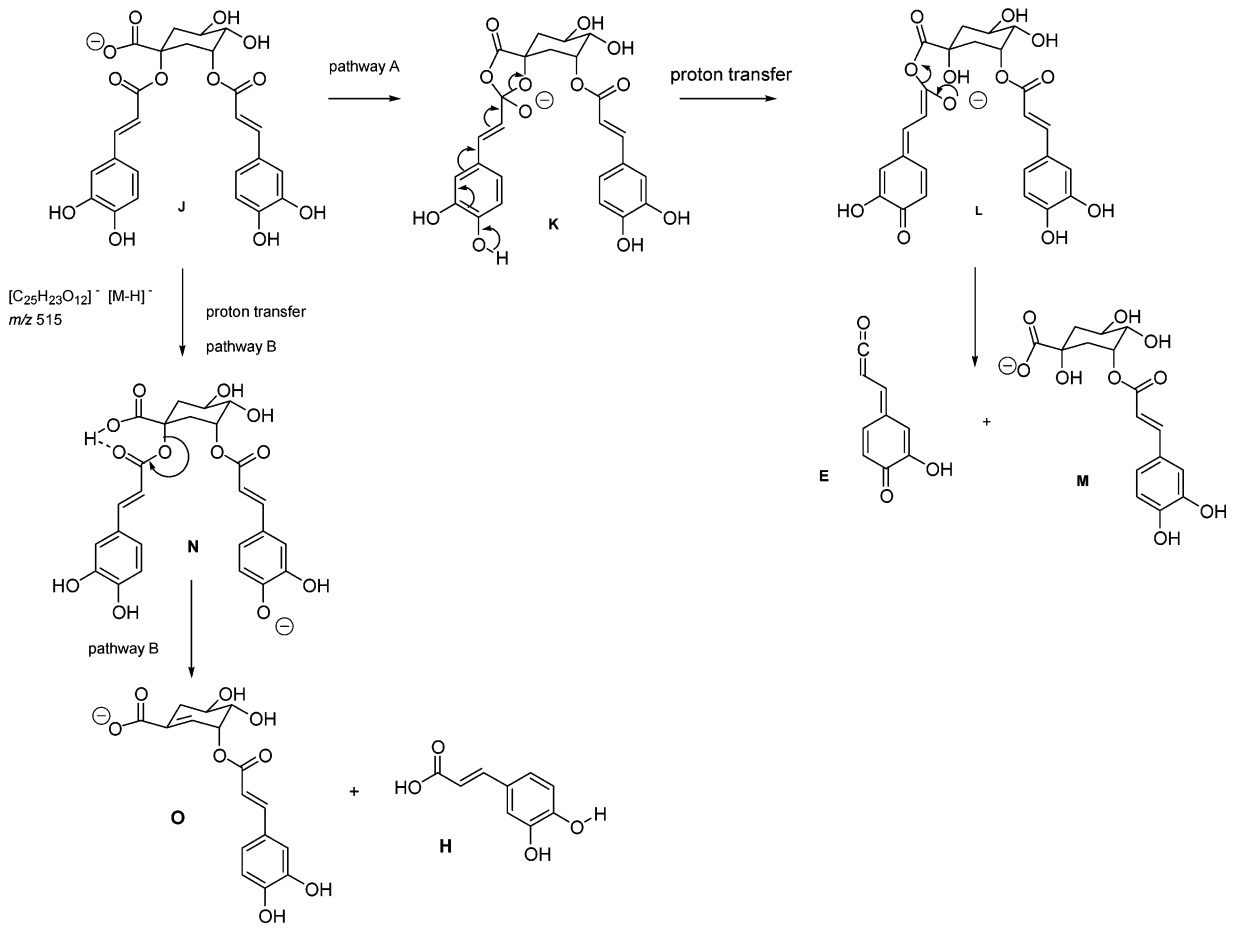
Preliminary studies indicated that 1,5-diCQA (**VII**) was rapidly and extensively converted to 1,3-diCQA (**V**) and 5-CQA (**III**) by treatment with tetramethylammonium hydroxide (TMAH), the substrate having almost disappeared after 1 min at room temperature (22 °C). Because logically conversion of 1,5-diCQA (**VII**) to 1,3-diCQA (**V**) must proceed through 1,4-diCQA (**VI**), the transesterification reaction conditions were optimized by trial and error. The relative yields of the major products as a function of time of treatment are presented in **Figure 2**. These were identified as 1,3-diCQA (**V**) by comparison with an authentic standard and a second, less intense, peak that on C_{18} eluted before 1,5-diCQA (**VII**) (~45 min) but after 1,5-diCQA (**VII**) on the phenylhexyl packing (~55 min). This new product had a parent ion at *m*/*z* 515 and MS^3 and MS^4 base peaks at *m*/*z* 173 and 93, respectively. Such fragments have previously been shown to be diagnostic for all 4-substituted monoacyl and diacyl CGA so far examined (*5*).

Spiking of the TMAH-treated 1,5-diCQA (**VII**) into the green coffee extract established that this peak was chromatographically distinct from 3,4-diCQA (**VIII**) and 4,5-diCQA (**X**) on both the C_{18} and the phenylhexyl packings, indicating that it must be the expected 1,4-diCQA (**VI**) (**Figure 3**). The different sequences of elution were not expected. In general, those CGA with a greater number of free equatorial hydroxyl groups in the

Scheme A



Scheme B



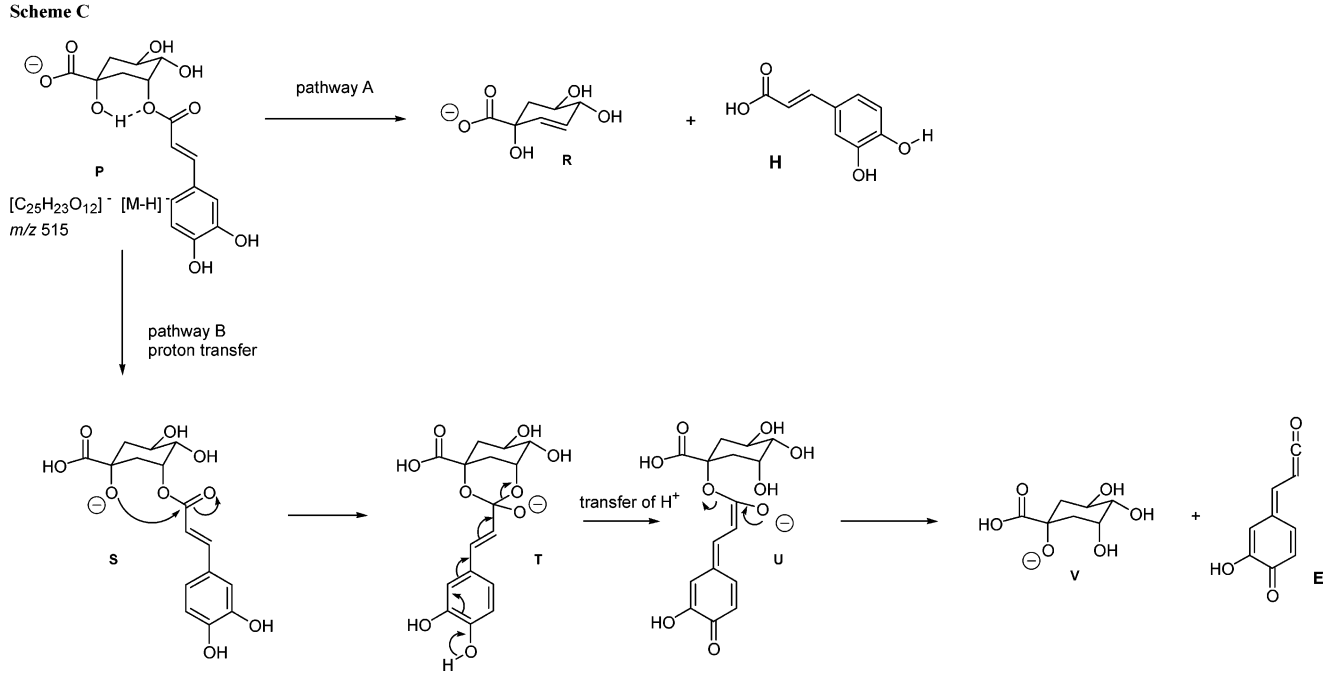


Figure 9. Schemes showing the reactive intermediates.

quinic acid residue are more hydrophilic than those with a greater number of free axial hydroxyl groups (*13*), but it is known that on some packings 4-CQA (**IV**) precedes 5-CQA (**III**), whereas on others the reverse occurs (*4*).

Preliminary studies indicated that treatment of 1,3-diCQA (**V**) with hydrochloric acid (2 M, 2 h, 100 °C) caused extensive degradation. Trial and error optimization of the acid treatment (**Figure 2b**) with LC-MS of the products established that shorter acid treatments produced 1,4-diCQA (**VI**) and three isomeric CQA, each in a low yield.

1,5-DiCQA (**VII**) and 5-CQA (**III**) could not be detected among the products. Two CQA isomers eluted from the phenylhexyl packing at ~12.8 and ~14.5 min, respectively, well in advance of 4-CQA (**IV**) at ~22.7 min (**Figure 3c**). MS^3 fragmentation (**Figure 4**) indicated that the peak at 14.5 min was undistinguishable from 3-CQA (**II**) (*5*), and accordingly the earlier eluting peak was assigned by a process of elimination as the little studied 1-CQA (**I**).

Fragmentation of 1-CQA and the 1-Acyl DiCQA. The MS^n fragmentation data are summarized in **Tables 1** and **2** for CQA and diCQA, respectively. It is not possible to reliably distinguish between 1-CQA and 5-CQA by their fragmentation. Fortunately, 5-CQA is readily available from commercial sources, and 1-CQA can be easily resolved from this, so, in practice, discrimination is straightforward.

The fragmentations observed for 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA (**VIII**−**X**) were identical to those previously reported (*5*), but as discussed below reference is made in this paper to some minor fragments not previously considered to be important. All three 1-acyl diCQA (**V**−**VII**) gave the expected molecular ion [diCQA − H$^+$]$^-$ at *m*/*z* 515 and MS^2 base peak [diCQA-cinnamate − H$^+$]$^-$ at *m*/*z* 353. Consistent with previous studies (*5*) 1,3-diCQA (**V**) and 1,5-diCQA (**VII**) gave the expected MS^3 and MS^4 base peaks at *m*/*z* 191 and 85, respectively, whereas 1,4-diCQA (**VI**) yielded base peaks at *m*/*z* 173 and 93.

From **Table 2**, 1,4-diCQA (**VI**) is easily distinguished by its unique MS^2 fragments including comparatively intense (>50% base peak) ions at *m*/*z* 299 and 203 supported by less intense fragments at *m*/*z* 317 (~35%) and *m*/*z* 255 (~22%). The fragment at *m*/*z* 203 is produced weakly (<15% base peak) by all other isomers except 1,3-diCQA (**V**). The other three ions are unique to 4-acyl diCQA but, in 3,4-diCQA (**VIII**) and 4,5-diCQA (**X**), none exceed 15% of base peak. Because 3,4-diCQA (**VIII**) has a comparatively intense MS^2 fragment at *m*/*z* 335, which is not seen in the MS^2 spectrum of 4,5-diCQA (**X**) (in Figure 10 of ref *5*, this point is incorrectly stated but is correctly illustrated in Figure 7), these factors collectively discriminate between the three 4-acyl diCQA. The remaining three isomeric diCQA (**V**, **VII**, and **IX**) can be distinguished by the relative intensity of the MS^2 fragment at *m*/*z* 335 (1,3-diCQA, ~35%; 1,5-diCQA, ~7%; and 3,5-diCQA, not detectable). The MS^1, MS^2, MS^3, and MS^4 spectra are illustrated in **Figures 5**−**8**.

Previous studies (*5*) led to the conclusion that during diCQA fragmentation the C5 caffeoyl is the most easily removed and the C4 caffeoyl is the most stable, but the 1-acyl diCQA molecules were not examined, and the relative stability of the C1 caffeoyl could not be estimated. If the C1 caffeoyl were the most stable, then the MS^2 and MS^3 fragmentations for the three 1-acyl diCQA (**V**−**VII**) would be identical because the MS^2 base peak would be [1-CQA − H$^+$]$^-$ in each case. From **Table 1** and the discussion above, it is clear that this is not the case. Comparison of MS^2 and MS^3 fragmentation data (**Table 1**) for the CQA (**I**−**IV**) with the MS^3 and MS^4 fragmentation for the 1-acyl diCQA (**Table 2**) indicates perfect matches for 1,3-diCQA (**V**) with 3-CQA (**II**), for 1,4-diCQA (**VI**) with 4-CQA (**IV**), and for 1,5-diCQA (**VII**) with both 1-CQA (**I**) and 5-CQA (**III**). This demonstrates clearly that the caffeoyl moiety at C1 is more easily removed than the caffeoyl residues at C3 or C4, but because 1-CQA (**I**) and 5-CQA (**III**) fragment identically, it is not possible to determine whether the MS^2 base peak produced from 1,5-diCQA (**VII**) is [1-CQA − H$^+$]$^-$ or [5-CQA − H$^+$]$^-$, and thus one cannot judge on this basis which caffeoyl residue is more easily removed.

Mechanisms of Fragmentation. We have reconsidered the mechanisms of fragmentation and no longer favor the radical anion pathway originally proposed (*5*). We now suggest that in order to rationalize the observed fragmentations, two alternative

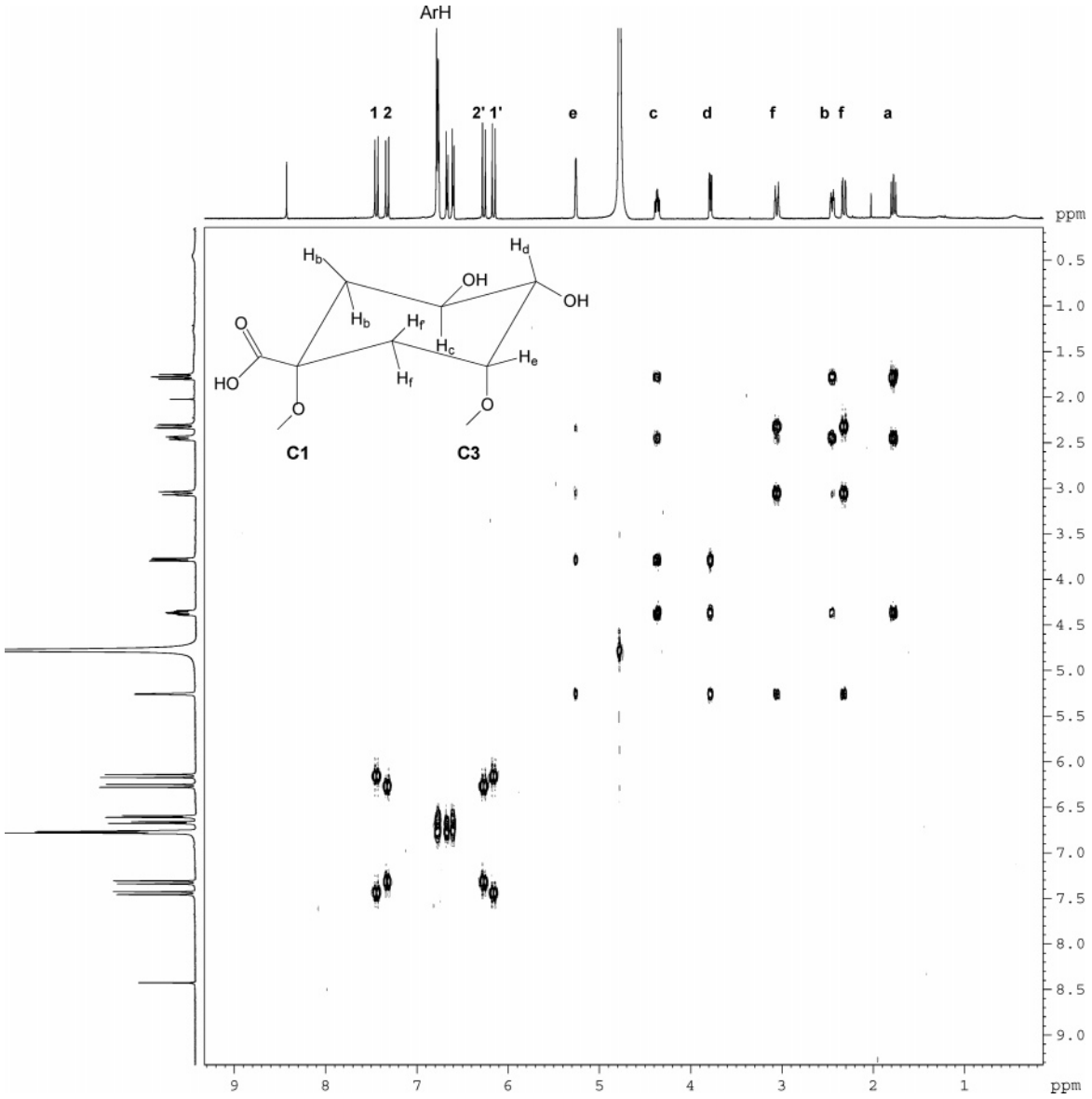


Figure 10. ^{1}H–^{1}H COSY NMR spectrum (500 MHz) of 1,3-diCQA (**V**) in D_2O.

pathways must be considered. Which pathway operates depends crucially upon the site of the negative charge within the molecular ion. Although the initial ionization will occur at the most acidic moiety within the molecule (the COOH in the case of all quinic acid derivatives), it may subsequently migrate. In either case it leads to the formation of an anion $[M - H^+]^-$ at *m*/*z* 515. Because a priori we cannot eliminate either possibility, we therefore present both pathways and discuss in detail their individual merits.

When the negative charge is located at the carboxyl in **A**, we suggest that the comparatively easy loss of the C5 caffeoyl residue can be rationalized by assuming an acyl transfer mechanism (**Figure 9**, **Scheme A**, pathway A). In a 1,3-*syn*-diaxial chair conformation the COO^- acts as a nucleophile and attacks the C5 acyl carbonyl to form a bicyclic tetrahedral intermediate **B**. Loss of a proton from the *p*-OH on the 5-acyl moiety (most likely via an intramolecular proton shift because no dianion fragments could be observed in any mass spectra) leads to a breakdown of the tetrahedral intermediate to give **C**. Further fragmentation leads to the formation of the deacylated quinic acid fragment ion **D** and most likely a neutral quinone ketene **E**.

The alternative scenario envisages fragmentation driven by an anion located other than on the quinic carboxyl. Although an initial ionization at this other site cannot be excluded unequivocally, the alternative anion is more likely to form after migration of the negative charge from the carboxyl by inter- or intramolecular proton transfer. As a representative example **Figure 9**, **Scheme A**, shows the negative charge located on a particular phenolic oxygen **F**, but other sites for the negative charge, such as **G**, cannot be excluded. Such proton transfers to non-carboxylic sites have been postulated (*15*) for serine, threonine, or tyrosine residues during ESI mass spectrometry of proteins. The C1 carboxyl and the C5 caffeoyl again have the 1,3-*syn*-diaxial arrangement (**Figure 9**, **Scheme A**, pathway B), and the comparatively easy loss of the C5 caffeoyl can be rationalized (*5*) by transfer of the proton from the C1 carboxyl, while in an inverted chair, to the acyl oxygen of the C5 substituent as shown in **F** and **G**. Deacylation facilitated by protonation of the C5 acyl substituent would give neutral caffeic acid **H** and, after proton transfer, anionic fragment **I** with *m*/*z* 335.

The comparatively easy loss of the C1 acyl group could be explained by an analogous mechanism (**Figure 9**, **Scheme B**,

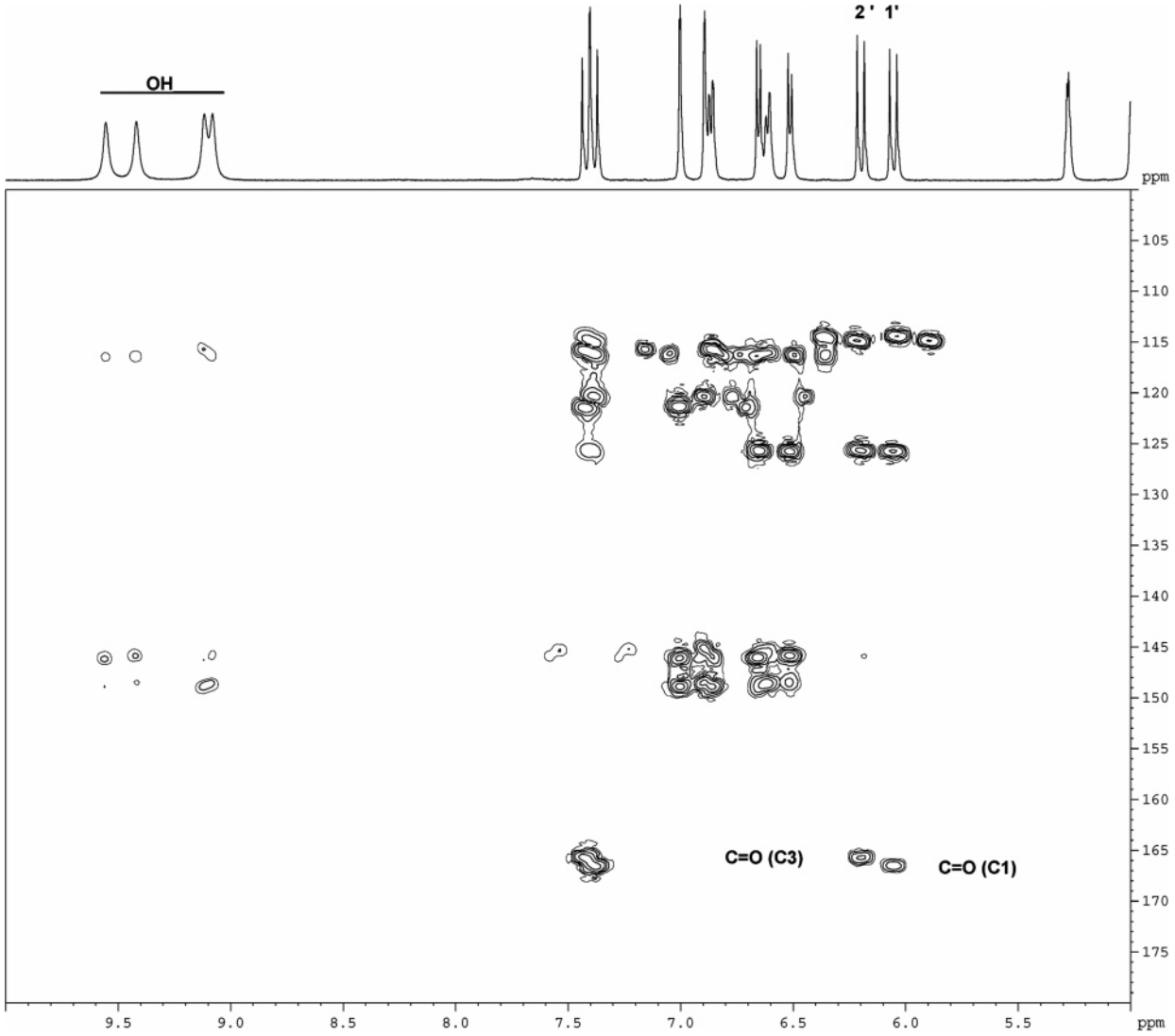


Figure 11. ^{1}H–^{13}C HMBC NMR (500 MHz) of 1,3-diCQA (**V**) in DMSO-d_6 expanded to show aromatic, olefinic, and phenolic protons.

pathway A). Again, after initial formation of COO^- to give **J**, nucleophilic attack at the C1 acyl carbonyl leads to the formation of a spirocyclic tetrahedral intermediate **K**, which after breakdown of the tetrahedral carbon gives **L**. Loss of a proton (again presumably via an intramolecular proton shift) gives a deacylated fragment ion **M** and a neutral quinone ketene **E**. Both modes of attack (7-*exo-trig* for attack at the C5 acyl moiety and 5-*exo-trig* for attack at the C1 acyl moiety) are allowed by Baldwin rules. Because we never observed a fragment ion at *m*/*z* 161, corresponding to $[\mathbf{E} - \mathbf{H}]^-$, it can be inferred that the negative charge in **C** and **L** is not transferred to the ketene **E**.

In the 1-acyl diCQA (**V**−**VII**) we suggest again as an alternative pathway that the C1 carboxyl can, after proton transfer to give **N**, protonate the C1 caffeoyl group (**Figure 9**, **Scheme B**, pathway B). Because this occurs in the preferred carboxy-equatorial conformation of the quinic acid moiety, the C1 caffeoyl might be more rapidly removed than the C5 caffeoyl that requires the thermodynamically less favored carboxy-axial conformation. Deacylation from **N** is again facilitated by protonation of the C1 acyl moiety and gives fragment ion **O** and neutral caffeic acid **H**.

This hypothesis is supported by a close inspection of NMR and IR spectral data for 1,3-diCQA (**V**) (*16*). In the ^{1}H NMR and ^{13}C NMR spectra of 1,3-diCQA (**V**) in a variety of solvents, such as D_2O, DMSO-d_6 and MeOH-d_4, differences are observed

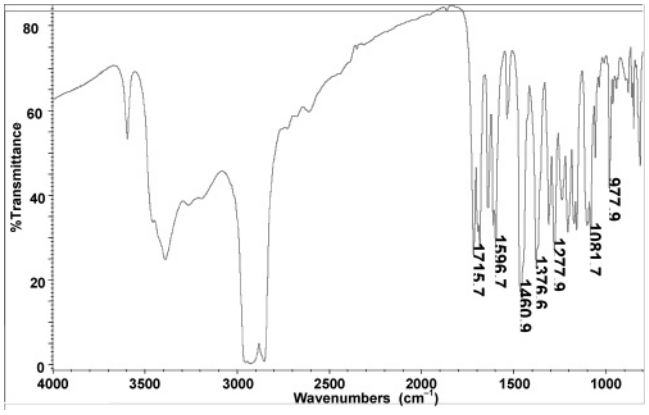


Figure 12. Infrared spectrum (4000 to 800 cm^{-1}) of 1,3-diCQA (**V**) in Nujol.

in the chemical shifts for the two acyl moieties. In each of the NMR spectra the resonances for the two caffeoyl residues appear at distinct chemical shifts. ^{1}H−^{1}H COSY analysis (**Figure 10**) and ^{1}H−^{13}C HMBC spectra (**Figure 11**) clearly indicate that the olefinic signals of one caffeoyl residue are shifted significantly downfield compared with the signals for the second caffeoyl residue. This can be interpreted in terms of hydrogen bonding of the C1 carboxyl to one of the caffeoyl residues (*17*,

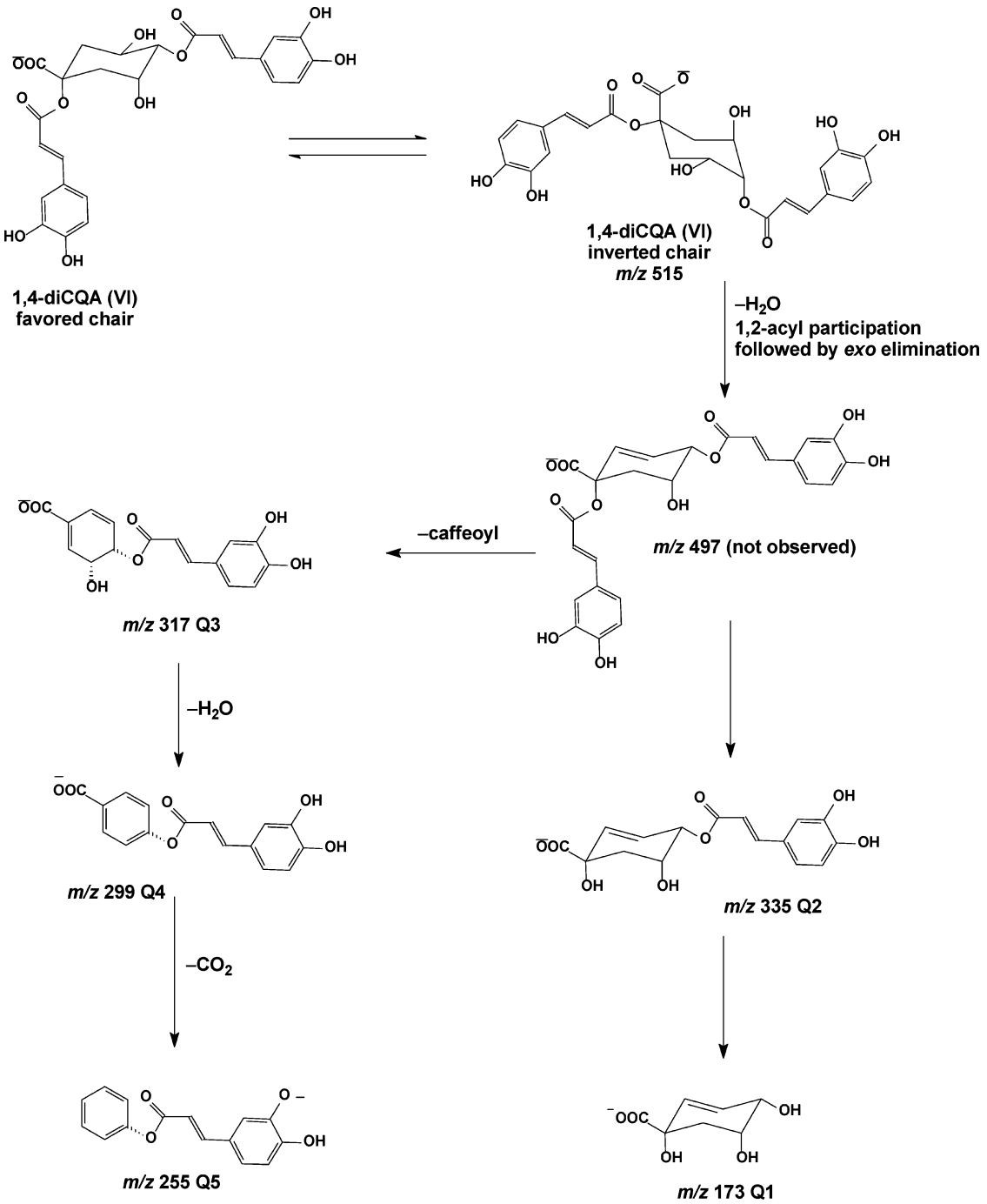


Figure 13. Structures of the dicaffeoylquinic acid derived fragments.

18). Unfortunately, the low resolution of the $^{1}H-^{13}C$ HMBC spectrum does not allow unambiguous identification of the two C═O resonances connected to the olefinic moieties. In the ^{1}H NMR spectrum in DMSO-d_6, the resonance for the carboxyl COOH proton is observed at 13.8 ppm, considerably downfield compared with this signal in quinic acid (**Q**) and 3-CQA (**II**), again indicating hydrogen bonding (*17*, *18*). Molecular modeling at the MM-2 level indicates that for 1-CQA (**I**) there are two minimized energy structures involving hydrogen bonds between the equatorial C1 carboxyl (COOH) and the axial C1 caffeoyl residue that differ by only 0.5 kJ·mol^{-1}. In the first structure, the hydrogen bonds to the C═O carbonyl oxygen and in the second to the O−C═O ester oxygen.

Furthermore, the IR spectrum (**Figure 12**) of 1,3-diCQA (**V**) is consistent with a hydrogen bond between the equatorial C1 carboxyl and the axial C1 caffeoyl residue. In the OH region, four broad bands around 3390 cm^{-1} and a sharp band at 3596 cm^{-1} are observed. Although hydrogen bonding is usually associated with broadened bands in the IR spectrum, there are a series of literature examples in which strong intramolecular hydrogen bonds are manifested by sharp bands at higher wavenumbers (*19*−*21*). It is worth noting that this sharp band is absent in the IR spectrum of 5-CQA (**III**) (*22*). The carbonyl region in the IR spectrum of 1,3-diCQA (**V**) strongly supports the hypothesis of a C1 carboxyl-ester oxygen hydrogen bond. Three C═O absorptions (**Figure 12**) are observed in the carbonyl region at 1715, 1683, and 1638 cm^{-1}. The absorption at 1715 cm^{-1} can be assigned to the ester C═O of the C3 caffeoyl by comparison with the C═O absorption of *trans*-methylcinnamate at 1722 cm^{-1} (*23*). The absorption at 1638 cm^{-1} can be assigned to the C═O absorption of the C1 carboxyl by comparison with the C═O absorption in 5-CQA (**III**) at 1640

	Criterion	Identification	Action
1	Parent ion *m/z* 337	***p*-Coumaroylquinic acids**	***Go to reference (5)***
	Parent ion *m/z* 353	**Caffeoylquinic acids**	***Go to 2***
	Parent ion *m/z* 367	**Feruloylquinic acids**	***Go to reference (5)***
	Parent ion *m/z* 515	**Dicaffeoylquinic acids**	***Go to 3***
	Parent ion *m/z* 529	**Caffeoyl-feruloylquinic acids**	***Go to reference (5)***
2	MS^2 base peak *m/z* 191, and relatively intense (*ca* 50% base peak) MS^2 ion at *m/z* 179	**3-Caffeoylquinic acid (II)**	
	MS^2 base peak *m/z* 173	**4-Caffeoylquinic acid (IV)**	
	MS^2 base peak *m/z* 191, and weak or undetectable (<5% base peak) MS^2 ion at *m/z* 179	**5-Caffeoylquinic acid (III) or 1-Caffeoylquinic acid (I)**	**Distinguish by retention time on reverse phase packing**
3	MS^3 base peak *m/z* 173	**4-Acyl dicaffeoylquinic acids**	***Go to 4***
	MS^3 base peak *m/z* 191	**Dicaffeoylquinic acids NOT substituted at position 4**	***Go to 5***
4	Strong (>50% base peak) MS^1 ions at *m/z* 299 and *m/z* 203	**1,4-Dicaffeoylquinic acid (VI)**	
	Weak (*ca* 15%) MS^1 ion at *m/z* 335 and strong (>50% base peak) MS^2 ion at *m/z* 179	**3,4-Dicaffeoylquinic acid (VIII)**	
	MS^1 ion at *m/z* 335 undetectable, strong (>50% of base peak) MS^2 ion at *m/z* 179	**4,5-Dicaffeoylquinic acid (X)**	
5	MS^1 ion at *m/z* 335 (>30% of base peak) and strong (>50% of base peak) MS^2 ion at *m/z* 179	**1,3-Dicaffeoylquinic acid (V)**	
	Weak MS^1 ion at *m/z* 335 (<10% of base peak) and weak MS^2 ion at *m/z* 179 (<10% of base peak)	**1,5-Dicaffeoylquinic acid (VII)**	
	MS^1 ion at *m/z* 335 undetectable, strong (<50% of base peak) MS^2 ion at *m/z* 179	**3,5-Dicaffeoylquinic acid (IX)**	

Figure 14. Hierarchical key for the identification by LC-MS^n of caffeoylquinic and dicaffeoylquinic acids including those substituted at position 1.

cm^{-1}. Therefore, by a process of elimination, the third C═O absorption at 1683 cm^{-1} has to be assigned to the C═O absorption of the C1 caffeoyl residue. From the observed wavenumber it clearly follows by comparison with *trans*-methylcinnamate and 5-CQA that the absorption is shifted toward lower wavenumbers, indicative of a weakening of the C═O bond and hence hydrogen bonding between the equatorial C1 carboxyl and the axial C1 caffeoyl residue.

Additional information on the two alternative fragmentation mechanisms for loss of a caffeoyl moiety from either C1 or C5 has been obtained from an experiment in which the pH value was varied. For a proton transfer process the intensities of fragment ions observed should increase at a lower pH. In contrast, for a COOH deprotonation ionization mechanism the intensities of fragment ions observed should increase at a higher pH of the ESI solution. By omitting the acetic acid cosolvent from the LC-MS experiment, the chromatographic resolution of the CQAs was affected; however, no change in the relative intensities of the fragment ions was observed within experimental error.

Although the fragmentations observed for 1-CQA (**I**) and 5-CQA (**III**) are indistinguishable one from the other, they are quite different from the fragmentations previously observed for 3-CQA (**II**) and 4-CQA (**IV**) (*5*). This is in keeping with the fragmentation of 3-CQA (**II**) and 4-CQA (**IV**) occurring via mechanisms quite different from nucleophilic attack by COO^- or protonation by the quinic acid carboxyl operative for the caffeoyl residues at C1 and C5.

Fragmentations of 3-CQA and related diCQA can be explained by arguments similar to those above (**Figure 9**, **Scheme C**, pathway A). The only 1,3-*syn*-diaxial arrangement available to the caffeoyl substituent in 3-CQA (**II**) involves the C1 hydroxyl (**Figure 9**, **Scheme C**, pathway A), and the high p*K* of the hydroxyl (pK >15) relative to the carboxyl (pK ≈ 3.5) explains why 3,5-diCQA (**IX**) loses the residue at C5 prior to the residue at C3. Starting from the $[M - H]^-$ ion **P**, protonation of the C3 acyl group by the C1 hydroxyl group would give fragment ion **R** and neutral caffeic acid **H**. Alternatively, after intramolecular proton transfer, the negative charge could be located at the C1 oxygen in **S**, which after nucleophilic attack on the C3 acyl carbonyl carbon would give tetrahedral intermediate **T**. Breakdown of this intermediate would lead via **U** to the deacylated anion **V** and quinone ketene **E**. It is worth pointing out that the fragmentation mechanisms suggested here constitute more detailed extensions of those reported previously (*5*).

In summary, the ease of loss for caffeoyl moieties esterified to quinic acid is determined by the relative stereochemical relationships between the caffeoyl substituents and the other functionalities, in particular the C1 carboxyl and C1 hydroxyl. The comparatively facile loss of a caffeoyl moiety from C1 or C5 of the diCQAs can be rationalized in terms of an acyl transfer mechanism initiated by a nucleophilic carboxylate COO^- or alternatively by a protonation mechanism involving the acidic COOH at C1. The comparatively more difficult loss of a caffeoyl moiety from C3 can be rationalized in terms of protonation of the C3 caffeoyl substituent by the C1 hydroxyl or alternatively by an acyl transfer mechanism involving the nucleophilic C1 alcoholate. Loss of water is preferred for a caffeoyl substituent at C4 as discussed previously (*5*). All proposed mechanisms are in agreement with the observed mass spectra and constitute a very useful tool for the prediction of fragmentation patterns in structurally related molecules. Further experiments are required to distinguish unambiguously between alternative mechanisms.

Whatever the pathway(s), the formation of "dehydrated" fragments [**Figure 13** *m/z* 335 ($\mathbf{Q_2}$) and *m/z* 173 ($\mathbf{Q_1}$)] is characteristic of 4-acyl CGA (*5*), and the fragments at *m/z* 317

(**Figure 13**, $\mathbf{Q_3}$) and *m*/*z* 299 seen clearly during fragmentation of 1,4-diCQA (**V**) continue this sequence, leading to full aromatization of the quinic acid residue and a caffeoylbenzoic acid structure (**Figure 13**, $\mathbf{Q_4}$). Such dehydration has been postulated (*24*) during pyrolysis of 5-CQA (**III**). The fragment at *m*/*z* 255 probably arises by subsequent loss of carbon dioxide (**Figure 13**, $\mathbf{Q_5}$) from the caffeoylbenzoic acid. The fragment at *m*/*z* 203 was trapped and further fragmented, yielding progressively ***m*/*z*** 175, 147, and 119 as base peaks, the latter accompanied by *m*/*z* 129 (~45%).

The peak at *m*/*z* 203 could result from loss of C_4H_4 from $\mathbf{Q_5}$. This fragmentation is highly unusual and has no literature precedent to our knowledge. It might involve a rearrangement followed by a retro-Diels−Alder process. The fragments at *m*/*z* 175, 147, and 119 could form from the sequential consequential loss of three CO units. Loss of CO from esters and phenols is well precedented in EI mass spectrometry. The peak at *m*/*z* 129 should occur due to loss of water from *m*/*z* 147.

Dehydration accompanying elimination of a moiety at C4 is thought to involve a conformer that allows 1,2-acyl participation by the *trans*-vicinal acyl moiety on C4, thus facilitating the formation of a bicyclic oxonium radical anion by loss of OH from C5 assisted by protonation in the inverted chair conformation and subsequent loss of the caffeoyl at C4 or C1. For chemical arguments we favor elimination of the caffeoyl at C1. Loss of the C4 caffeoyl would lead to an enolether−enone pair of tautomers, which are not amenable to further dehydration and aromatization. Furthermore, retention of the caffeoyl at C1 would require caffeoyl migration prior to aromatization. In contrast, loss of the C1 caffeoyl would, after subsequent dehydrations involving loss of the C3 OH from fragment ($\mathbf{Q_3}$), produce the benzoic acid ($\mathbf{Q_4}$) fragment ion. To obtain a final proof of this hypothesis would require independent synthesis and investigations into the fragmentation of $\mathbf{Q_2}-\mathbf{Q_5}$, and is beyond the scope of the present investigation.

Revised Hierarchical Key for DiCQA. Figure 14 incorporates revisions to the hierarchical key (*5*) developed for CGA not substituted at position 1. It is clear that all six of the diCQA can be distinguished by LC-MS^3 with further confirmation furnished at MS^4. 1-CQA and 5-CQA cannot be distinguished by MS fragmentation, but the greater hydrophobicity of 5-CQA and its commercial availability ensure that the two can be distinguished by retention time on reversed phase packings.

ACKNOWLEDGMENT

Technical assistance from H. Roozendaal is gratefully acknowledged.

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Received for review January 10, 2005. Revised manuscript received March 14, 2005. Accepted March 23, 2005.

JF050046H