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Profiling and Characterization by LC-MS^n of the Galloylquinic Acids of Green Tea, Tara Tannin, and Tannic Acid

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Green tea, tara tannin, and tannic acid have been profiled for their contents of galloylquinic acids using LC-MS^8. These procedures have provided evidence for the first observation of (i) 1-galloylquinic acid (**11**), 1,3,5-trigalloylquinic acid (**22**), 4-(digalloyl)quinic acid (**28**), 5-(digalloyl)quinic acid (**29**), and either 3-galloyl-5-(digalloyl)quinic acid (**32**) or 3-(digalloyl)-5-galloylquinic acid (**33**) from any source; (ii) 4-galloyl-5-(digalloyl)quinic acid (**34**), 5-galloyl-4-(digalloyl)quinic acid (**35**), 3-(digalloyl)-4,5-digalloylquinic acid (**41**), 4-(digalloyl)-3,5-digalloylquinic acid (**40**), 5-(digalloyl)-3,4-digalloylquinic acid (**39**), and 1,3,4-trigalloylquinic acid (**21**) from tara tannin; and (iii) 3-galloylquinic acid (**12**) and 4-galloylquinic acid (**14**) from green tea. The first mass spectrometric fragmentation data are reported for galloylquinic acids containing between five and eight gallic acid residues. For each of these mass ranges at least two isomers based on the 1,3,4,5-tetragalloylquinic acid core (**25**) and at least three based on the 3,4,5-trigalloylquinic acid core (**24**) were observed. Methanolysis of tara tannin yielded methyl gallate, methyl digallate, and methyl trigallate, demonstrating that some of these galloylquinic acids contained at least one side chain of up to four galloyl residues.

KEYWORDS: Caesalpinia; depsides; digalloylquinic acids; galloylquinic acids; green tea; LC-MS^n; polygalloylquinic acids; tannic acid; tara tannin; tetragalloylquinic acids; trigalloylquinic acids

INTRODUCTION

Classically, chlorogenic acids are a family of esters formed between quinic acid and certain *trans*-cinnamic acids, most commonly caffeic, *p*-coumaric, and ferulic (*1*−*3*). In the IUPAC system (−)-quinic acid is defined as 1L-1(OH),3,4/5-tetrahydroxycyclohexane carboxylic acid, but Eliel and Ramirez (*4*) recommend 1α,3*R*,4α,5*R*-tetrahydroxycyclohexane carboxylic acid. Structurally related, but much less studied, are similar esters between quinic acid and gallic acid (3,4,5-trihydroxybenzoic acid). Structures are shown in **Figure 1**. The most extensively studied sources of galloylquinic acids are tara tannin (from the pods of the leguminous tree *Caesalpinia spinosa*) (*5*−*7*) and green and black tea (leaves of *Camellia sinensis*) (*8*, *9*). Other genera in which they have been found are *Bersonima* (*10*), *Castanopsis (11*), *Galphimia* (*12*), *Guiera* (*13*, *14*), *Lepidobotrys (15*), *Myrothamnus (16*), *Pistacia (17*, *18*), and *Quercus (19*, *20*), where they may contribute to the pharmacological properties of derived herbal preparations (*21*). Galloylquinic acids have also been isolated from Chinese and Turkish galls (*22*) that classically are considered to be rich in galloylglucoses rather than galloylquinic acids (*5*, *23*). In tara tannin, acylation of the quinic acid residue has been reported only at C3, C4, and C5 (*6*), but in galls (*22*), *Guiera (13*), *Galphimia (12*), *Lepidobotrys* (*15*), and some (*19*) but not all (*20*) *Quercus* spp. acylation has been reported also at C1.

Galloylquinic acids are potentially more complex than the classic chlorogenic acids because not only may gallic acid residues be attached at each of the four hydroxyls in the quinic acid moiety but these may form aryl ester(s) (depsides) with one or more additional gallic acid residues (*5*, *6*, *18*). Chains of up to three gallic acid residues, of which two are depsidic, have been reported (*6*), and a given quinic acid may bear more than one depside chain (*22*). Theoretically, depsidic linkages may be either meta or para, but it has been demonstrated that in aqueous solution the depsidic residues may move easily between meta and para positions, existing as an equilibrium mixture (*23*).

LC-MS^n has been used with considerable success for characterizing the classic chlorogenic acids, for example, discriminating between six caffeoylferuloylquinic acid isomers (*24*) and the six dicaffeoylquinic acids (*25*), and structure−diagnostic hierarchical keys have been developed and used to characterize seven new classes of chlorogenic acids in coffee (*25*, *26*). In this study we applied these methods to the profiling and characterization of galloylquinic acids in green tea, tara tannin, and tannic acid.

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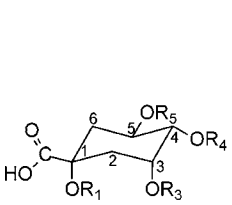

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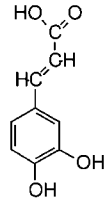

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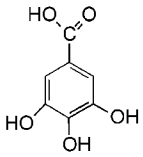

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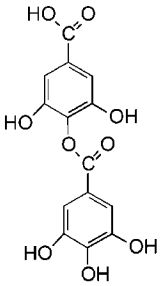
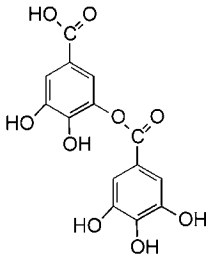


p-diG



m-diG

Name	Number	R_1	R_3	R_4	R_5	M_r	Detected[a]
1-*O*-caffeoylquinic acid	1	C	H	H	H	354	
3-*O*-caffeoylquinic acid	2	H	C	H	H	354	
5-*O*-caffeoylquinic acid	3	H	H	H	C	354	
4-*O*-caffeoylquinic acid	4	H	H	C	H	354	
1,3-di-*O*-caffeoylquinic acid	5	C	C	H	H	516	
1,4-di-*O*-caffeoylquinic acid	6	C	H	C	H	516	
1,5-di-*O*-caffeoylquinic acid	7	C	H	H	C	516	
3,4-di-*O*-caffeoylquinic acid	8	H	C	C	H	516	
3,5-di-*O*-caffeoylquinic acid	9	H	C	H	C	516	
4,5-di-*O*-caffeoylquinic acid	10	H	H	C	C	516	
1-*O*-galloylquinic acid	11	G	H	H	H	344	+
3-*O*-galloylquinic acid	12	H	G	H	H	344	+
5-*O*-galloylquinic acid	13	H	H	H	G	344	+
4-*O*-galloylquinic acid	14	H	H	G	H	344	+
1,3-di-*O*-galloylquinic acid	15	G	G	H	H	496	
1,4-di-*O*-galloylquinic acid	16	G	H	G	H	496	
1,5-di-*O*-galloylquinic acid	17	G	H	H	G	496	
3,4-di-*O*-galloylquinic acid	18	H	G	G	H	496	+
3,5-di-*O*-galloylquinic acid	19	H	G	H	G	496	+
4,5-di-*O*-galloylquinic acid	20	H	H	G	G	496	+
1,3,4-tri-*O*-galloylquinic acid	21	G	G	G	H	648	(+)
1,3,5-tri-*O*-galloylquinic acid	22	G	H	G	G	648	(+)
1,4,5-tri-*O*-galloylquinic acid	23	G	H	G	G	648	
3,4,5-tri-*O*-galloylquinic acid	24	H	G	G	G	648	
1,3,4,5-tetra-*O*-galloylquinic acid	25	G	G	G	G	800	
1-*O*-(digalloyl)quinic acid	26	diG	H	H	H	344	
3-*O*-(digalloyl)quinic acid	27	H	diG	H	H	344	
4-*O*-(digalloyl)quinic acid	28	H	H	diG	H	344	
5-*O*-(digalloyl)quinic acid	29	H	H	H	diG	344	
3-*O*-galloyl, 4-*O*-(digalloyl)quinic acid	30	H	G	diG	H	648	
4-*O*-galloyl, 3-*O*-(digalloyl)quinic acid	31	H	diG	G	H	648	
3-*O*-galloyl, 5-*O*-(digalloyl)quinic acid	32	H	G	H	diG	648	(+)
5-*O*-galloyl, 3-*O*-(digalloyl)quinic acid	33	H	diG	H	G	648	

Name	Number	R_1	R_3	R_4	R_5	M_r	Detected[a]
4-*O*-galloyl, 5-*O*-(digalloyl)quinic acid	34	H	H	G	diG	648	(+)
5-*O*-galloyl, 4-*O*-(digalloyl)quinic acid	35	H	H	diG	G	648	
3,4-di-*O*-(digalloyl)quinic acid	36	H	diG	diG	H	800	
3,5-di-*O*-(digalloyl)quinic acid	37	H	diG	H	diG	800	
4,5-di-*O*-(digalloyl)quinic acid	38	H	H	diG	diG	800	
3,4-di-*O*-galloyl, 5-*O*-(digalloyl)quinic acid	39	H	G	G	diG	800	(+)
3,5-di-*O*-galloyl, 4-*O*-(digalloyl)quinic acid	40	H	G	diG	G	800	(+)
3-*O*-(digalloyl), 4,5-di-*O*-galloylquinic acid	41	H	diG	G	G	800	(+)

Q = quinic acid, C = caffeic acid, G = gallic acid, diG = digalloylquinic acid

Figure 1. Structures of selected chlorogenic acids and galloylquinic acids (IUPAC nomenclature) (*1*). [a] Substances detected during this study for which strong evidence is presented are marked "+". The symbol "(+)" indicates that the assignment is tentative.

MATERIALS AND METHODS

Samples for Analysis. A commercial preparation of green tea polyphenols (Hunan Kinglong Bioresource Co. Ltd., Hunan, China), commercial tara tannin (Roy Wilson Dickson, Alrewas, Burton-on-Trent, Staffordshire, U.K.), and tannic acid of unspecified botanical source (Sigma, Gillingham, U.K.) were used as convenient sources of galloylquinic acids. The tea polyphenols and tannic acid were dissolved in solvent A [water/acetonitrile/glacial acetic acid (980:20:5 v/v, pH 2.68) at 1 mg/mL. The crude commercial tara tannin (1 g) was extracted with 70% v/v aqueous methanol as previously described (*24*) (4 × 25 mL, 25 min each) using an HT-1043 solid−liquid continuous extraction system (Tecator, Bristol, U.K.). The bulked extracts were treated with Carrez reagents (1 mL of reagent A plus 1 mL of reagent B) (*27*) to precipitate colloidal material, diluted to 100 mL with 70% v/v aqueous methanol, and filtered through a Whatman no. 1 filter paper. The methanol was removed by evaporation with nitrogen and the aqueous extract stored at −12 °C until required, thawed at room temperature, centrifuged (1360*g*, 10 min), and used directly for LC-MS.

Methanolyzed samples of tara tannin were prepared by refluxing 1 g of tara tannin in a 50:50 mixture of methanol and 0.05 M acetate buffer (pH 5.5) (*23*). Samples were taken at 5, 10, 20, 30, and 60 min and thereafter at hourly intervals up to 6 h. The samples were filtered, the methanol was removed under nitrogen, and the aqueous extract was stored at −12 °C until required, thawed at room temperature, centrifuged (1360*g*, 10 min), and used directly for LC-MS.

LC-MS^n. The LC equipment (ThermoFinnigan) 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 data-dependent, full-scan, MS^n mode to obtain fragment ion *m*/*z*. For better discrimination of isomers additional targeted MS^n experiments were performed that focused only on compounds producing a particular parent ion, for example, at *m*/*z* 343 for galloylquinic acids, at *m*/*z* 495 for digalloylquinic acids, at *m*/*z* 647 for trigalloylquinic acids, and up to and including *m*/*z* 1559 for nonagalloylquinic acids. Searches were also made for ellagoylquinic acids (*m*/*z* 493, 645, 795, 797, etc.).

MS operating conditions (negative ion) had been optimized originally using 5-caffeoylquinic acid (**3**) with a collision energy of 35%, an ionization voltage of 3.5 kV, a capillary temperature of 350 °C, a sheath gas flow rate of 65 arbitrary units, and an auxiliary gas flow rate of 10 arbitrary units (*24*). These conditions were evaluated for trigalloylquinic acids (*m*/*z* 647) using the methanolyzed tara tannin and found to be satisfactory.

Galloylquinic acid separations were achieved on a 150 × 3 mm column containing Luna 5 μm phenylhexyl packing (Phenomenex, 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, beginning in 100% A and changing linearly to 70% A at 100 min and 0% A at 105 min. The column was washed for 5 min, returned to 100% A in 5 min, and held for 5 min to re-equilibrate.

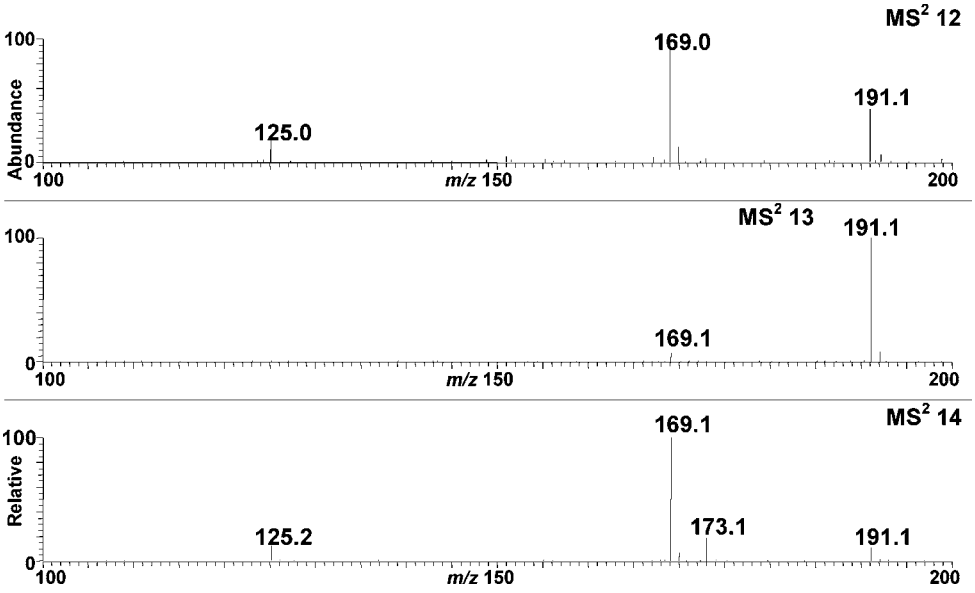


Figure 2. MS^2 spectra of the putative galloylquinic acids (**12**–**14**) in an extract of green tea.

Table 1. MS^3 Fragmentation Data for Galloylquinic Acids (**11**–**14**)

				MS^2 secondary ions											MS^3 secondary ions	
compd	*N*[a]	parent ion *m/z*	MS^2 base peak *m/z*	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	MS^3 base peak *m/z*	*m/z*	intensity
11	3	343.0	169.0	191.0	2			bp[b]	100	150.9	8	125.1	15			
12	3	343.1	169.0	191.1	50			bp	100	150.9	8					
14	3	343.3	169.1	191.1	10	173.1	15	bp	100			125.1	10	125.1		
13	3	343.1	191.1	bp	100			169.0	10					85.1	127.0	95

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

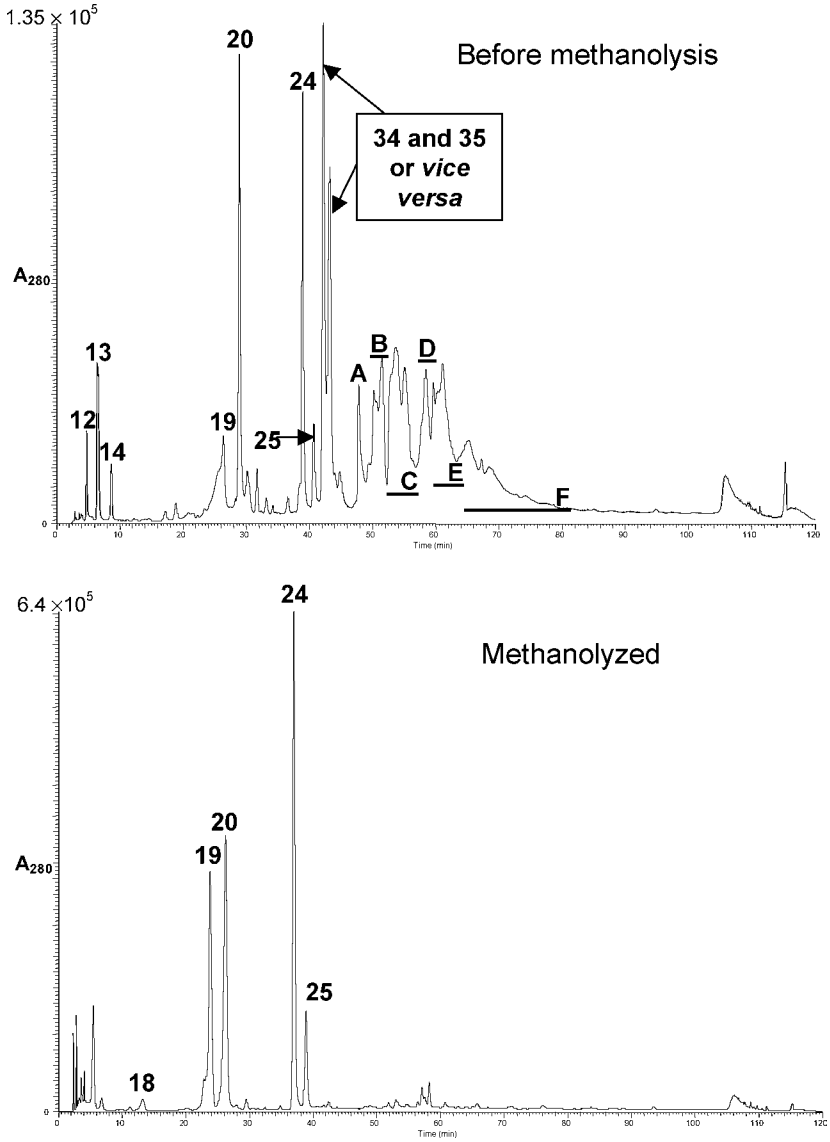


Figure 3. Profile (A_{280}) of a tara tannin before and after methanolysis. Numbered peaks are as in **Figure 1**. A, depsidic tetragalloylquinic acids based on 3,4,5-trigalloylquinic acid core; B, depsidic pentagalloylquinic acids based on 1,3,4,5-tetragalloylquinic acid core; C, depsidic pentagalloylquinic acids based on 3,4,5-trigalloylquinic acid core; D, depsidic hexagalloylquinic acids based on 1,3,4,5-tetragalloylquinic acid core; E, depsidic hexagalloylquinic acids based on 3,4,5-trigalloylquinic acid core; F, hepta- and octagalloylquinic acids. Because of complexity and overlap, the retention times of assignments A–F are approximate.

RESULTS AND DISCUSSION

General LC-MS Chromatographic and Spectroscopic Data. All data for galloylquinic acids presented in this paper use the recommended IUPAC numbering system (*1*), and structures are presented in **Figure 1**. When necessary, for example, refs *7*, *10*, *11*, and *22*, previously published non-IUPAC data have been amended to ensure consistency and avoid ambiguity. Because of the known complexity of the galloylquinic acids in tara tannin, initial studies used a commercial extract of green tea as a convenient source of 5-galloylquinic acid (theogallin) (**13**) (*9*) and methanolyzed tara tannin as a convenient source of mono-, di-, tri-, and tetragalloylquinic acids from which all depsidic gallic acid residues had been removed as methyl gallate (*6*). Peak assignments have been made on the

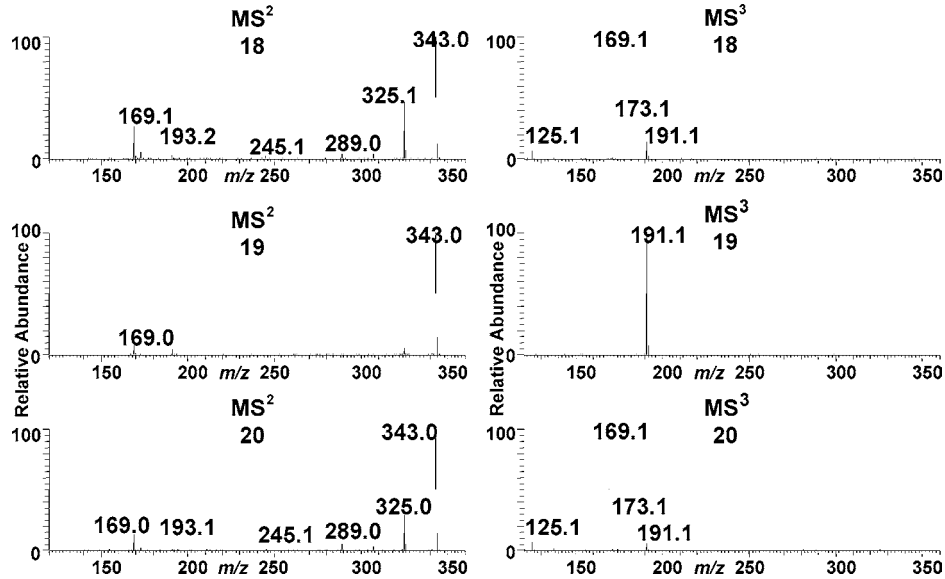


Figure 4. MS^2 and MS^3 spectra of the putative digalloylquinic acids (**18**–**20**) in methanolyzed tara tannin.

Table 2. MS^3 Fragmentation Data for Digalloylquinic Acids (**18**–**20**, **28**, and **29**)

				MS^2 secondary ions									MS^3 secondary ions					
compd	N^a	parent ion *m/z*	MS^2 base peak *m/z*	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	MS^3 base peak *m/z*	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity
18	3	495.0	343.0	325.0	45					169.0	37	169.1	191.1	15	173.1	15	bp^b	100
19	3	495.1	343.0	325.0	5			191.2	5	169.0	12	191.1	bp	100			169.0	8
20	3	495.1	343.0	325.0	20	193.1	15			169.0	20	169.0	191.1	10	173.1	15	b.p.	100
29	3	495.0	343.0									191.1	bp	100			169.0	10
28	3	495.0	343.1									169.0			173.1	15	bp	100

^{a} Number of LC-MS analyses used to collect MS data. ^{b} Occurs as base peak.

basis of the patterns of fragmentation established for various chlorogenic acids (*24*−*26*, *28*) and the relationship between isomer hydrophilicity and the relative number of free equatorial and axial hydroxyl groups in the quinic acid residue (*24*−*26*, *28*−*30*). Ellagoylquinic acids were sought but could not be found.

Galloylquinic Acids in Green Tea. The green tea extract contained three peaks that yielded a parent ion at *m/z* 343 (**Figure 2**; **Table 1**). The dominant isomer produced an MS^2 base peak at *m/z* 191 and an MS^3 base peak at *m/z* 85, consistent with the fragmentation behavior of 5-galloylquinic acid (**13**) as predicted from our previous studies of 5-acyl chlorogenic acids (*24*, *25*, *28*). 5-Galloylquinic acid (**13**) has been previously reported in green tea (*9*). The fastest eluting isomer yielded an MS^2 base peak at *m/z* 169 and a strong (50% of base peak) secondary ion at *m/z* 191 and, accordingly, was assigned as 3-galloylquinic acid (**12**) by analogy with the behavior of 3-caffeoylquinic acid (**2**) (*24*, *28*). The most hydrophobic isomer yielded an MS^2 base peak at *m/z* 169 accompanied by fragment ions at *m/z* 173 (10%) and 191 (10%), suggesting that this is 4-galloylquinic acid (**14**). Although its MS^2 base peak differs from that produced by the four 4-acyl chlorogenic acids previously examined (*m/z* 173) (*24*, *25*), it is the only galloylquinic acid to produce this ion, and this assignment seems to be reasonable. Although all three isomers are known (*11*, *13*, *17*−*20*, *31*), only 5-galloylquinic acid (**13**) has been reported previously in green or black tea. A search in the tea extract for digalloylquinic acids and higher homologues was unsuccessful. Sannomiya et al. (*10*), using a similar mass spectrometer, reported that they could not observe any differences in the fragmentation behavior of the galloylquinic acid isomers. It should be noted that they tuned the mass spectrometer using a flavonoid rather than a chlorogenic acid and used a higher ionization voltage (5 kV compared with 3.5 kV) and a lower collision energy (25% compared with 35%), and these operating differences may have masked the subtle differences between isomers that we have observed consistently (*24*−*26*, *28*, *32*).

Galloylquinic and Digalloylquinic Acids in Methanolyzed Tara Tannin. The methanolyzed tara tannin (6 h) produced a relatively simple chromatogram at 280 nm in which four galloylquinic acids, three digalloylquinic acids, three trigalloylquinic acids, one tetragalloylquinic acid, and methyl gallate were identified by their parent ions. The five major components were compounds **18**−**20**, **24**, and **25** (**Figure 3**). Methyl gallate (*m/z* 183) is formed as depsidic galloyl residues are removed: methyl digallate (*m/z* 335) and methyl trigallate (*m/z* 487) also were observed in samples taken at up to 30 min and indicate that chains of at least four gallic acid residues were present prior to methanolysis. These longer depsidic chains have not previously been reported in tara tannin. Methyl tetragallate was sought but not observed. The fastest eluting galloylquinic acid in methanolyzed tara tannin eluted before 3-galloylquinic acid (**12**) and was not present in the green tea extract. Because there are only four possible locations for methanolysis-stable quinic acid residues, and three of these have already been accounted for, it was assigned as 1-galloylquinic acid (**11**) (**Table 1**), which has not previously been reported so far as we are aware.

The three digalloylquinic acids produced MS^2 base peaks at *m/z* 343 indicating the loss of one galloyl residue. The MS^3 spectra for the fastest and slowest eluting isomers (**Figure 4**; **Table 2**) corresponded exactly with the MS^2 spectrum assigned above to 4-galloylquinic acid (**14**), whereas for the remaining

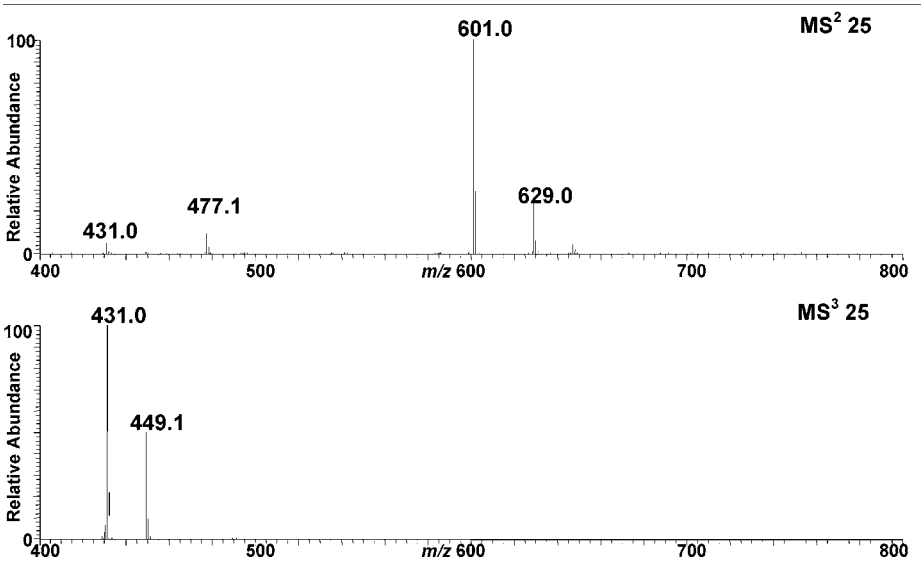


Figure 5. MS^2 and MS^3 spectra of the putative 1,3,4,5-tetragalloylquinic acid (**25**) in methanolyzed tara tannin.

Table 3. MS^5 Fragmentation Data for Tetragalloylquinic Acids (**25** and **39**–**41**)

				MS^2 secondary ions						MS^3 secondary ions											
compd	N^a	parent ion *m/z*	MS^2 base peak *m/z*	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	MS^3 base peak *m/z*	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity
25	6	799.0	601.1	629.0	25			477.1	15	431.0			449.1	45					279.0	15	261.0	18
	6	799.0	647.1			495.0	4			495.0	477.0	6			343.1	6	325.0	4				
39–**41**	6	799.0	647.1			495.1	2			495.1	477.0	8			343.1	4	325.1	6				
	6	799.0	647.1			495.1	2			495.1	477.0	8			343.1	5	325.1	4				

			MS^4 secondary ions															
compd	*N*	MS^4 base peak *m/z*	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity
25	6	167.9							279.1	55	261.0	60	217.2	25			124.1	13
	6	343.0	325.1	40	289.0	6	245.2	5							193.2	10		
39–**41**	6	343.0	325.1	40	289.1	4	245.2	3										
	6	343.0	325.1	42	289.0	4	245.2	4										

			MS^5 secondary ions							
compd	*N*	MS^5 base peak *m/z*	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity
25	6	124.1					140.6	30		
	6	169.1	191.2	16	173.1	2			125.1	14
39–**41**	6	169.0	191.1	16	173.1	12			125.2	20
	6	169.1	191.3	20	173.1	8			125.1	18

[a] Number of LC-MS analyses used to collect MS data.

isomer (**Figure 4**) it corresponded exactly with that produced by 5-galloylquinic acid (**13**). This clearly identified the middle isomer as 3,5-digalloylquinic acid (**19**) and the other two as *vic*-digalloylquinic acids. At MS^2, these both produced a significant (30−40% of base peak) "dehydrated" fragment ion at *m/z* 325, accompanied by weak fragment ions at *m/z* 289 and 245 (∼5%) and 193 (15−30%) [equivalent to *m/z* 299, 255, and 203 in the dicaffeoylquinic acid spectra (*28*)], indicating progressive aromatization of the quinic acid residue and its subsequent decarboxylation. Accordingly, the first and last eluting isomers have been assigned as 3,4-digalloylquinic acid (**18**) and 4,5-digalloylquinic (**20**), respectively. These three isomers have been reported previously (*11*, *13*, *19*, *20*, *31*), but only 3,4-digalloylquinic acid (**18**) has been reported in tara tannin (*7*). Other digalloylquinic acids were sought but could not be found. 1,3-Digalloylquinic acid (**15**) has been reported in *Guiera* (*13*), and 1,4-digalloylquinic acid (**16**) has been reported in *Quercus myrsinaefolia* (*19*).

Trigalloylquinic and Tetragalloylquinic Acids in Methanolyzed Tara Tannin. Three trigalloylquinic acids (*m/z* 647) and one tetragalloylquinic acid (*m/z* 799) were observed. The tetragalloylquinic acid (accounting at 280 nm for ∼6% of the methanolysis products) and two minor hydrophilic trigalloylquinic acids fragmented similarly, whereas the major hydrophobic trigalloylquinic acid (accounting at 280 nm for ∼40% of the methanolysis products) fragmented quite differently. Because 1,3,4,5-tetragalloylquinic acid (**25**) has previously been isolated from tara tannin (*6*, *22*) and because there can be only one methanolysis-stable tetragalloylquinic acid, the assignment of the signal at *m/z* 799 cannot be in doubt, and its fragmentation (**Figure 5**; **Table 3**) is discussed first.

It produces an MS^2 base peak at *m/z* 601 (rather than 647) accompanied by dehydrated fragment ions at *m/z* 629 ([tetragalloylquinic acid − gallic acid − H_2O − H^+]$^-$, 25% of base peak) and 477 ([tetragalloylquinic acid − (2 × gallic acid) − H_2O − H^+]$^-$, 15% of base peak). The MS^3 base peak (*m/z* 431)

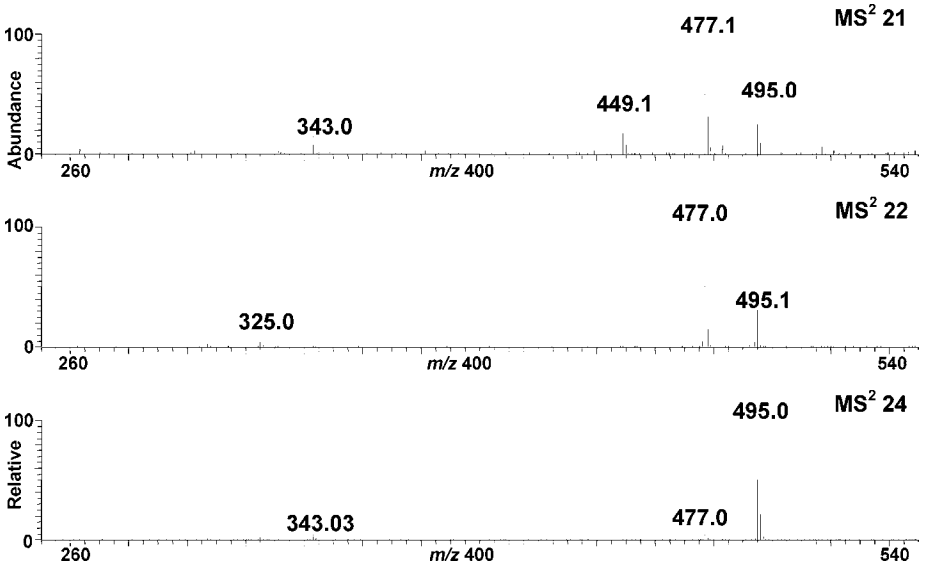


Figure 6. MS^2 spectra of the putative 1,3,4-trigalloylquinic acid (**21**), 1,3,5-trigalloylquinic acid (**22**), and 3,4,5-trigalloylquinic acid (**24** in methanolyzed tara tannin.

Table 4. MS^4 Fragmentation Data for Trigalloylquinic Acids (**21**–**24** and **32**–**35**)

				MS^2 secondary ions							MS^3 secondary ions							
compd	N[a]	parent ion *m/z*	MS^2 base peak *m/z*	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	MS^3 base peak *m/z*	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity
21	6	647.0	477.0	bp[b]	100	449.0	90			325.1	bp	100						
22	6	647.0	477.0	bp	100					325.0	bp	100	307.0	30	289.1	30	193.1	20
23																		
24	3	647.0	495.0	477.1	10			343.0	10	343.0	325.1	35						
32 or **33**	6	647.0	495.0					343.0	10	343.0	325.1	10			289.1	5	193.2	12
34 or **35**	6	647.0	495.1							343.0	325.1	40			289.1	5	193.1	10
35 or **34**	6	647.0	495.0					343.0	10	343.0	325.1	10						

			MS^4 secondary ions															
compd	N	MS^4 base peak *m/z*	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity	*m/z*	intensity
21	6	169.0			245.0	50	219.0	30	191.1	10	173.0	25	bp	100				
22	6	193.0	289.0	55									169.0	40				
23																		
24	3	169.1							191.1	20	173.1	10	bp	100				
32 or **33**	6	169.0							191.1	50			bp	100	150.9	8		
34 or **35**	6	169.1							191.1	10	173.1	12	bp	100			125.1	10
35 or **34**	6	169.1							191.1	10	173.1	15	bp	100			125.1	10

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

was accompanied by fragment ions at *m/z* 449 (35%), 261 (20%), 169 (12%), and 279 (10%), and at MS^4 the *m/z* 168 base peak was accompanied by strong secondary fragment ions at *m/z* 261 (95%), 279 (70%), 217 (37%), and 125 (27%). This last ion became the base peak at MS^5 and was assigned as [galloyl − COOH − H$^+$]$^-$.

All of these fragments can be derived from the parent ion (*m/z* 799) by various permutations of three eliminations: (i) a deprotonated galloyl residue (152 amu), (ii) a deprotonated galloyl residue with simultaneous dehydration (170 amu), and (iii) a deprotonated galloyl residue with simultaneous decarboxylation (197 amu) with deprotonation of the resultant fragment if necessary. Quinic acid decarboxylation and full aromatization (−3 × H_2O) have been observed previously in 4-substituted dicaffeoylquinic acids, most prominently in the 1,4-isomer (**6**) (*28*).

The first-eluting minor trigalloylquinic acid produced (**Figure 6**; **Table 4**) a dehydrated MS^2 base peak at *m/z* 477 [trigalloylquinic acid − gallic acid − H_2O − H$^+$]$^-$, accompanied by a "decarboxylated" MS^2 fragment ion at *m/z* 449. The MS^2 base peak fragmented to an *m/z* 325 MS^3 base peak followed at MS^4 by *m/z* 169 along with *m/z* 173 (25%) (suggestive of [4-galloylquinic acid − H$^+$]$^-$), accompanied by *m/z* 219 (30%) and *m/z* 245 (50%). The fragment at *m/z* 245 is equivalent to *m/z* 255 seen during the fragmentation of 4-substituted dicaffeoylquinic acids (*28*). A fragment-targeted MS^3 experiment established that the fragment ion at *m/z* 449 produced *m/z* 279. The propensity to dehydrate and decarboxylate, the production of [4-galloylquinic acid − H$^+$]$^-$, and *m/z* 245 at MS^4 all indicate a close affinity with 1,3,4,5-tetragalloylquinic acid (**25**) and behavior closely resembling that of 1,4-dicaffeoylquinic acid (**6**) (*28*). The second-eluting minor trigalloylquinic acid produced the dehydrated MS^2 base peak (*m/z* 477), but the decarboxylated fragment ion (*m/z* 449) was not detectable. Further fragmentation produced *m/z* 325 as MS^3 base peak, corresponding to the loss

of another galloyl residue, and an *m*/*z* 193 MS^4 base peak accompanied by *m*/*z* 289 (55%) and *m*/*z* 169 (40%).

Because these two trigalloylquinic acids are stable even to 6 h of methanolysis, they do not contain depsidic galloyl residues. They are comparatively hydrophilic, eluting at ~20 and 22 min compared with the more dominant isomer at ~37 min. This behavior is reminiscent of 1,3-dicaffeoylquinic acid (**5**) (with two free equatorial quinic acid hydroxyls) that on the same column packing elutes at ~27 min compared with ~47 min for the next most hydrophilic dicaffeoylquinic acid isomer (with one free equatorial and one free axial hydroxyl) (*28*). The only trigalloylquinic acids possessing a free equatorial hydroxyl are 1,3,4-trigalloylquinic acid (**21**) and 1,3,5-trigalloylquinic acid (**22**). We suggest tentatively that **21** is the isomer that decarboxylates and dehydrates and that **22** is the isomer that dehydrates but does not decarboxylate. The comparatively hydrophilic nature of 1,3-digalloylquinic acid (**11**) and 1,3,4-trigalloylquinic acid (**21**) reported by Bouchet et al. (*14*) is consistent with these assignments.

The dominant trigalloylquinic acid isomer yielded a parent ion at *m*/*z* 647 and an MS^2 base peak at *m*/*z* 495 accompanied by a secondary dehydrated ion at *m*/*z* 477 (10% of base peak). Subsequent fragmentation produced an MS^3 base peak at *m*/*z* 343 with a secondary dehydrated ion at *m*/*z* 325 (35% of base peak), followed by an MS^4 spectrum corresponding to [4-galloylquinic acid − H^+]$^-$. These fragmentations (**Figure 6**; **Table 4**) indicate successive loss of three galloyl residues from a relatively hydrophobic 4-substituted isomer. Two possibilities remain, 1,4,5-trigalloylquinic acid (**23**) and 3,4,5-trigalloylquinic acid (**24**), both possessing a single free axial hydroxyl. The absence of strong signals at *m*/*z* 477 and 449 argues against the 1,4-substituted **23**. That its MS^3 spectrum resembles the MS^2 spectrum of 3,4-digalloylquinic acid (**18**) argues in favor of **24**. Accordingly, this component is assigned as 3,4,5-trigalloylquinic acid (**24**). It is the most widely reported galloylquinic acid (*11*, *13*, *15*−*18*, *20*, *31*), occurring as a major component of tara tannin both before and after methanolysis (*6*, *7*). So far as we are aware, **22** has not previously been reported, but 1,3,4-trigalloylquinic acid (**21**) has been isolated from *Guiera (14*) and 1,4,5-trigalloylquinic acid (**23**) has been isolated from *Quercus myrsinaefolia* and *Quercus mongolica* (*19*). A search in the tara tannin extract for 1,4,5-trigalloylquinic acid (**23**) was unsuccessful.

Time Course of Methanolysis. Methyl esters were not detectable at zero time. Methyl trigallate (*m*/*z* 487) was produced rapidly, giving a strong signal at 5 min, whereas methyl gallate and methyl digallate were not detectable until 20 min. By 60 min only methyl gallate was detectable. The octagalloylquinic and heptagalloylquinic acids had disappeared by 5 and 15 min, respectively. In contrast, the hexagalloylquinic, pentagalloylquinic, and two trigalloylquinic acid depsides increased initially but had disappeared by 60 min.

The progressive increase in 1,3,4,5-tetragalloylquinic acid (**25**), 3,4,5-trigalloylquinic acid (**24**), 3,5-digalloylquinic acid (**19**), 4,5-digalloylquinic acid (**20**), and 5-galloylquinic acid (**13**) and their stability once all despides had been removed indicate that these are all "core" depside-bearing structures.

Depside-Containing Galloylquinic Acids in Untreated Tara Tannin. The methanolysis-stable galloylquinic acids, as discussed above, were observed in the tara tannin prior to methanolysis and will not be discussed further in this section. Those galloylquinic acids present before methanolysis and destroyed by it must, by definition, contain at least one depsidic galloyl residue. The retention time increased with increasing number of galloyl residues, but with a depsidic residue having a greater effect than a gallic acid residue attached directly to the quinic acid (**Table 5**). Many of these substances are poorly resolved in the UV traces and can be located only by SIM and characterized only by LC-MS^n fragmentation. The depsidic galloylquinic acids are structurally complex, and in the absence of purified standards also characterized by CD and NMR, the structure assignments proposed below must be considered tentative.

Table 5. Approximate Retention Times for Galloylquinic Acids as a Function of the Number and Nature of the Galloyl Residues

no. and nature of gallic acid residues			
total	non-depsidic	depsidic	approximate[a] retention time (min)
1	1		<10
2	2		12–27
2	1	1	14–28
3	3		19–37
3	2	1	39–43
4	4		39
4	3	1 (3,4,5-core)[b]	46–49
5	4	1 (1,3,4,5-core)[c]	49–52
5	3	2 (3,4,5-core)	52–57
6	4	2 (1,3,4,5-core)	58–60
6	3	3 (3,4,5-core)	60–64
7+			>65

[a] The precise retention time is influenced by the age of the column. [b] Based on a 3,4,5-trigalloylquinic acid core. [c] Based on a 1,3,4,5-tetragalloylquinic acid core.

Depside-Containing Digalloylquinic Acids in Untreated Tara Tannin. SIM at *m*/*z* 495 identified two depsidic digalloylquinic acids. The first-eluting yielded an MS^2 base peak at *m*/*z* 343 accompanied by *m*/*z* 191 (10%) but without any dehydrated fragment ions, and MS^3 produced [5-galloylquinic acid − H^+]$^-$ as its base peak. Accordingly, this was assigned as 5-(digalloyl)quinic acid (**29**). The second isomer lacked the *m*/*z* 191 MS^2 secondary fragment ion and produced [4-galloylquinic acid − H^+]$^-$ as its MS^3 base peak. Accordingly, it was assigned as 4-(digalloyl)quinic acid (**28**). The corresponding 1-(digalloyl)quinic acid (**26**) and 3-(digalloyl)quinic acid (**27**) were sought but could not be identified.

The elimination solely of the depsidic galloyl residue was unexpected because the aryl ester should be more difficult to cleave than the alicyclic ester, but consistent with this interpretation it was not possible to detect ions at *m*/*z* 321 and 483, the "dimer" and "trimer" analogues of *m*/*z* 169 produced by gallic acid. The expectation that the alicyclic ester should be more readily cleaved arises from our previous observation on the fragmentation of chlorogenic acids, through which activation by hydrogen bonding or intramolecular acyl transfer renders the ester residues at C5 of quinic acid very labile (*24*−*26*, *28*). In the case of the depsidic digalloyl derivatives it appears that the aryl ester is chemoselectively cleaved in preference to the alicyclic ester, presumably due to activation through a hydrogen bond of the carbonyl C═O to its adjacent phenolic OH group.

Depside-Containing Trigalloylquinic Acids in Untreated Tara Tannin. SIM at *m*/*z* 647 identified two substantial and one minor depsidic trigalloylquinic acid. The first-eluting depsidic isomer produced an MS^2 base peak at *m*/*z* 495 accompanied by a secondary fragment ion at *m*/*z* 343 (10% of base peak). It subsequently yielded an MS^3 base peak at *m*/*z* 343 with only a weak dehydrated ion (10%). At MS^4 the fragmentation was identical with that observed for 3-galloylquinic acid (**12**), suggesting that there is no galloyl residue at C4.

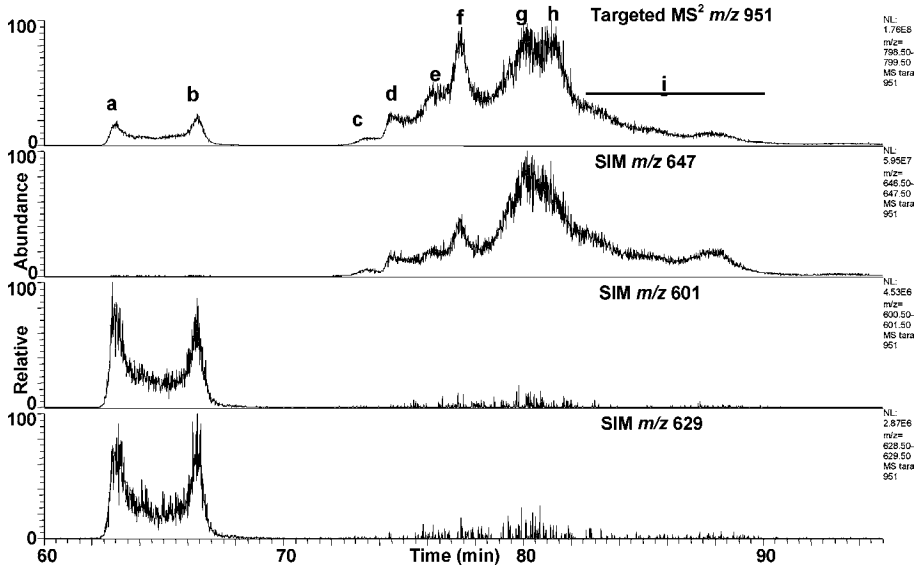


Figure 7. Targeted MS^2 (*m*/*z* 951 + 799) of the pentagalloylquinic acids in an untreated methanolic extract of tara tannin to discriminate between the depsides based on the 1,3,4,5-tetragalloylquinic acid core (*m*/*z* 601 and 629) and the later-eluting depsides based on 3,4,5-trigalloylquinic acid core (*m*/*z* 647). At least some of the signals observed beyond 80 min arise from in-source fragmentation of the hexagalloylquinic acids.

Accordingly, it has been assigned as one of the two possible depsidic trigalloylquinic acids with substituents at C3 and C5 (**32**, **33**).

The second-eluting depsidic isomer produced an MS^2 base peak at *m*/*z* 495 without any secondary fragment ions and an MS^3 base peak at *m*/*z* 343 accompanied by a strong (40% of base peak) dehydrated fragment ion at *m*/*z* 325. At MS^4 the fragmentation was identical to that observed for 4-galloylquinic acid (**14**). This behavior is consistent with one of the four possible *vic*-diacyl trigalloylquinic acids with substituents at C3 and C4 (**30**, **31**) or at C4 and C5 (**34**, **35**), probably the latter because it elutes later than the 3,5-isomer(s) (**32**, **33**).

The third-eluting depsidic isomer also produced a [4-galloylquinic acid − H$^+$]$^-$ base peak at MS^4, and because of its hydrophobicity it would appear to be the second *vic*-diacyl trigalloylquinic acid with substituents at C4 and C5 (**34**, **35**). 4-Galloyl-5-(digalloyl)quinic acid (**34**) and 4-(digalloyl)-5-galloylquinic acid (**35**) have been isolated from tannic acid (*31*) but have not previously been reported in tara tannin.

Depside-Containing Tetragalloylquinic Acids in Untreated Tara Tannin. Three depsidic tetragalloylquinic acids were detected with retention times ∼20−30% longer than that of the methanolysis-stable **25**. They each lost galloyl residues, sequentially producing an *m*/*z* 647 MS^2 base peak (without any dehydrated fragment ion), an *m*/*z* 495 MS^3 base peak accompanied by *m*/*z* 477 (10%) and 343 (5%), followed by an *m*/*z* 343 MS^4 base peak accompanied by 325 (40%), and finally yielded [4-galloylquinic acid − H]$^-$ at MS^5. This behavior after the elimination of the first galloyl residue is identical to that of 3,4,5-trigalloylquinic acid (**24**), thus implying that these isomers are not 1-substituted.

Because dehydration does not occur at MS^2, one may deduce, as for the depsidic digalloylquinic acids (**28** and **29**), that it is the depsidic galloyl residue that is eliminated first because elimination of either a galloyl or a digalloyl residue directly from C5 (or even C3), by analogy with the fragmentation of 3,4-digalloylquinic acid (**18**), 4,5-digalloylquinic (**20**), and 3,4,5-trigalloylquinic acid (**24**), would have resulted in a significant *m*/*z* 629 or 477 at MS^2. By the same argument we can exclude 3,4-di(digalloyl)quinic acid (**36**) and 4,5-di(digalloyl)quinic acid (**38**). 3,5-Di(digalloyl)quinic acid (**37**) can be excluded because this would ultimately produce [3-galloylquinic acid − H$^+$]$^-$ not [4-galloylquinic acid − H$^+$]$^-$ at MS^5. 3,4-Digalloyl-5-(digalloyl)quinic acid (**39**), 3,5-digalloyl-4-(digalloyl)quinic acid (**40**), and 3-(digalloyl)-4,5-digalloylquinic acid (**41**) have previously been isolated from tannic acid (*22*, *31*) but not from tara tannin. Because the fragmentations observed are compatible with these structures and because other possibilities have been excluded, it is suggested that the three depside-containing tetragalloylquinic acids are **39**, **40**, and **41**, but confirmation must await isolation and NMR characterization.

Depside-Containing Pentagalloylquinic Acids in Untreated Tara Tannin. Mass spectrometric fragmentation data have not previously been obtained for galloylquinic acids containing more than four gallic acid residues. By targeting *m*/*z* 951 at least eight peaks (a−h) were detected (**Figure 7**), and this material was assigned as pentagalloylquinic acids. The poorly resolved region labeled (i) includes hexagalloylquinic acids that have suffered in-source fragmentation. Although pentagalloylquinic acids, and higher mass analogues, must contain at least one depsidic galloyl residue, they are not necessarily based on the 1,3,4,5-tetragalloylquinic acid skeleton. The two most hydrophilic isomers (a, b) were chromatographically well resolved. They produced MS^2 base peaks at *m*/*z* 799 accompanied by a secondary fragment ion at *m*/*z* 601 (15% of base peak) followed by a decarboxylated MS^3 base peak at *m*/*z* 601 with secondary fragment ions at *m*/*z* 629 (25%) and *m*/*z* 477 (20%). These isomers clearly resemble 1,3,4,5-tetragalloylquinic acid (**25**), and because this resemblance is apparent after the elimination of only one galloyl residue, one may deduce that they contain only one depsidic galloyl residue. If two depsidic galloyl residues were present on a trigalloylquinic acid core, decarboxylation would not have been expected until MS^4. The observed fragmentation strongly suggests that these two hydrophilic isomers are based on the 1,3,4,5-tetragalloylquinic acid skeleton, but the position(s) of the additional galloyl residue(s) cannot be defined on the basis of the present evidence.

The more hydrophobic isomers (c−h) produced MS^2 base peaks at *m*/*z* 799 accompanied by a secondary fragment ion at *m*/*z* 647 (20% of base peak) and followed by *m*/*z* 647 at MS^3, *m*/*z* 495 with 477 (10%) at MS^4, *m*/*z* 343 with 325 (35%) at MS^5, and [4-galloylquinic acids − H$^+$]$^-$ at MS^6. These isomers

are thus quite different from 1,3,4,5-tetragalloylquinic acid (**25**), but clearly resemble 3,4,5-trigalloylquinic acid (**24**) and the depside-containing tetragalloylquinic acids (**39**−**41**) containing this core. Therefore, they must possess at least two depsidic galloyl residues.

Depside-Containing Hexagalloylquinic Acids in Untreated Tara Tannin. At least five hexagalloylquinic acid isomers, identified by their parent ion at *m*/*z* 1103, eluted at ∼58−65 min. The two more hydrophilic isomers produced MS^2 and MS^3 base peaks at *m*/*z* 951 and 799, respectively. The MS^4 base peak at *m*/*z* 601 produced *m*/*z* 431 at MS^5 followed by *m*/*z* 168 and 124 (40%) at MS^6. The formation of *m*/*z* 168 and 124 at approximately twice the intensity of *m*/*z* 169 and 125, respectively, also is reminiscent of the fragmentation of 1,3,4,5-tetragalloylquinic acid (**25**). This suggests that these isomers are derived from **25** by the addition of two depsidic galloyl residues. In contrast, the more hydrophobic isomers that were incompletely resolved and mass spectrometrically homogeneous, repeatedly lost 152 amu, leading eventually to $[4\text{-galloylquinic acid} - H^+]^-$ at MS^7. Fragment ions at *m*/*z* 168 and 124 were not detected in these spectra. These isomers would appear to be derived from a trigalloylquinic acid, probably **24**, by the addition of three depsidic galloyl residues.

Depside-Containing Heptagalloylquinic Acids in Untreated Tara Tannin. At least five heptagalloylquinic acid isomers, identified by their parent ion at *m*/*z* 1255, eluted at ∼65−80 min. Fragmentation followed the two patterns observed for the pentagalloylquinic acids and hexagalloylquinic acids with the two more hydrophilic isomers producing *m*/*z* 601 at MS^5 with significant MS^7 signals at *m*/*z* 168 and 124, suggesting that these are derived from the tetragalloylquinic acid (**25**) bearing three depsidic galloyl residues. The more hydrophobic isomers fragmented via *m*/*z* 647, producing dehydrated secondary fragment ions at MS^6 (*m*/*z* 477, ∼10%) and MS^7 (*m*/*z* 325, 20−50%), finally yielding $[4\text{-galloylquinic acid} - H^+]^-$ at MS^8, suggesting a trigalloylquinic acid bearing four depsidic galloyl residues. Because methyl tetragallate was not detected, these hydrophobic heptagalloylquinic acids must carry depsides at two positions.

Depside-Containing Octagalloylquinic Acids and Nonagalloylquinic Acids in Untreated Tara Tannin. A weak but very broad signal was observed at *m*/*z* 1407, in which a comparatively narrow *m*/*z* 601 fragment ion could be recognized at MS^6. Despite the poor chromatographic and mass spectral resolution (at MS^1), it is evident that octagalloylquinic acids are based on the same core structures as the heptagalloylquinic acids. There was no significant signal at *m*/*z* 1559, indicating the effective absence of nonagalloylquinic acids.

Galloylquinic Acids in Tannic Acid. Up to the hexagalloylquinic acids, the commercial tannic acid produced the same qualitative profile of galloylquinic acids as tara tannin, but the heptagalloylquinic acids produced very weak signals and the octagalloylquinic acids could not be detected.

Discussion. This investigation has demonstrated that the LC-MS^4 procedures previously developed for the characterization of individual isomers of classic diacyl chlorogenic acids (*24*−*26*, *28*, *32*) can be extended to LC-MS^8 procedures able to provide at least partial characterization of galloylquinic acids containing up to eight gallic acid residues, at least four of which occur as depsides. The structural detail achieved declines progressively when the parent molecule contains more than four gallic acid residues, in part because some more complex isomers do not necessarily have unique fragmentation patterns but also because of constraints on their chromatographic resolution. Our interpretation of the mass spectral data is based upon LC-MS^n investigation of over 20 pure chlorogenic acids (*24*−*26*, *28*, *32*), but the structures proposed for the depsides must be treated with caution until such time as pure standards are available for investigation and the arguments can be tested. Nevertheless, the fragmentation behavior defines the structure of these substances to an extent not previously possible, and this is achieved much more rapidly by LC-MS^n than by the traditional approach of isolation and purification followed by NMR and CD spectroscopy.

These procedures have provided the first evidence for the existence of (a) 1-galloylquinic acid (**11**), 1,3,5-trigalloylquinic acid (**22**), 4-(digalloyl)quinic acid (**28**), 5-(digalloyl)quinic acid (**29**), and either 3-galloyl-5-(digalloyl)quinic acid (**32**) or 3-(digalloyl)-5-galloylquinic acid (**33**) from any source; (b) 4-galloyl-5-(digalloyl)quinic acid (**34**), 5-galloyl-4-(digalloyl)-quinic acid (**35**), 3-(digalloyl)-4,5-digalloylquinic acid (**41**), 4-(digalloyl)-3,5-digalloylquinic acid (**40**), 5-(digalloyl)-3,4-galloylquinic acid (**39**), and 1,3,4-trigalloylquinic acid (**21**) from tara tannin; and (c) 3-galloylquinic acid (**12**) and 4-galloylquinic acid (**14**) from green tea. These ion trap LC-MS^n procedures have also generated the first mass spectrometric fragmentation data for galloylquinic acids containing between five and eight gallic acid residues, demonstrating for these four mass ranges two distinct groups of isomers. These are (a) two chromatographically distinct peaks each containing one or more isomers based on the 1,3,4,5-tetragalloylquinic acid core (**25**) and (b) at least three distinct but incompletely resolved peaks each containing one or more isomers based on the 3,4,5-trigalloylquinic acid core (**24**). The galloylquinic acids of tara tannin can thus be divided into three groups: (i) mono-, di, tri-, and tetragalloylquinic acids that lack depsidic galloyl residues; (ii) depsides related to the 3,4,5-trigalloyl structure previously considered to be the major components of tara tannin (*6*); and (iii) depsides related to the 1,3,4,5-tetragalloylquinic acid.

The generation of quantitative data was not a primary objective of this investigation, but by comparison of the area of the MS^1 signals for each group of isomers, and assuming that each group ionizes with equal efficiency, one can conclude that the trigalloylquinic acids and tetragalloylquinic acids are the major molecular mass fractions, with a substantial contribution also from the pentagalloylquinic acids. Within the trigalloylquinic acids, the major contributors, in order of decreasing peak area, are 4-galloyl-5-(digalloyl)quinic acid and 5-galloyl-4-(digalloyl)quinic acid (**34** and **35**), 3,4,5-trigalloylquinic acid (**24**), and 3-galloyl-5-(digalloyl)quinic acid and 5-galloyl-3-(digalloyl)quinic acid (**32** and **33**). After methanolysis of tara tannin, the yields were 40% 3,4,5-trigalloylquinic acid (**24**), 26% 4,5-digalloylquinic acid (**20**), 16% 3,5-digalloylquinic acid (**19**), 6% 1,3,4,5-tetragalloylquinic acid (**25**), 1.5% 3,4-digalloylquinic acid (**18**), 1.5% galloylquinic acids (**11**−**14**), and 9% methylgallate. Tara tannin has a much greater content of galloylquinic acids than tannic acid.

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