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Identification and Characterization of Two New Derivatives of Chlorogenic Acids in Arnica (*Arnica montana* L.) Flowers by High-Performance Liquid Chromatography/Tandem Mass Spectrometry

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Supporting Information

ABSTRACT: *Arnica montana* is a medicinally important plant due to its broad health effects, and it is used in Ayurvedic, Homeopathic, Unani, and folk medicines. We have used LC−MS^n (n = 2−5) to detect and characterize in Arnica flowers 11 quantitatively minor fumaric and methoxyoxalic acid-containing chlorogenic acids, nine of them not previously reported in nature. These comprise 1,5-dicaffeoyl-3-methoxyoxaloylquinic acid, 1,3-dicaffeoyl-4-methoxyoxaloylquinic acid, 3,5-dicaffeoyl-4-methoxyoxaloylquinic acid, and 1-methoxyoxaloyl-4,5-dicaffeoylquinic acid (M_r 602); 3-caffeoyl-4-feruloyl-5-methoxyoxaloylquinic acid and 3-feruloyl-4-methoxyoxaloyl-5-caffeoylquinic acid (M_r 616); 1,5-dicaffeoyl-4-fumaroyl and 1,5-dicaffeoyl-3-fumaroylquinic acid (M_r 614); 3,5-dicaffeoyl-1,4-dimethoxyoxaloylquinic acid (M_r 688); and 1-methoxyoxaloyl-3,4,5-tricaffeoylquinic acid and 1,3,4-tricaffeoyl-5-methoxyoxaloylquinic acid (M_r 764). All of the structures have been assigned on the basis of LC−MS^n patterns of fragmentation, relative hydrophobicity, and analogy of fragmentation patterns if compared to caffeoylquinic acids. This is the first time when fumaric acid-containing chlorogenic acids are reported in nature.

KEYWORDS: Chlorogenic acids, Arnica, dicaffeoylquinic acids, feruloyl-caffeoyl-methoxyoxaloylquinic acids, dicaffeoyl-methoxyoxaloylquinic acids, dicaffeoyl-fumaroylquinic acids, tricaffeoylquinic acids, dicaffeoyl-dimethoxyoxaloylquinic acid, tricaffeoyl-methoxyoxaloylquinic acids, *Arnica montana*, LC−MS^n

INTRODUCTION

Classically, chlorogenic acids (CGAs) are a family of esters formed between quinic acid and certain *trans*-cinnamic acids, most commonly caffeic, *p*-coumaric, and ferulic acids[1−3] and sometimes dimethoxycinnamic, trimethoxycinnamic, and sinapic acids.[4−6] Representative structures are shown in Figure 1. In the IUPAC system, (−)-quinic acid is defined as 1L-1(OH),3,4/5-tetrahydroxycyclohexane carboxylic acid, but Eliel and Ramirez[7] recommend 1α,3*R*,4α,5*R*-tetrahydroxycyclohexane carboxylic acid.

Several pharmacological activities of CGAs including antioxidant activity,[8] ability to increase hepatic glucose utilization,[9] inhibition of the HIV-1 integrase,[10] antispasmodic activity,[11] and inhibition of the mutagenicity of carcinogenic compounds[12] have been revealed by studies so far. *Arnica montana* L. roots and flowers are used in Ayurvedic, Homeopathic, Unani, and folk medicines in India for the treatment of skin disorders, swelling, stomach and muscle pains, hair loss, and wound healing. Arnica flowers are used as a flavor additive in food materials. Arnica flowers are also used as an ingredient to Benedictine liquor.[13] In Europe, Arnica flowers are used to treat skin infections, bruises, and muscle pains. Arnica is a source of sesquiterpenes, flavonoids, and phenolic acids, especially chlorogenic acids.[14−18] On no occasion have the chlorogenic acids in Arnica flowers been analyzed by tandem mass spectrometry.[14,15,17,18]

Recently, LC−MS^n has been used to characterize cinnamoyl−amino acid conjugates[19] and to discriminate between individual isomers of monoacyl, diacyl, and triacyl chlorogenic acids.[4−6,20−25] The MS fragmentation patterns, found to be significantly different for all regioisomers of CGAs, based on fragmentation preferences induced by characteristic hydrogen-bonding arrays within the gas-phase ions of CGAs in tandem MS spectra, UV spectrum, retention times, and relative hydrophobicity have been utilized to develop structure-diagnostic hierarchical keys for the identification of chlorogenic acids. In this study, we expand on methods developed previously for the structure elucidation of CGAs to the qualitative profiling of chlorogenic acids in Arnica flower extract. In previous work, we have shown that our structure diagnostic hierarchical key can be applied to cinnamoyl and aryl-substituted esters of quinic acid. In this study, we report on its extension to quinic acid esters bearing a functionalized aliphatic side chain such as methoxyoxalic acid and fumaric acid. Because these side chains carry further substituents such as carboxylic acids, which are able to form further hydrogen bonds within the gas-phase ions, we address the question on whether our previous observation can after critical evaluation be applied to these new derivatives of CGAs.

MATERIALS AND METHODS

All of the chemicals (Analytical grade) were purchased from Sigma-Aldrich (Bremen, Germany). Dry Arnica flowers were purchased from a Pharmacy in Bremen (Germany).

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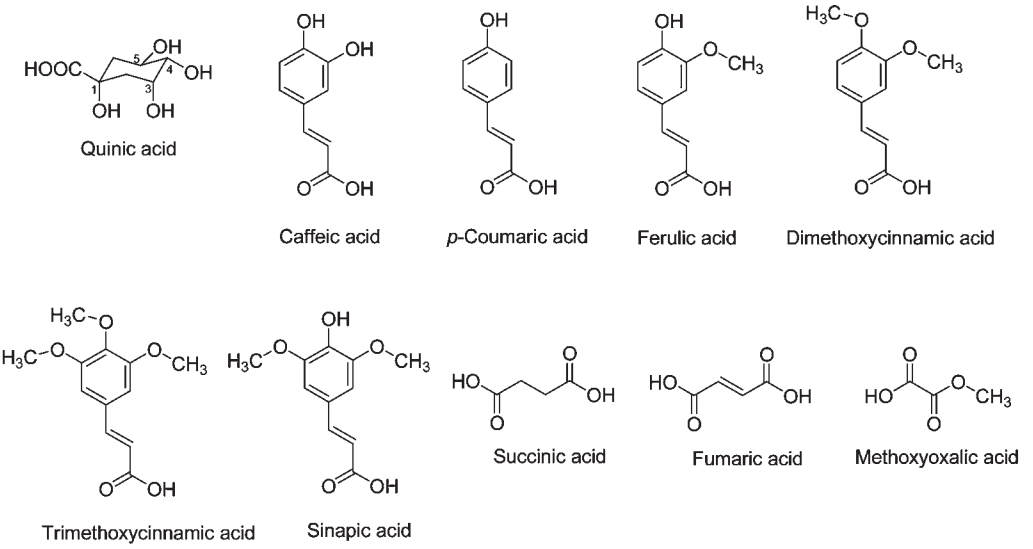

No.	Name	Abbreviation	R^1	R^3	R^4	R^5
1	1-*O*-caffeoylquinic acid	1-CQA	C	H	H	H
2	3-*O*-caffeoylquinic acid	3-CQA	H	C	H	H
3	5-*O*-caffeoylquinic acid	5-CQA	H	H	H	C
4	4-*O*-caffeoylquinic acid	4-CQA	H	H	C	H
5	*Cis*-5-*O*-feruloylquinic acid [a]	*Cis*-5-CQA	H	H	H	C
6	1,5-di-*O*-caffeoylquinic acid	1,5-diCQA	C	H	H	C
7	3,5-di-*O*-caffeoylquinic acid	3,5-diCQA	H	C	H	C
8	A *cis*-3,5-di-*O*-caffeoylquinic acid [a]	A *cis*-3,5-diCQA	H	C	H	C
9	A *cis*-3,5-di-*O*-caffeoylquinic acid [a]	A *cis*-3,5-diCQA	H	C	H	C
10	3,4-di-*O*-caffeoylquinic acid	3,4-diCQA	H	C	C	H
11	4,5-di-*O*-caffeoylquinic acid	4,5-diCQA	H	H	C	C
12	3,4,5-tri-*O*-caffeoylquinic acid	3,4,5-triCQA	H	C	C	C
13	1,3,5-tri-*O*-caffeoylquinic acid [a]	1,3,5-triCQA	C	C	H	C
14	5-*O*-feruloylquinic acid	5-FQA	H	H	H	F
15	1-*O*-caffeoyl-5-*O*-feruloylquinic acid [a, b]	1-C-5-FQA	C	H	H	F
16	1,5-di-*O*-caffeoyl-3-*O*-methoxyoxaloylquinic acid	1,5-diC-3-MOQA	C	MO	H	C
17	1,3-di-*O*-caffeoyl-4-*O*-methoxyoxaloylquinic acid [a, b]	1,3-diC-4-MOQA	C	C	MO	H
18	3,5-di-*O*-caffeoyl-4-*O*-methoxyoxaloylquinic acid [a, b]	3,5-diC-4-MOQA	H	C	MO	C
19	1-*O*-methoxyoxaloyl-4,5-di-*O*-caffeoylquinic acid	1-MO-4,5-diCQA	MO	H	C	C
20	3-*O*-caffeoyl-4-*O*-feruloyl-5-*O*-methoxyoxaloylquinic acid [a, b]	3-C-4-F-5-MOQA	H	C	F	MO
21	3-*O*-feruloyl-4-*O*-methoxyoxaloyl-5-*O*-caffeoylquinic acid [a, b]	3-F-4-MO-5-CQA	H	F	MO	C
22	1,5-di-*O*-caffeoyl-4-*O*-fumaroylquinic acid [a, b]	1,5-diC-4-FuQA	C	H	Fu	C
23	1,5-di-*O*-caffeoyl-3-*O*-fumaroylquinic acid [a, b]	1,5-diC-3-FuQA	C	Fu	H	C
24	3,5-di-*O*-caffeoyl-1-4-di-*O*-methoxyoxaloylquinic acid [a, b]	3,5-diC-1,4-diMOQA	MO	C	MO	C
25	1-*O*-methoxyoxaloyl-3,4,5-tri-*O*-caffeoylquinic acid [a, b]	1-MO-3,4,5-triCQA	MO	C	C	C
26	1,3,4 -tri-*O*-caffeoyl-5-*O*-methoxyoxaloylquinic acid [a, b]	1,3,5-triC-4-MOQA	C	C	MO	C

Figure 1. Structures, numbering, nomenclature, and substituents of chlorogenic acids and their derivatives.

Methanolic Extract of Arnica Flowers. Arnica flowers (5 g) were ground to a fine powder, stirred with aqueous methanol (90%, 100 mL) at room temperature for 12 h, and ultrasonicated for 5 min. The methanol and the water were removed in vacuo, and the residue was stored at −20 °C until required, thawed at room temperature, dissolved in methanol (50 mg/10 mL), filtered through a membrane filter, and then used for LC−MS.

LC-MS^n. The LC equipment (Agilent, Karlsruhe, Germany) was comprised of a binary pump, an auto sampler with a 100 μL loop, and a DAD detector with a light-pipe flow cell (recording at 320 and 254 nm and scanning from 200 to 600 nm). This was interfaced with an ion-trap mass spectrometer fitted with an ESI source (Bruker Daltonics, Bremen, Germany) operating in full scan, Auto MS^n mode to obtain fragment ion m/z. As necessary, MS^2, MS^3, and MS^4 fragment-targeted experiments

Table 1. Negative Ion MS^5 Fragmentation Data for the Chlorogenic Acids[a]

			MS^2							MS^3						
		MS^1	base peak		secondary peak					base peak		secondary peak				
no.	compd	pseudomolecular ion	m/z	m/z	int	m/z	int	m/z	int	m/z	m/z	int	m/z	int	m/z	int
13	1,3,5-triCQA	677.1	497.0	353.0	45	335.0	40			335.0	172.7	18				
15	1-C-5-FQA	529.0	367.0	353.0	30	335.1	25	190.8	25	109.7						
16	1,5-diC-3-MOQA	601.0	515.0	557.1	60	439.0	47	395.0	95	353.1	190.9	30				
17	1,3-diC-4-MOQA	601.0	395.1	557.2	30	439.1	5	515.1	55	232.9	335.0	5	172.9	10		
18	3,5-diC-4-MOQA	601.0	395.1	557.2	46	439.1	10	515.1	54	232.9			172.9	10		
19	1-MO-4,5-diCQA	601.0	515.0	557.1	30	439.0	5	395.0	20	353.0	299.0	5	255.0	5	190.8	8
20	3C-4F-5MOQA	615.1	409.1	571.1	65	349.0	10			349.1	232.9	50	172.8	10		
21	3F-4MO-5CQA	615.1	409.1	571.1	40	349.0	10			232.9	349.1	40	172.9	18		
22	1,5-diC-4FuQA	613.1	515.1	577.1	20	433.0	70	353.0	40	353.1	190.9	20				
23	1,5-diC-3-FuQA	613.1	515.1	577.1	25	436.6	25	353.0	50	353.1						
24	3,5-diC-1,4-diMOQA	687.1	601.1							395.1	557.2	40	439.1	12	515.1	12
25	1-MO-3,4,5-triCQA	763.1	677.1	719.1	5	557.1	10	395.0	5	515.0	497.0	80	469.0	50		
26	1,3,5-triC-4-MOCQA	763.0	677.1	719.1	25	557.1	70	395.0	60	515.0	497.0	60	469.0	25		

		MS^4							MS^5				
		base peak		secondary peak					base peak		secondary peak		
no.	compd	m/z	m/z	int	m/z	int	m/z	int	m/z	m/z	int	m/z	int
13	1,3,5-triCQA	172.7	178.9	6	134.7	6							
15	1-C-5-FQA												
16	1,5-diC-3-MOQA	190.8											
17	1,3-diC-4-MOQA	172.9											
18	3,5-diC-4-MOQA	172.9											
19	1-MO-4,5-diCQA	172.9	178.9	55	191.0	30							
20	3-C-4-F-5-MOQA	192.8	173.0	65									
21	3-F-4-MO-5-CQA	172.8											
22	1,5-diC-4-FuQA	190.9											
23	1,5-diC-3-FuQA	190.8											
24	3,5-diC-1,4-diMOQA	232.9							172.8				
25	1-MO-3,4,5-triCQA	353.0	298.9	5	254.8	5	172.7	10	172.8	179.0	42	191.0	38
26	1,3,5-triC-4-MOCQA	353.0	298.9	9	202.8	8			172.8			191.0	12

[a] C = caffeoyl; F = feruloyl; Fu = fumaroyl; MO = methoxyoxaloyl; H = hydrogen; QA = quinic acid.

were performed to focus only on compounds producing a pseudomolecular ion at m/z 353, 367, 515, 529, 601, 613, 615, 677, 687, and 763. Tandem mass spectra were acquired in the Auto-MS^n mode (smart fragmentation) using a ramping of the collision energy. Maximum fragmentation amplitude was set to 1 V, starting at 30% and ending at 200%. The MS operating conditions (negative mode) had been optimized using 5-caffeoylquinic acid **3** with a capillary temperature of 365 °C, a dry gas flow rate of 10 L/min, and a nebulizer pressure of 10 psi. High-resolution LC−MS was carried out using the same HPLC equipped with a MicrOTOF Focus mass spectrometer (Bruker Daltonics, Bremen, Germany) fitted with an ESI source, and internal calibration was achieved with 10 mL of a 0.1 M sodium formate solution injected through a six port valve prior to each chromatographic run. Calibration was carried out using the enhanced quadratic mode, and the mass error was below 5 ppm.

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

RESULTS AND DISCUSSION

Preliminary Assessment of Data. All of the data for chlorogenic acids presented in this Article use the recommended IUPAC numbering system,[1] with the structures being presented in Figure 1. When necessary, previously published data have been amended to ensure consistency and avoid ambiguity.

In general, new CGAs can be identified in an all tandem mass spectrum EIC (extracted ion chromatogram) by their unique fragments at m/z 173 and 191. Selected ion monitoring at m/z 353, 367, 515, 529, 601, 613, 615, 677, 687, and 763 immediately located 26 chromatographic peaks eluting between 12 and 55 min,

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each with a UV spectrum typical of chlorogenic acids [λ_{max} 320 nm]. During this study, we did not observe any aliphatic acid-containing monoacyl, diacyl, and triacyl or tetraacyl quinic acid isomer.

Four CGAs were identified showing an m/z value in the negative ion mode at 601. Two CGAs produced pseudomolecular ions at m/z 615, two CGAs produced pseudomolecular ions at m/z 763, and one chlorogenic acid (CGA) produced pseudomolecular ion at m/z 687. All of these produced MS^n peaks by the loss of an acyl residue of mass unit 86 Da corresponding to loss of a methoxyoxaloyl moiety. Two CGAs produced MS^n peaks by the loss of an acyl residue of mass unit 98 Da corresponding to a fumaroyl moiety. In nature, only fumaric acid (*trans*-isomer) is present. The elemental composition of all compounds at the m/z values above was determined by high resolution LC-ESI-TOF mass spectrometry. All compounds identified displayed mass errors below 5 ppm.

The chemical nature of the non caffeoyl side chains was established first using high resolution in source fragmentation experiments in negative ion mode showing fragment ions with the neutral losses of 97.9998 (−0.4 ppm error) and 85.9998 (0.5 ppm error) Da for the fumaroyl and methoxyoxaloyl residues, respectively. Furthermore, a hydrolysis experiment, in which the Arnica extract was treated for 1 h with 1 N NaOH, followed by HPLC analysis, revealed the presence of fumaric acid (rather than its isomer maleic acid) and methoxyoxalic acid by comparison to authentic standards.

On first inspection of tandem mass spectra, all compounds were deviating from the typical fragmentation behavior observed for triacyl chlorogenic acids.[5,6] In five MS^2 spectra (**15**, **16**, **19**, **22**, and **23**), the base peaks were observed at m/z 515 or 367 (Table 1), indicating a facile loss of the alkoyl residue if compared to the caffeoyl residues. This observation indicates that an internal hydrogen bond from the free carboxylic acid moiety of the alkoyl residue activates the alkoyl residue for facile fragmentation. In the other four MS^2 spectra (**17**, **18**, **20**, and **21**), base peaks were observed at m/z 395 or 409 (Table 1), indicating a facile loss of the caffeoyl residue. Hence, an uncritical adaptation of our previous structure assignment tools is not possible here and requires modification. We propose here to adopt the following general regiochemistry assignment strategy: Targeted MS^3 experiments of the ion at m/z 515 and MS^4 experiments of the ions at m/z 353 allow unambiguous determination of the regiochemistry of the ions corresponding to dicaffeoyl quinic acid, where ions observed are identical to ions formed from the compounds originally reported, thus allowing the definition of the caffeoyl regiochemistry. Once the two positions of caffeoyl regiochemistry are defined, the further substituents must form an ester bond to any one of the two remaining quinic acid alcohol groups. Should the substitution pattern remaining correspond to *n*,4 (*n* being one of the free alcohol and the 4-OH being the other one), we apply our previous observation that a 4-substituted derivative loses its acyl side chain most reluctantly, producing a dehydrated quinic acid fragment ion at m/z 173 in the process. Should this feature be observed in the spectra, the third substituent occupies position 4 of the quinic acid; if not, it must occupy position *n*. If positions 1,3-, 1,5-, or 3,5 are unoccupied after definition of caffeoyl regiochemistry, assignment of aliphatic side chain regiochemistry is more difficult and is only reliable if tandem mass spectra of both remaining regioisomers are available that allow a direct comparison of the ease of fragmentation of the aliphatic side chain, where we apply our previous rule that fragmentation occurs in the order of 1 after 5 after 3 after 4 position. Should experimental tandem mass spectra for both regioisomers not be available, further supporting arguments including relative hydrophobicity, comparison of fragmentation patterns of chemically similar side chains (e.g., fumaroyl and methoxyoxaloyl), or phytochemical arguments (e.g., *Asteraceae* are rarely acylating at C1 of the quinic acid) are employed in a more tentative assignment of regiochemistry.

Characterization of Caffeoylquinic Acids (M_r 354), Dicaffeoylquinic Acids (M_r 516), and Tricaffeoylquinic Acids (M_r 678). Five caffeoylquinic acids eluting from ∼12−24 min were easily located and assigned using the hierarchical keys previously developed[4,21] as the well-known 1-caffeoylquinic acid (**1**), 3-caffeoylquinic acid (**2**), 5-caffeoylquinic acid (**3**), 4-caffeoylquinic acid (**4**), and *cis*-5-caffeoylquinic acid (**5**) with the 5-isomer (**3**) dominant. Similarly, six dicaffeoylquinic acids eluting from ∼37−49 min were assigned as 1,5-dicaffeoylquinic acid (**6**), 3,5-dicaffeoylquinic acid (**7**), a A *cis*-3,5-dicaffeoylquinic acid (**8**), A *cis*-3,5-dicaffeoylquinic acid (**9**), 3,4-dicaffeoylquinic acid (**10**), and 4,5-dicaffeoylquinic acid (**11**).[21] Isomers **7** and **8** were assigned as A *cis*-3,5-dicaffeoylquinic acid because in these isomers one of the caffeoyl residues was *cis* (A *cis*). Detailed mass spectra have been published previously[4,20,21] and are not repeated here.

A search for tricaffeoylquinic acids as previously reported in various *Asteraceae* plants[26−30] located four signals at m/z 677 in the extract. Two of them eluted before 4,5-dicaffeoylquinic acid (**11**), suggesting that they are too hydrophilic to be tricaffeoylquinic acids, although they do show characteristic peaks of caffeic acid derivatives. These compounds were not investigated further.

The most hydrophobic of the two minor components that eluted after 4,5-dicaffeoylquinic acid (**11**) was assigned as 3,4,5-tricaffeoylquinic acid (**12**), which we have recently reported in maté[4] and green coffee beans.[5,6] The most hydrophilic isomer produced an MS^2 base peak at m/z 497 accompanied by m/z 515 (18% of base peak) (Table 1). Its MS^3 base peak at m/z 335 was accompanied by m/z 203 and 173. The absence of secondary peaks at m/z 255 and 299 in MS^3 spectra suggests the 1,3,5-tricaffeoylquinic acid.[21] Targeted MS^3 experiment on m/z 677 + 353 produced MS^2 base peak at m/z 191 accompanied by m/z 179 and 135, which is characteristic of 3-caffeoylquinic acid (**2**).[20] On the basis of the above arguments, this triacaffeoylquinic acid was tentatively assigned as 1,3,5-tricaffeoylquinic acid **13**.

Characterization of Feruloylquinic Acids (M_r 368) and Putative Feruloyl-caffeoylquinic Acid (M_r 530). The feruloylquinic acid isomer **14** eluting at ∼29 min was assigned as 5-feruloylquinic acid.[20] The isomer **15** produced the pseudomolecular ion at m/z 529 (∼42 min) and was tentatively assigned as caffeoyl-feruloylquinic acid. This isomer produced the MS^2 base peak at m/z 367 ([feruloylquinic acid − H^+]$^-$) by the loss of a caffeoyl residue and a secondary peak at m/z 353 ([caffeoylquinic acid − H^+]$^-$) (30% of base peak) (Table 1). It showed an MS^3 base peak at m/z 191, which is consistent with the MS^2 spectra of 5-feruloylquinic acid **14**.[20] From our previous studies, it is known that the loss of the C1 acyl residue takes place easier than that of the C5 acyl residue.[20−23] On the basis of the above arguments, isomer **15** was tentatively assigned as 1-caffeoyl-5-feruloylquinic acid.

Characterization of Putative Dicaffeoyl-methoxyoxaloylquinic Acids (M_r 602). Four peaks were located (∼42−52 min) in the extracted ion chromatogram, and they produced each the pseudomolecular ion at m/z 601. These isomers were tentatively assigned as dicaffeoyl-methoxyoxaloylquinic acids **16**−**19**. Isomers **16** and **19** produced MS^2 base peaks at m/z 515

([dicaffeoylquinic acid − H^+]$^-$), MS^3 base peaks at m/z 353 ([caffeoylquinic acid − H^+]$^-$), and MS^4 base peaks at m/z 191 ([quinic acid − H^+]$^-$) and 173 ([quinic acid−H_2O − H^+]$^-$), respectively (Table 1). Their MS^3 and MS^4 spectra are identical to the MS^2 and MS^3 spectra of 1,5-dicaffeoylquinic acid (**6**) and 4,5-dicaffeoylquinic acid (**11**),[21] respectively. Isomer **16** produced MS^2 secondary peaks at m/z 395 ([caffeoyl-methoxyoxaloylquinic acid−O_2 − H^+]$^-$) by the loss of a caffeoyl residue and a CO_2 molecule, and at m/z 439 ([caffeoyl-methoxyoxaloylquinic acid − H^+]$^-$) by the loss of a caffeoyl residue (Table 1). Isomer **16** loses its methoxyoxaloyl residue before the caffeoyl residue, but the intensity is almost equivalent (95%) to the one of the MS^2 base peak; from our previous studies, it is known that the loss of the C3 and the C5 acyl residue is almost the same.[20−23] Consequently, if this is the case, then isomer **16** was tentatively assigned as 1,5-dicaffeoyl-3-methoxyoxaloylquinic acid. Isomer **19** also produced MS^2 secondary peaks at m/z 395 and 439, but the intensities are very low if compared to isomer **16**. On the basis of the above points, the methoxyoxaloyl residue should thus be present at C1, and isomer **19** was tentatively assigned as 1-methoxyoxaloyl-4,5-dicaffeoylquinic acid. Isomers **16** and **19** were previously reported in Arnica flowers, but there were no reasonable arguments for the assignment of regiochemistry of the acyl residues.[17]

Isomers **17** and **18** produced MS^2 base peaks at m/z 395 ([caffeoyl-methoxyoxaloylquinic acid-CO_2 − H^+]$^-$) by the loss of a caffeoyl residue and a CO_2 molecule and secondary peaks at m/z 515 ([dicaffeoylquinic acid − H^+]$^-$) and 557 ([dicaffeoyl-methoxyoxaloylquinic acid-CO_2 − H^+]$^-$). They produced identical MS^3 base peaks at m/z 233 by the loss of the second caffeoyl residue and MS^4 base peaks at m/z 173 ([quinic acid-H_2O − H^+]$^-$) (Table 1). An MS^4 base peak at m/z 173 suggests that both isomers have a methoxyoxaloyl residue at C4,[20] and they are dicaffeoyl-4-methoxyoxaloylquinic acids. Isomer **18** produced a more intense MS^2 secondary peak at m/z 439 by the loss of a caffeoyl residue if compared to isomer **17**. From this, it is clear that they both have at least one caffeoyl residue, which is at a different position. They have identical MS^3 and MS^4 spectra, which suggested that they have a second caffeoyl residue at the same position. The location of the caffeoyl residues was investigated by fragment-targeted MS^3 experiments at m/z 601 + 353 and 601 + 515. Isomer **17** produced MS^3 peaks in the favor of 1,3-dicaffeoylquinic acid, and isomer **18** produced MS^3 base peaks in the favor of 3,5-dicaffeoylquinic acid. On the basis of the above points, isomers **17** and **18** were tentatively assigned as 1,3-dicaffeoyl-4-methoxyoxaloylquinic acid and 3,5-dicaffeoyl-4-methoxyoxaloylquinic acid, respectively.

Characterization of Putative Caffeoyl-feruloyl-methoxyoxaloylquinic Acids (M_r 616). Two peaks eluting between ∼48−51 min produced the pseudomolecular ion at m/z 615 and were tentatively assigned as feruloyl-caffeoyl-methoxyoxaloylquinic acids. The most hydrophilic isomer **20** produced an MS^2 base peak at m/z 409 by the loss of a caffeoyl residue and a CO_2 molecule from the methoxyoxaloyl residue and secondary peaks at m/z 571 (70% of the base peak) (loss of a CO_2 molecule) and 529 (5% of the base peak) (loss of a methoxyoxaloyl residue) (Table 1). In the MS^3 spectrum, it produced the base peak at m/z 349 ([feruloylquinic acid-H_2O − H^+]$^-$) by the loss of a H_2O molecule and an acetyl residue. The base peak at m/z 349 in the MS^3 spectrum is characteristic of the feruloyl residue at position C4 of the quinic acid moiety in a 3,4-heterodiacyl CGA.[20] The MS^4 base peak at m/z 193 is also in agreement with the assignment. The location of the caffeoyl residue was investigated by a fragment-targeted MS^3 experiment at m/z 615 + 353. At MS^2, it produced m/z 191 ([3-caffeoylquinic acid-caffeoyl − H^+]$^-$) and a secondary peak at m/z 179 ([caffeic acid − H^+]$^-$) (30% of the base peak).[21] From this, it is clear that the caffeoyl residue is at position C3 and the methoxyoxaloyl residue is at position C5 of the quinic acid moiety. In this case, compound **20** loses CO_2 preferentially to the methoxyoxaloyl residue. On the basis of the above points, isomer **20** was tentatively assigned as 3-caffeoyl-4-feruloyl-5-methoxyoxaloylquinic acid.

Second eluting isomer **21** produced the MS^2 base peak at m/z 409 by the loss of a caffeoyl residue and secondary peaks at m/z 571 and 367 (Table 1). It produced the MS^3 base peak at m/z 233 by the loss of a feruloyl residue and a secondary peak at m/z 349. In the MS^4 spectrum, it produced the base peak at m/z 173 ([quinic acid-H_2O − H^+]$^-$) by the loss of an acetyl residue and a H_2O molecule. The MS^n base peaks at m/z 173 are characteristic of C4 acylated quinic acid, which means the methoxyoxaloyl residue is at C4 of the quinic acid moiety. Targeted MS^3 experiments of m/z 615 + 353 and 615 + 367 produced the MS^2 base peak at m/z 191, which suggested that the caffeoyl and the feruloyl residues are at C1 and C3, respectively.[20,21] On the basis of the above arguments, isomer **21** was tentatively assigned as 3-feruloyl-4-methoxyoxaloyl-5-caffeoylquinic acid.

Characterization of Putative Dicaffeoyl-fumaroylquinic Acids (M_r 614). Two chromatographic peaks were detected at m/z 613 eluting between ∼37−39 min, and their LC−MS data are summarized in Table 1. Both isomers **22** and **23** produced MS^2 base peaks at m/z 515 ([dicaffeoylquinic acid − H^+]$^-$) by the loss of a fumaroyl residue. First, eluting isomer **22** produced an MS^2 secondary peak at m/z 433 by the loss of a caffeoyl residue and a H_2O molecule, which is completely absent in the second eluting isomer (Table 1). Both isomers have MS^3 and MS^4 spectra identical to MS^2 and MS^3 spectra of 1,5-dicaffeoylquinic acid. Accordingly, these isomers are 1,5-dicaffeoyl-fumaroylquinic acids. In both cases, the fumaroyl residue cannot be at the C3 or C4 position. From our previous studies, it has been proven that the loss of the C4 acyl residue is more difficult than that of the C1, C3, and C5 residues.[20] The first eluting isomer **22** produced an MS^2 secondary peak at m/z 433 by the loss of a caffeoyl residue, while this peak was absent for the second isomer **23.** Given this, it is clear that for the first isomer the loss of the fumaroyl residue at C4 competes with the loss of the caffeoyl residue at C1. On the basis of the above arguments, isomers **22** and **23** were tentatively assigned as 1,5-dicaffeoyl-4-fumaroylquinic acid and 1,5-dicaffeoyl-3-fumaroylquinic acid, respectively.

Characterization of Putative Dicaffeoyl-dimethoxyoxaloylquinic Acid (M_r 688). Isomer **24** produced the pseudomolecular ion at m/z 687 (∼48 min) and was tentatively assigned as dicaffeoyl-dimethoxyoxaloylquinic acid. This isomer produced the MS^2 base peak at m/z 601 ([dicaffeoyl-methoxyoxaloylquinic acid − H^+]$^-$) by the loss of a methoxyoxaloyl residue, which suggested that one of the two methoxyoxaloyl residues is found at the C1 position of the quinic acid moiety (Table 1). It additionally showed the MS^3 base peak at m/z 395 ([caffeoyl-methoxyoxaloylquinic acid-CO_2 − H^+]$^-$) by the loss of a caffeoyl residue and a CO_2 molecule from the second methoxyoxaloyl residue, which suggested that one of the caffeoyl residues was at C5. In the MS^4 spectrum, it produced the base peak at m/z 233 by the loss of another caffeoyl residue, which suggested the second caffeoyl residue at C3. This isomer produced the MS^4 base peak at m/z 173 ([quinic acid-H_2O − H^+]$^-$), which is

characteristic of the 4-acylated chlorogenic acids.[20] For further evidence, a targeted MS^3 experiment of m/z 687 + 515 produced the base peak at m/z 353 and secondary peaks at m/z 191 and 179, which are specific to 3,5-diCQA **7**.[21] A similar experiment of m/z 395 + 233 produced the MS^3 base peak at m/z 173, which is characteristic of C4-acylated quinic acid.[20] On the basis of the above arguments, isomer **24** was tentatively assigned as 3,5-dicaffeoyl-1,4-dimethoxyoxaloylquinic acid.

Characterization of Putative Tricaffeoyl-methoxyoxaloyl-quinic Acids (M_r 764). Two chromatographic peaks were detected at m/z 763 eluting between ~51−55 min, and their LC−MS data are summarized in Table 1. These isomers were tentatively assigned as tricaffeoyl-methoxyoxaloylquinic acids. Both isomers (**25** and **26**) have comparable MS^4 and MS^5 spectra, which are similar to the MS^2 and MS^3 spectra of 4,5-dicaffeoylquinic acid (Table 1).[21] Both isomers produced MS^2 base peaks at m/z 677 and secondary peaks at m/z 557 and 719 with different intensities (isomer **26** showed higher intensities for both secondary peaks). This suggests that isomer **26** loses its caffeoyl residue and a CO_2 molecule (from the methoxyoxaloyl residue) faster than does isomer **25** (MS^2 secondary peaks). On the basis of the above arguments, isomers **25** and **26** were tentatively assigned as 1-methoxyoxaloyl-3,4,5-tricaffeoylquinic acid and 1,3,4-tricaffeoyl-5-methoxyoxaloylquinic acid, respectively.

■ ASSOCIATED CONTENT

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

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