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Profiling the Chlorogenic Acids and Other Caffeic Acid Derivatives of Herbal Chrysanthemum by LC−MS^n

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Four samples of herbal chrysanthemum have been profiled qualitatively by LC−MS^5 to identify their component chlorogenic acids and partially characterize other caffeic acid derivatives. The chlorogenic acids were minor components, and the four samples varied markedly in profile. Three *p*-coumaroylquinic acids, three feruloylquinic acids, four caffeoylquinic acids, six dicaffeoylquinic acids, and two tricaffeoylquinic acids were detected, 13 for the first time from this source. Partial characterization of minor components suggested the presence of five caffeoyl-hexose esters and caffeic acid-4-β-D-glucose that have not previously been reported from this source, and eight caffeoylquinic acid glycosides and 16 dicaffeoylquinic acid glycosides that have not previously been reported in nature. Succinic acid-containing chlorogenic acids and chlorogenic acids based on *epi*-quinic acid, previously reported in *Chrysanthemum* spp., were not detected in these samples.

KEYWORDS: Asteraceae; Beijuhua; caffeic acid glycosides; caffeoyl-hexoses; caffeoylquinic acids; caffeoylquinic acid glycosides; chlorogenic acids; *Chrysanthemum*; *p*-coumaroylquinic acids; cynarin; *Dendranthema*; dicaffeoylquinic acid glycosides; dicaffeoylquinic acids; disuccinoyl-caffeoylquinic acids; feruloylquinic acids; Garland; Gongju; Ju Hua; LC−MS^n; succinoyl-caffeoylquinic acids; succinoyl-dicaffeoylquinic acids; tricaffeoylquinic acids

INTRODUCTION

Cinnamic acid conjugates are known to occur widely in dicotyledenous plants (*1*). The best known and most extensively studied are the chlorogenic acids (*2*−*4*). Classically, chlorogenic acids are a family of esters formed between one or more residues of certain *trans*-cinnamic acids (for example, *p*-coumaric, ferulic, or caffeic) and quinic acid (*3*, *4*). In the IUPAC system, (−)-quinic acid is defined as 1l-1(OH),3,4/5-tetrahydroxycyclohexane carboxylic acid (*5*), but Eliel and Ramirez (*6*) now propose 1α,3*R*,4α,5*R*-tetrahydroxycyclohexane carboxylic acid. Structurally related, but much less studied, are similar esters containing one or more succinic acid residues in addition to one or more caffeic acid residues, as observed in some Asteraceae, for example *Chrysanthemum* spp. (*7*−*9*) and *Arctium* spp (*10*). These species produce chlorogenic acids in which all four quinic acid hydroxyl groups may be acylated (*10*, *11*) and a wide range of chlorogenic acids may be anticipated, but at the start of this study only 5-caffeoylquinic acid (**3**), three dicaffeoylquinic acids (1,5-dicaffeoylquinic acid (**7**), 3,5-dicaffeoylquinic acid (**9**), and 4,5-dicaffeoylquinic acid (**10**)), and five succinic acid-containing tri-acyl and tetra-acyl chlorogenic acids (1,5-dicaffeoyl-3-succinoylquinic acid (**11**), 1,5-dicaffeoyl-4-succinoylquinic acid (**12**), 3,5-dicaffeoyl-4-succinoylquinic acid (**13**), 1,5-dicaffeoyl-3,4-disuccinoylquinic acid (**14**), and 1,3,5-tricaffeoyl-4-succinoylquinic acid (**15**)) had been reported (*7*−*13*). Garland (*C. coronarium*) is eaten as a vegetable (*7*), and extracts of both chrysanthemum (*14*, *15*) and burdock (*10*) are used as herbal teas and/or medicines in Asia.

Recently, LC−MS^n has been used to characterize cinnamoyl−amino acid conjugates (*16*) and to discriminate between individual isomers of mono-acyl and di-acyl chlorogenic acids, for example, between six caffeoyl-feruloylquinic acid isomers (*17*), between the six dicaffeoylquinic acids (*18*), six chlorogenic acids with M_r 544 (*19*), and six *p*-coumaroyl-caffeoylquinic acids (*20*). The MS fragmentation data have been utilized to develop structure-diagnostic hierarchical keys for the identification of chlorogenic acids. In this study, we applied these methods to the qualitative profiling of chlorogenic acids and other caffeic acid derivatives in extracts from plants known commonly as chrysanthemum (*Chrysanthemum morifolium* and *Dendranthema morifolium*).

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MATERIALS AND METHODS

Plant Material. Four samples of chrysanthemum were obtained, three from Changsha and one from the UK. Sample 1 was Huangshan gongju, the flower of *Dendranthema morifolium* cv. *Gongju* (*Dendranthema morifolium* (Ramat) Tzvel. cv. *Gongju*) from Huangshan in Anhui Province. Traditionally, the flowers are soaked in boiled water to prepare an herbal tea, to flavor wine, and as an ingredient of traditional Chinese medicines. Sample 2, Beijuhua, the flower of *Dendranthema morifolium* cv. *Beijuhua* (*Dendranthema morifolium* (Ramat) Tzvel. cv. *Beijuhua*) (also known as florist's chrysanthemum), from the same geographic origin is used mainly as a herbal tea. Sample 3, Hangzhou Ju Hua from Hangzhou in Zhejiang Province, is used mainly as an ingredient in traditional Chinese medicines. Sample 4 was purchased in the UK (Mayway UK Co. Ltd., Hanwell, London, UK) and is described as a concentrated Ju Hua powder. Ju Hua, also known as florist's chrysanthemum, is prepared from the flowers of *C. morifolium*.

Extraction. Methanolic extracts were prepared as previously described (*17*). Samples (1 g) were extracted (4 × 25 mL, 25 min each) with 70% v/v aqueous methanol using an HT-1043 solid−liquid continuous extraction system (Tecator, Bristol, UK). The bulked extracts were treated with Carrez reagents (1 mL of reagent A plus 1 mL of reagent B) (*21*) to precipitate colloidal material, diluted to 100 mL with 70% v/v aqueous methanol, and filtered through Whatman No. 1 filter paper. The methanol was removed by evaporation with 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, autosampler with 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*. Based upon experience gained in previous studies (*17*, *18*, *22*), MS^5 experiments were used initially to locate compounds producing fragment ions, for example, *m/z* 85, 93, 173, or 191, characteristic of the quinic acid moiety of mono-acyl, di-acyl, tri-acyl, or tetra-acyl chlorogenic acids, and *m/z* 179 characteristic of a caffeoyl substituent. For greater sensitivity and better discrimination of isomers, additional MS^n experiments were performed that focused only on compounds producing a particular parent ion, for example, at *m/z* 291 for succinoylquinic acids, *m/z* 453 for succinoyl-caffeoylquinic acids, *m/z* 553 for disuccinoyl-caffeoylquinic acids, *m/z* 615 for succinoyl-dicaffeoylquinic acids, *m/z* 653 for trisuccinoyl-caffeoylquinic acids, *m/z* 677 for tricaffeoylquinic acids, *m/z* 715 for disuccinoyl-dicaffeoylquinic acids, and *m/z* 777 for succinoyl-tricaffeoylquinic acids. MS operating conditions (negative ion) had been optimized using 5-caffeoylquinic acid (**3**) with a collision energy of 35%, ionization voltage of 3.5 kV, capillary temperature 350 °C, sheath gas flow rate 65 arbitrary units, and auxiliary gas flow rate 10 arbitrary units.

Chlorogenic acid separations were achieved on a 150 × 3 mm column containing Kromasil phenylhexyl packing (Phenonemex, Macclesfield, UK). 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. The gradient profile for chlorogenic acid characterization was 4% B to 33% B linearly in 90 min, a linear increase to 100% B at 95 min, followed by 5 min isocratic, and a return to 4% B at 105 and 5 min isocratic to re-equilibrate. The gradient profile for characterization of the non-chlorogenic acid components was 4% B to 33% B linearly in 45 min, a linear increase to 100% B at 50 min, followed by 5 min isocratic, and a return to 4% B at 60 and 5 min isocratic to re-equilibrate.

RESULTS AND DISCUSSION

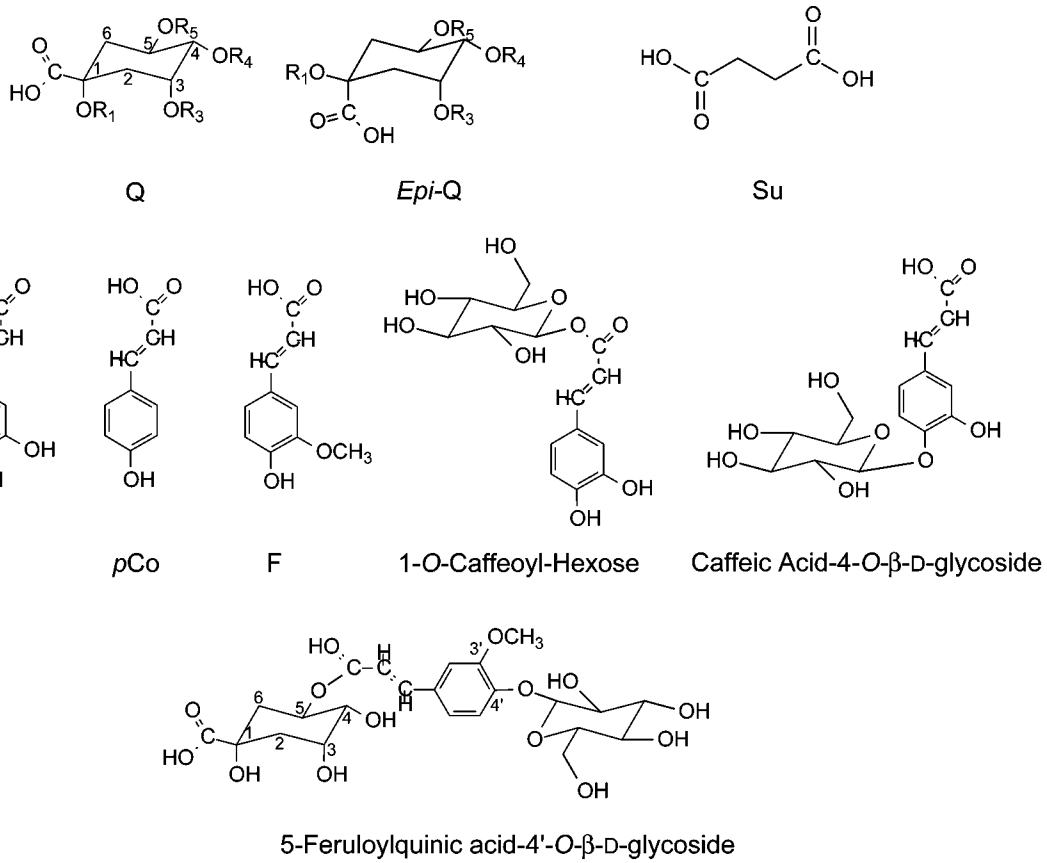
Preliminary Assessment of Data. All data for chlorogenic acids presented in this Article use the recommended IUPAC numbering system (*5*), and structures are presented in **Figure 1**. Where necessary, previously published data have been amended to ensure consistency and avoid ambiguity. Peak assignments have been made on the basis of the structure-diagnostic hierarchical keys previously developed, supported by examination of the UV spectrum and retention time relative to 5-caffeoylquinic acid (**3**) (*17*, *18*). For quantitatively minor components, more sensitive and more selective fragment-targeted MS^n experiments were used. For certain chlorogenic acids, such experiments generate spectra that differ slightly from those used in the original diagnostic keys. For example, 4-caffeoylquinic acid (**4**) yields nearly equal *m/z* 173 and *m/z* 179 ions at MS^2 compared with an *m/z* 173 base peak and *m/z* 179 at ∼70% of base peak (*22*), but the diagnostic keys remain valid.

Initial screening using targeted MS^2 experiments failed to locate molecular ions corresponding to succinoylquinic acids, disuccinoyl-dicaffeoylquinic acids, trisuccinoyl-caffeoylquinic acids, and succinoyl-tricaffeoylquinic acids, and it was concluded that these classes of chlorogenic acids were not present, although disuccinoyl-dicaffeoylquinic acids and succinoyl-tricaffeoylquinic acids have been reported in burdock (*10*).

It was evident from the chromatograms recorded at 320 nm that chlorogenic acids were not the dominant components of these extracts. The four chrysanthemum extracts differed significantly in composition (**Figure 2**). This variation was greater than could be accounted for by typical between-sample inhomogeneity and may indicate that the plants from which these extracts have been prepared are not as close genetically as use of the term chrysanthemum implies, but the possibility also of artefacts arising from commercial preparation should not be discounted. Interestingly, sample 4 purchased in the UK (described as concentrated Ju Hua) more closely resembled sample 2 (Beijuhua purchased in China) rather than sample 3 (Ju Hua purchased in China).

Characterization of Caffeoylquinic Acids (M_r 354) and Dicaffeoylquinic Acids (M_r 516). Four caffeoylquinic acids were easily located in the extracts from all four samples and assigned using the hierarchical keys previously developed (*17*, *18*) as the well-known 1-caffeoylquinic acid (**1**), 3-caffeoylquinic acid (**2**), 4-caffeoylquinic acid (**4**), and 5-caffeoylquinic acid (**3**), with the latter isomer dominating this group in every case (**Figure 2**). In samples 2 and 4, 5-caffeoylquinic acid (**3**) was the dominant component at 320 nm: it was subdominant in sample 1, but a comparatively minor component in sample 3 (∼0.25% the amount in sample 4).

Similarly, six dicaffeoylquinic acid isomers were easily identified by their *m/z* 515 parent ion and assigned as 1,3-dicaffeoylquinic acid (**5**), 1,4-dicaffeoylquinic acid (**6**), 1,5-dicaffeoylquinic acid (**7**), 3,4-dicaffeoylquinic acid (**8**), 3,5-dicaffeoylquinic acid (**9**), and 4,5-dicaffeoylquinic acid (**10**) (*17*, *18*). 1,5-Dicaffeoylquinic acid (**7**) dominated this subgroup in all samples, followed by 3,5-dicaffeoylquinic acid (**9**) and 4,5-dicaffeoylquinic acid (**10**): 1,5-Dicaffeoylquinic acid (**7**) was the second strongest component (A_{320}) in sample 4, a comparatively minor component in sample 1, and only a trace was detected in sample 3. When this investigation commenced, only 5-caffeoylquinic acid (**3**), 1,5-dicaffeoylquinic acid (**7**), 3,5-dicaffeoylquinic acid (**9**), and 4,5-dicaffeoylquinic acid (**10**) had been reported (*7*, *9*, *12*, *13*) in *Chrysanthemum* spp., but 1,3-dicaffeoylquinic acid (**5**) has since been reported in the flowers of *C. morifolium* (*23*). These workers also reported 1,3-dicaffeoyl-*epi*-quinic acid (**27**) and 3,5-dicaffeoyl-*epi*-quinic acid (**28**), but these isomers in which the quinic acid carboxyl is axial were not detected in the current study.



Name and abbreviation	Number	R_1	R_3	R_4	R_5
1-*O*-caffeoylquinic acid	1	C	H	H	H
3-*O*-caffeoylquinic acid	2	H	C	H	H
5-*O*-caffeoylquinic acid	3	H	H	H	C
4-*O*-caffeoylquinic acid	4	H	H	C	H
1,3-di-*O*-caffeoylquinic acid	5	C	C	H	H
1,4-di-*O*-caffeoylquinic acid	6	C	H	C	H
1,5-di-*O*-caffeoylquinic acid	7	C	H	H	C
3,4-di-*O*-caffeoylquinic acid	8	H	C	C	H
3,5-di-*O*-caffeoylquinic acid	9	H	C	H	C
4,5-di-*O*-caffeoylquinic acid	10	H	H	C	C
1,5-di-*O*-caffeoyl, 3-*O*-succinoylquinic acid[a]	11	C	Su	H	C
1,5-di-*O*-caffeoyl, 4-*O*-succinoylquinic acid[a]	12	C	H	H	C
3,5-di-*O*-caffeoyl, 4-*O*-succinoylquinic acid[a]	13	H	C	Su	C
1,5-di-*O*-caffeoyl, 3,4-di-*O*-succinoylquinic acid[a]	14	C	Su	Su	C
1,3,5-tri-*O*-caffeoyl, 4-*O*-succinoylquinic acid[a]	15	C	C	Su	C

Name and abbreviation	Number	R_1	R_3	R_4	R_5
1-*O*-feruloylquinic acid	16	F	H	H	H
3-*O*-feruloylquinic acid	17	H	F	H	H
5-*O*-feruloylquinic acid	18	H	H	H	F
4-*O*-feruloylquinic acid	19	H	H	F	H
1-*O*-*p*-coumaroylquinic acid	20	*p*Co	H	H	H
3-*O*-*p*-coumaroylquinic acid	21	H	*p*Co	H	H
5-*O*-*p*-coumaroylquinic acid	22	H	H	H	*p*Co
4-*O*-*p*-coumaroylquinic acid	23	H	H	*p*Co	H
1,3,4-tri-*O*-caffeoylquinic acid	24	C	C	C	H
1,3,5-tri-*O*-caffeoylquinic acid	25	C	C	H	C
1,4,5-tri-*O*-caffeoylquinic acid	26	C	H	C	C
3,4,5-tri-*O*-caffeoylquinic acid	27	H	C	C	C
1,3-di-*O*-caffeoyl-*epi*-quinic acid[a]	28	C	C	H	H
3,5-di-*O*-caffeoyl-*epi*-quinic acid[a]	29	H	C	H	C

Figure 1. Structures of selected chrysanthemum chlorogenic acids (IUPAC numbering) (*5*), glucose esters, and glycosides. Q, quinic acid; *epi*-Q, *epi*-quinic acid; C, caffeic acid; *p*Co, *p*-coumaric acid; F, ferulic acid; Su, succinic acid. (a) Reported in the literature (*7–10*, *23*) but not detected in the current study.

Characterization of Putative Succinic Acid-Containing Chlorogenic Acids. Targeted MS^2 experiments located 12 parent ions at *m*/*z* 453 (putative succinoyl-caffeoylquinic acids), five parent ions at *m*/*z* 553 (putative dicaffeoylquinic-succinoylquinic acids), and 20 parent ions at *m*/*z* 615 (putative succinoyl-dicaffeoylquinic acids) at retention times ranging from 8 to 70 min. Although there were some variations in the relative intensity of individual peaks, most of these parent ions were located in all samples, but none had UV spectra typical of chlorogenic acids.

A series of MS^3 fragment-targeted experiments were performed to seek fragments that might have been expected to form from succinoyl-caffeoylquinic acids, such as (*m*/*z* 453 + 353) and (*m*/*z* 453 + 291); from disuccinoyl-caffeoylquinic acids, such as (*m*/*z* 553 + 453), (*m*/*z* 553 + 391), and (*m*/ *z* 553 + 353); or from succinoyl-dicaffeoylquinic acids, such as (*m*/*z* 615

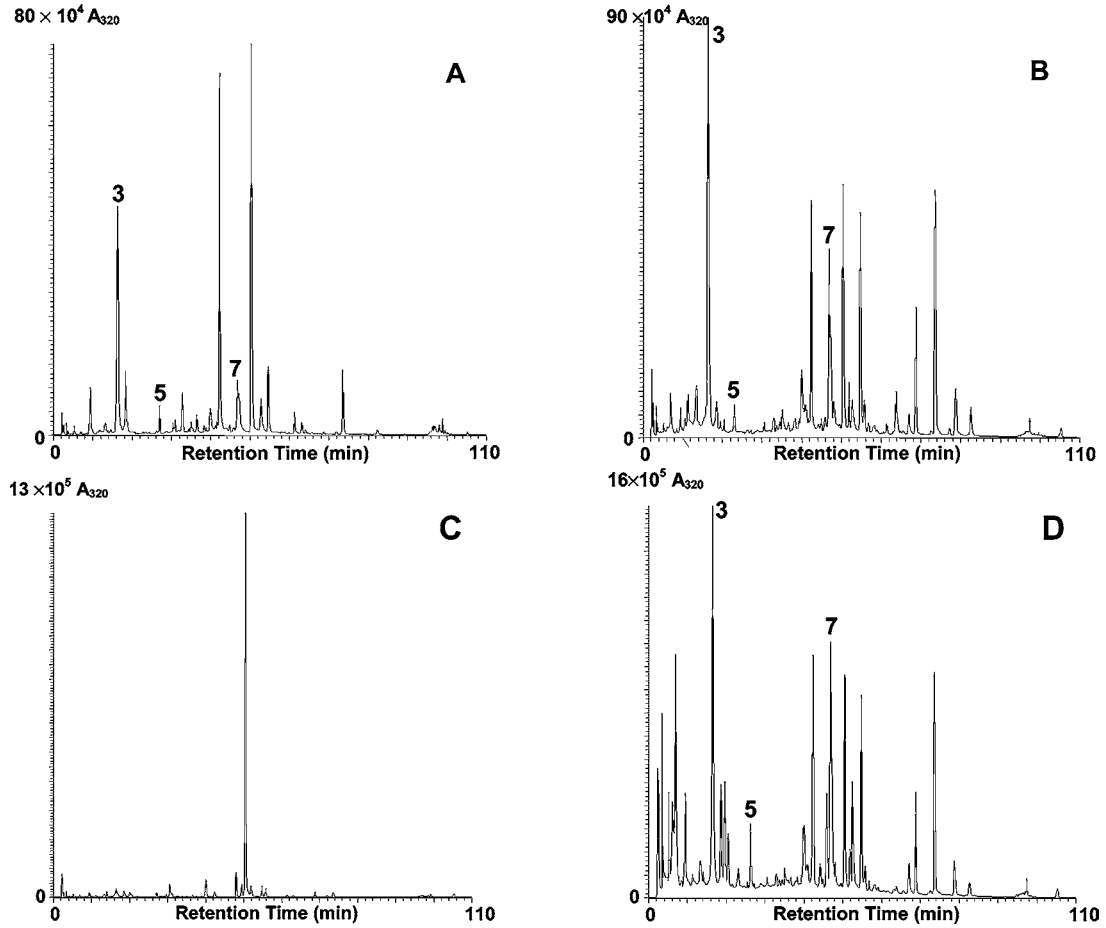


Figure 2. Profiles (A_{320}) of methanolic extracts of four samples of chrysanthemum: (**A**) sample 1 = Huangshan gongju; (**B**) sample 2 = Beijuhua; (**C**) sample 3 = Hangzhou Ju Hua; (**D**) sample 4 = concentrated Ju Hua. Peak 3 = 5-caffeoylquinic acid. Peak 5 = 1,3-dicaffeoylquinic acid. Peak 7 = 1,5-dicaffeoylquinic acid.

+ 515) and *m*/*z* (615 + 453). These spectra contained no peaks significantly different from the baseline noise, and it was concluded that the succinic acid-containing chlorogenic acids were not present in these four samples of chrysanthemum. A recent study of *C. morifolium* flowers also failed to observe succinic acid-containing chlorogenic acids (*23*). Previously, 3,5-dicaffeoyl-4-succinoylquinic acid (**13**) has been reported in *C. coronarium* (Tong hao) leaves, but the content varied approximately 3-fold with cultivar, declined (often markedly) with maturation of the plant, and the content in the stem was only ~5−10% that of the young leaves. It is rapidly leached into boiling water during domestic cooking (*7*, *9*). We have previously observed (unpublished data) the analogous 3,5-dicaffeoyl-4-(3-hydroxy,3-methyl)glutaroyl)quinic acid in *Gardenia* extracts without difficulty, suggesting that the sample preparation and analysis methods are appropriate, and our failure to detect succinic acid-containing chlorogenic acids might genuinely reflect their absence from the species studied (samples 1−3) or losses during the commercial preparation of the plant material analyzed (sample 4).

Characterization of Feruloylquinic Acids (M_r 368) and *p*-Coumaroylquinic Acids (M_r 338). A targeted MS^2 experiment at *m*/*z* 337 applied to extract sample 4 located three minor components that were identified by their fragmentation (*17*, *22*) as the well-characterized 3-*p*-coumaroylquinic acid (**21**), 5-*p*-coumaroylquinic acid (**22**), and 4-*p*-coumaroylquinic acid (**23**). The analogous experiment at *m*/*z* 367 similarly located 3-feruloylquinic acid (**17**), 5-feruloylquinic acid (**18**), and 4-feruloylquinic acid (**19**). These six components were clearly present also in sample 2, but were detected only as traces in samples 1 and 3. These six chlorogenic acids were recently reported (*24*) in the flower buds of *Aster ageratoides* Turcz., but the cis isomer of 5-*p*-coumaroylquinic acid (**22**) prominent in aster was not observed in chrysanthemum. Despite finding 1-caffeoylquinic acid (**1**), a search for the corresponding 1-*p*-coumaroylquinic acid (**20**) and 1-feruloylquinic acid (**16**) was unsuccessful. Full chromatographic and mass spectrometric fragmentation data have been presented previously (*17*, *22*, *24*).

Characterization of Putative Tricaffeoylquinic Acids (M_r 678). A search for tricaffeoylquinic acids as previously reported in various Asteraceae (*25*−*30*) located 19 signals at *m*/*z* 677 in the extract from sample 4. Sixteen eluted before 4,5-dicaffeoylquinic acid (**10**), suggesting that they are too hydrophilic to be tricaffeoylquinic acids, and their characterization is dealt with separately.

The most hydrophobic of the three minor components that eluted after 4,5-dicaffeoylquinic acid (**10**) (**Figure 3**) progressively lost caffeoyl residues and produced a [4-caffeoylquinic acid − H^+]$^-$ ion at MS^3, thus clearly eliminating 1,3,5-tricaffeoylquinic acid (**25**) that would have produced [3-caffeoylquinic acid − H^+]$^-$. It produced only weak signals at *m*/*z* 299, 255, and 203 (less than 7% of base peak), suggesting by analogy with 1,4-dicaffeoylquinic acid (**6**) (*18*) that it is not 1,3,4-tricaffeoylquinic acid (**24**) or 1,4,5-tricaffeoylquinic acid (**26**) (*18*). Accordingly, it was assigned tentatively as 3,4,5-tricaffeoylquinic acid (**27**) that has been reported in several Asteraceae (*26*,

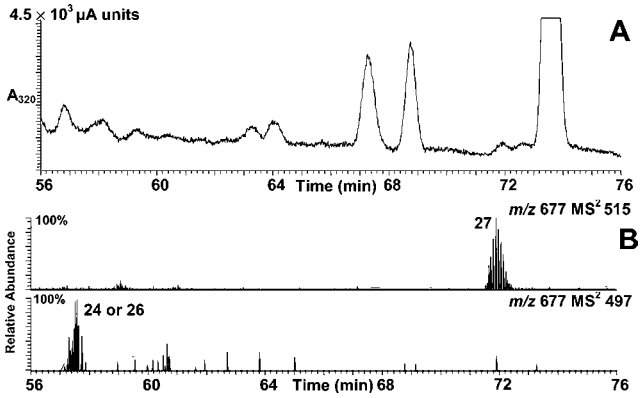


Figure 3. Targeted negative ion LC–MS (long gradient) for putative tricaffeoylquinic acids in the extract prepared from sample 4 (concentrated Ju Hua): (**A**) A_{320} and (**B**) SIM *m*/*z* 677 traces. Peak 24 = 1,3,4-tricaffeoylquinic acid. Peak 26 = 1,4,5-tricaffeoylquinic acid. Peak 27 = 3,4,5-tricaffeoylquinic acid.

29, *30*). The presence of only one free axial hydroxyl in the quinic acid moiety is consistent with its pronounced hydrophobicity. The most hydrophilic of the three components with M_r 678, at 57.5 min, produced an *m*/*z* 497 MS^2 base peak accompanied by *m*/*z* 515 (60% of base peak). Its MS^3 base peak (*m*/*z* 335) was accompanied by *m*/*z* 299 and 255 (7% and 5% of base peak, respectively). Targeted MS^4 (*m*/*z* 677 + 515 + 353) yielded [4-caffeoylquinic acid − H^+]$^-$, suggesting either 1,3,4-tricaffeoylquinic acid (**24**) or 1,4,5-tricaffeoylquinic acid (**26**). The latter has been reported in *Arnica* spp. (Asteraceae) (*28*). The third late-eluting component with an *m*/*z* 677 base peak was not a chlorogenic acid or other caffeic acid derivative, and it was not investigated further.

Miscellaneous Caffeic Acid-Containing Compounds (M_r 342, 516, and 678). Although mass spectrometrically distinct, many of these hydrophilic compounds were poorly resolved chromatographically, and in general it was not possible to obtain good UV spectra. These were investigated only in sample 4, using the shorter chromatographic procedure.

***M*$_r$ 342.** Eleven signals were observed at *m*/*z* 341. Five of these, observed between 10 and 16 min, gave an MS^2 base peak at *m*/*z* 215 but did not yield *m*/*z* 179 and were not investigated further. At MS^2, the other six (**Figure 4A**) produced *m*/*z* 179 followed by *m*/*z* 135 at MS^3 consistent with the presence of a caffeic acid residue. The most hydrophobic, eluting at ∼16 min, appeared to be identical to caffeic acid-4-*β*-D-glucoside and was tentatively assigned as such. It has a widespread occurrence (*31*−*34*) but has not previously been reported in Asteraceae so far as we are aware.

The MS^2 spectra of the other five, eluting between 10 and 15.5 min, were characterized by fragment ions at *m*/*z* 311, 281, 251, and 221, and 233 and 203, which could be produced by the loss of units based on −CHOH. This behavior is analogous to the behavior of purified digalloylglucoses that produce MS^3 fragments at *m*/*z* 301, 271, 241, and 211, and essentially identical to that reported previously for two caffeoylglucoses in Maté (*Ilex paraguariensis* = *I. paraguayensis*, Aquifoliaceae) (*35*). We tentatively assign these as isomeric caffeoyl-hexoses. In contrast to the putative caffeoyl esters, the spectra of caffeic acid-4-*β*-D-glucoside and caffeic acid-3-*β*-D-glucoside did not show any evidence of sugar fragmentation, and these differences in behavior may allow glycosides to be distinguished from sugar esters. As we have demonstrated for the quinic acid esters (*17*−*20*, *22*, *24*), there is variation in the relative intensity of the

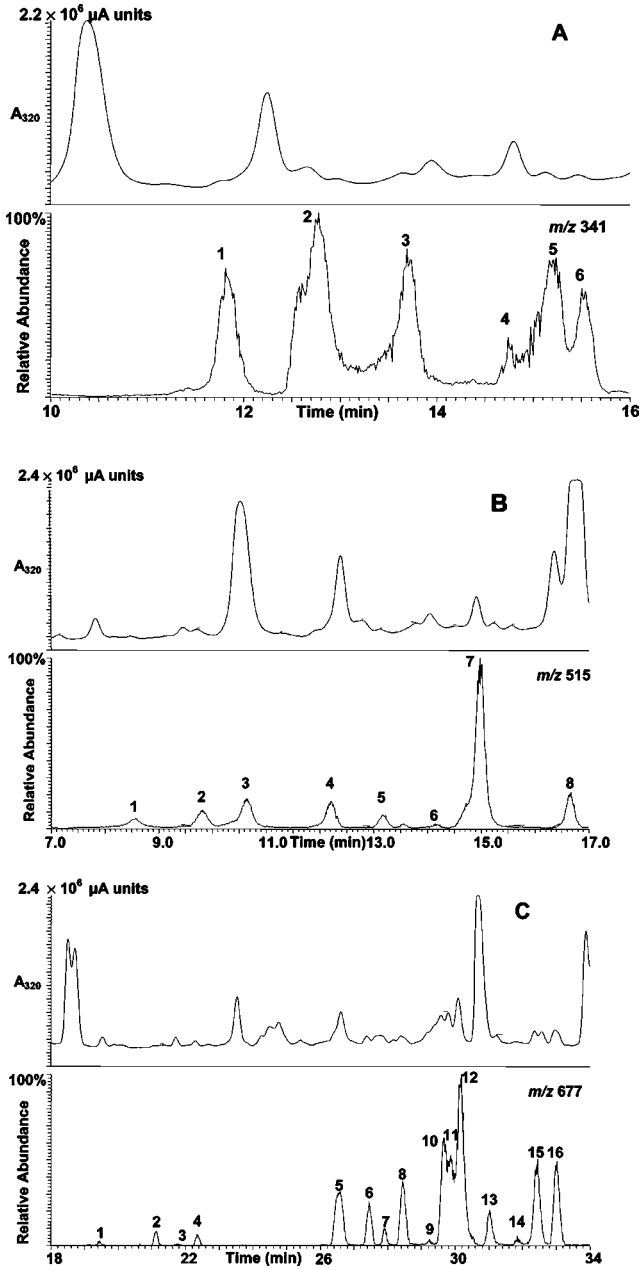


Figure 4. (**A**) Targeted negative ion LC–MS (short gradient, 10–16 min) A_{320} and SIM *m*/*z* 341 traces for putative caffeoyl-hexose esters and caffeic acid glycosides in the extract prepared from sample 4 (concentrated Ju Hua). For peak characteristics, see **Table 1A**. (**B**) Targeted negative ion LC–MS (short gradient, 7–17 min) A_{320} and SIM *m*/*z* 515 traces for putative caffeoylquinic acid glycosides in the extract prepared from sample 4 (concentrated Ju Hua). For peak characteristics, see **Table 1B**. (**C**) Targeted negative ion LC–MS (short gradient, 18–34 min) A_{320} and SIM *m*/*z* 677 traces for putative dicaffeoylquinic acid glycosides in the extract prepared from sample 4 (concentrated Ju Hua). For peak characteristics, see **Table 2**.

polyol fragment ions (**Table 1A**), and this may allow individual sugar esters to be distinguished, but confirmation must await the availability of appropriate pure standards. During the development of our hierarchical schemes for the identification of chlorogenic acids, we have argued that fragmentation is not necessarily driven directly by the nucleophilic attack of a carboxylate anion because this charge may subsequently migrate inter- or intramolecularly (*18*). The current observation of

Table 1. Mass Spectrometric Characteristics of the Hydrophilic Components Producing (**A**) an *m*/*z* 341 or (**B**) an *m*/*z* 515 Parent Ion

(**A**) *m*/*z* 341

peak number *m*/*z* 341 parent ion	MS^2 base peak	intensity of MS^2 fragment ions (% MS^2 base peak *m*/*z* 341)						
		m/*z* 311	*m*/*z* 281	*m*/*z* 251	*m*/*z* 233	*m*/*z* 221	*m*/*z* 203	*m*/*z* 179
1	179		5	5	55		20	100
2	251		20	100	35		15	50
3	281	10	100	80	25	20		50
4	251		37	100	10	35		40
5	251		80	100	8	25		40
6	179							100

(**B**) *m*/*z* 515

peak number *m*/*z* 515 parent ion	MS^2 base peak	*m*/*z* 341 as % MS^2 base peak	*m*/*z* 323 as % MS^2 base peak	identity of *m*/*z* 353
1	179	32	12	3-caffeoylquinic
2	323	45	100	1- or 5-caffeoylquinic
3	353	not detected	5	1- or 5-caffeoylquinic
4	341	100	8	3-caffeoylquinic
5	341	100	1	4-caffeoylquinic
6	323	8	75	1- or 5-caffeoylquinic
7	323	15	100	1- or 5-caffeoylquinic
8	341	100	2	4-caffeoylquinic

Table 2. Mass Spectrometric Characteristics of the Components Producing an *m*/*z* 677 Parent Ion

peak number	MS^2 base peak	MS^3 base peak	*m*/*z* 341 as % MS^3 base peak	*m*/*z* 323 as % MS^3 base peak	*m*/*z* 299 as % MS^3 base peak	identity of *m*/*z* 353
1	515	353	10			3-caffeoylquinic
2	515	341	100	5		3-caffeoylquinic
3	497	341	100	5		equivocal
4	515	353	10	3		3-caffeoylquinic
5	515	353	3			3-caffeoylquinic
6	515	179	35			3-caffeoylquinic
7	515	353			3	4-caffeoylquinic
8	515	353	3		15	4-caffeoylquinic
9	497	335	5		20	4-caffeoylquinic
10	515	353	3		10	4-caffeoylquinic
11	515	323	40	100	5	1- or 5-caffeoylquinic
12	515	323	80	100		3-caffeoylquinic
13	515	353	5		5	4-caffeoylquinic
14	515	353	5		5	4-caffeoylquinic
15	515	353	7		12	4-caffeoylquinic
16	515	341	100		10	4-caffeoylquinic

fragmentations in a sugar ester similar to those in a quinic acid ester indicates clearly that a carboxylate anion is not an absolute prerequisite.

Various mono- and di-caffeoyl-glucoses with acylation at C1, C2, C3, and C6 have been reported previously (*36*−*38*) and appear to be widespread (*34*, *39*). 4-Caffeoyl-glucose does not seem to have been reported per se, but C4 cinnamoyl esters of hexose sugars are known in complex anthocyanins, for example, pelargonidin-3-*O*-[(4″-*O*-(*trans*-*p*-coumaroyl)-α-l-6″-rhamnopyranosyl-*β*-D-glucopyranoside]-5-*O*-[*β*-D-glucopyranoside] (*40*).

***M*r 516.** Eight signals at *m*/*z* 515 were observed that eluted between 7 and 17 min (**Figure 4B**) well in advance of 1,3-dicaffeoylquinic acid (**5**) at 23 min, the most hydrophilic dicaffeoylquinic acid so far characterized by these LC−MS procedures (*18*). Ions corresponding to the loss of −CHOH units were not detected. At MS^2 (**Table 2**), all eight components produced *m*/*z* 353, 341, and the "dehydrated" ion *m*/*z* 323. A targeted MS^3 experiment (*m*/*z* 515 + 341) produced an *m*/*z* 179 base peak accompanied by *m*/*z* 135, indicating that the *m*/*z* 341 ion contained a caffeic acid moiety. Similarly, by using (*m*/*z* 515 + 353), it was established that two produced [3-caffeoylquinic acid − H^+]$^-$, two produced [4-caffeoylquinic acid − H^+]$^-$, and four produced the indistinguishable [1-caffeoylquinic acid − H^+]$^-$ and/or [5-caffeoylquinic acid − H^+]$^-$. These fragmentation patterns suggest the presence of a quinic acid residue, at least one caffeoyl residue, and a hexose sugar, with the caffeic acid attached to both the quinic acid (origin of *m*/*z* 353) and the hexose sugar (origin of *m*/*z* 341). Such a structure would exclude caffeoyl-sugar esters and suggests the presence of caffeoylquinic acid-glycosides. A 5-feruloylquinic acid-4′-*β*-D-glucoside has been isolated from *Hydrastis canadensis* (Ranunculaceae) (*41*), and the presence of the same or a similar compound suggested in artichoke (*Cynara scolymus*, Asteraceae) (*42*). Although glucosides of caffeic acid are well known (*31*−*33*), caffeoylquinic acid glycosides have not been reported so far as we are aware. Because both theoretical caffeic acid glucosides are known, and because *Chrysanthemum* spp. produce four caffeoylquinic acids (**1**−**4**), one might reasonably

expect eight caffeoylquinic acid glucosides. At MS^3, these glucosides would be expected to yield [1-caffeoylquinic acid − H$^+$]$^-$ and/or [5-caffeoylquinic acid − H$^+$]$^-$ on four occasions, [3-caffeoylquinic acid − H$^+$]$^-$ on two occasions, and [4-caffeoylquinic acid − H$^+$]$^-$ on two occasions, exactly as observed.

***M*$_r$ 678.** The 16 hydrophilic compounds, eluting between 18 and 34 min (**Figure 4C**), produced MS^4 fragment ions characteristic of a quinic acid residue and a caffeic acid residue, this latter confirmed at MS^5. Two of these relatively hydrophilic components produced an *m*/*z* 497 base peak at MS^2. Fourteen produced *m*/*z* 515, followed in nine cases by *m*/*z* 353 at MS^3, in two cases each by *m*/*z* 341 or 323, and once by *m*/*z* 179 (**Table 2**). A targeted MS^4 experiment (*m*/*z* 677 + 515 + 353) established that eight produced [4-caffeoylquinic acid − H$^+$]$^-$, six produced [3-caffeoylquinic acid − H$^+$]$^-$, and one produced [5-caffeoylquinic acid − H$^+$]$^-$ or [1-caffeoylquinic − H$^+$]$^-$, and there was one equivocal result. There was no evidence for sugar fragmentation, thus eliminating caffeoyl-sugar esters.

It is clear that these compounds are very similar to those with M_r 516 and appear to contain either an additional caffeic acid residue (dicaffeoylquinic acid glycosides) or an additional hexose residue (caffeoylquinic acid diglycosides/biosides). The observation of MS^3 fragment ions at *m*/*z* 299 and 255 strongly suggests that these are derivatives of dicaffeoylquinic acids rather than caffeoylquinic acids, and their intensity (at up to 20% of base peak) suggests that they are glycosidic derivatives of 3,4-dicaffeoylquinic acid (**8**) and 4,5-dicaffeoylquinic acid (**10**), but not 1,4-dicaffeoylquinic acid (**6**), which would be expected to produce these ions more intensely (*18*). So far as we are aware, such compounds have not previously been characterized unequivocally, but it has been suggested (*42*) that artichoke (*Cynara scolymus*, Asteraceae) might contain a dicaffeoylquinic acid glycoside. Theoretically, for any given sugar, one might expect 24 dicaffeoylquinic acid glycosides, and these on fragmentation would be expected at MS^4 to yield [4-caffeoylquinic acid − H$^+$]$^-$ on 12 occasions, [3-caffeoylquinic acid − H$^+$]$^-$ on eight occasions, and [1-caffeoylquinic acid − H$^+$]$^-$ and/or [5-caffeoylquinic acid − H$^+$]$^-$ on four occasions. The fragmentation data in **Table 2** suggest that the unassigned compound 3 and the missing compounds include four 1,4-dicaffeoylquinic acid glycosides, two that would produce [3-caffeoylquinic acid − H$^+$]$^-$ (1,3- and/or 3,5-dicaffeoylquinic acid glycosides), and three that would produce [1- and/or 5-caffeoylquinic acid − H$^+$]$^-$ (1,5-dicaffeoylquinic acid glycosides). Possibly these coelute, or are present below the limit of detection, but it may be that they are not produced by the plants. Whatever the explanation, our observations provide evidence for the occurrence in chrysanthemum of 16 isomeric dicaffeoylquinic acid glycosides.

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LITERATURE CITED

(1) Molgaard, P.; Ravn, H. Evolutionary aspects of caffeoyl ester distribution in dicotyledons. *Phytochemistry* **1988**, *27*, 2411−2421.

(2) Clifford, M. N. The analysis and characterization of chlorogenic acids and other cinnamates. In *Methods in Polyphenol Analysis*; Santos-Buelga, C., Williamson, G., Eds.; Royal Society of Chemistry: Cambridge, 2003; 337 pp.

(3) Clifford, M. N. Chlorogenic acids and other cinnamates − nature, occurrence, dietary burden, absorption and metabolism. *J. Sci. Food Agric.* **2000**, *80*, 1033−1042.

(4) Clifford, M. N. Chlorogenic acids and other cinnamates − nature, occurrence and dietary burden. *J. Sci. Food Agric.* **1999**, *79*, 362−372.

(5) IUPAC. Nomenclature of cyclitols. *Biochem. J.* **1976**, *153*, 23−31.

(6) Eliel, E. L.; Ramirez, M. B. (−)-Quinic acid: configurational (stereochemical) descriptors. *Tetrahedron: Asymmetry* **1997**, *8*, 3551−3554.

(7) Chuda, Y.; Suzuki, M.; Nagata, T.; Tsushida, T. Contents and cooking loss of three quinic acid derivatives from garland (*Chrysanthemum coronarium* L.). *J. Agric. Food Chem.* **1998**, *46*, 1437−1439.

(8) Chuda, Y.; Ono, H.; Ohnishi-Kameyama, M.; Nagata, T.; Tsushida, T. Structural identification of two antioxidant quinic acid derivatives from garland (*Chrysanthemum coronarium* L.). *J. Agric. Food Chem.* **1996**, *44*, 2037−2039.

(9) Takenaka, M.; Nagata, T.; Yoshida, M. Stability and bioavailability of antioxidants in garland (*Chrysanthemum coronarium* L.). *Biosci. Biotechnol. Biochem.* **2000**, *64*, 2689−2691.

(10) Maruta, Y.; Kawabata, J.; Niki, R. Antioxidative caffeoylquinic acid derivatives in the roots of burdock (*Arctium lappa* L.). *J. Agric. Food Chem.* **1995**, *43*, 2592−2595.

(11) Tsuchida, T.; Suzuki, M.; Kurogi, M. Evaluation of antioxidant activity of vegetable extracts and determination of some active compounds. *J. Jpn. Soc. Food Sci. Technol.* **1968**, *13*, 199−206.

(12) Beninger, C. W.; Abou-Zaid, M. M.; Kistner, A. L.; Hallett, R. H.; Iqbal, M. J.; Grodzinski, B.; Hall, J. C. A flavanone and two phenolic acids from *Chrysanthemum morifolium* with phytotoxic and insect growth regulating activity. *J. Chem. Ecol.* **2004**, *30*, 589−606.

(13) Murayama, T.; Yada, H.; Kobori, M.; Shinmoto, H.; Tsushida, T. Evaluation of three antioxidants and their identification and radical scavenging activities in edible chrysanthemums. *J. Jpn. Soc. Hortic. Sci.* **2002**, *71*, 236−242.

(14) Gins, V. K.; Kolesnikov, M. P.; Kononkov, P. F.; Trishin, M. E.; Gins, M. S. [Oxyanthraquinones and flavonoids from garland chrysanthenum]. *Prikl. Biokhim. Mikrobiol.* **2000**, *36*, 344−353.

(15) Lee, J. S.; Kim, H. J.; Lee, Y. S. A new anti-HIV flavonoid glucuronide from *Chrysanthemum morifolium*. *Planta Med.* **2003**, *69*, 859−861.

(16) Clifford, M. N.; Knight, S. The cinnamoyl−amino acid conjugates of green robusta coffee beans. *Food Chem.* **2004**, *87*, 457−463.

(17) Clifford, M. N.; Johnston, K. L.; Knight, S.; Kuhnert, N. A hierarchical scheme for LC−MS^n identification of chlorogenic acids. *J. Agric. Food Chem.* **2003**, *51*, 2900−2911.

(18) Clifford, M. N.; Knight, S.; Kuhnert, N. Discriminating between the six isomers of dicaffeoylquinic acid by LC−MS^n. *J. Agric. Food Chem.* **2005**, *53*, 3821−3832.

(19) Clifford, M. N.; Knight, S.; Surucu, B.; Kuhnert, N. Characterization by LC−MS^n of four new classes of chlorogenic acids in green coffee beans: dimethoxycinnamoylquinic acids, diferuloylquinic acids, caffeoyl-dimethoxycinnamoylquinic acids, and feruloyl-dimethoxycinnamoylquinic acids. *J. Agric. Food Chem.* **2006**, *54*, 1957−1969.

(20) Clifford, M. N.; Marks, S.; Knight, S.; Kuhnert, N. Characterization by LC−MS^n of four novel classes of *p*-coumaric acid-containing diacyl chlorogenic acids in green coffee beans. *J. Agric. Food Chem.* **2006**, *54*, 4095−4101.

(21) Egan, H.; Kirk, R. S.; Sawyer, R. *Pearson's chemical analysis of foods*; Churchill Livingstone: London, 1981.

(22) Clifford, M. N.; Wu, W.; Kuhnert, N. The chlorogenic acids of *Hemerocallis*. *Food Chem.* **2006**, *95*, 574−578.

(23) Kim, H. J.; Lee, Y. S. Identification of new dicaffeoylquinic acids from *Chrysanthemum morifolium* and their antioxidant activities. *Planta Med.* **2005**, *71*, 871−876.

(24) Clifford, M. N.; Wang, Z.; Kuhnert, N. Profiling the chlorogenic acids of aster by LC−MS^n. *Phytochem. Anal.* **2006**, *17*, 384−393.
(25) Agata, I.; Goto, S.; Hatano, T.; Ishibe, S.; Okuda, T. 1,3,5-Tri-*O*-caffeoylquinic acid from *Xanthium strumarium*. *Phytochemistry* **1997**, *33*, 508−509.
(26) dos Santos, M. D.; Gobbo-Neto, L.; Albarella, L.; de Souza, G. E.; Lopes, N. P. Analgesic activity of di-caffeoylquinic acids from roots of *Lychnophora ericoides* (Arnica da serra). *J. Ethnopharmacol.* **2005**, *96*, 545−549.
(27) Ma, Y. T.; Huang, M. C.; Hsu, F. L.; Chang, H. F. Thiazinedione from *Xanthium strumarium*. *Phytochemistry* **1998**, *48*, 1083−1085.
(28) Merfort, I. Caffeoylquinic acids from flowers of *Arnica montana* and *Arnica chamissonis*. *Phytochemistry* **1992**, *31*, 2111−2113.
(29) Peluso, G.; de Feo, V.; de Simone, F.; Bresciano, E.; Vuotto, M. L. Studies on the inhibitory effects of caffeoylquinic acids on monocyte migration and superoxide ion production. *J. Nat. Prod.* **1995**, *58*, 639−646.
(30) Timmermann, B. N.; Hoffmann, J. J.; Jolad, S. D.; Schram, K. H.; Klenk, R. E.; Bates, R. B. Constituents of *Chrysothamnus paniculatus*. 3. 3,4,5-tricaffeoylquinic acid (a new shikimate prearomatic) and 3,4-, 3,5- and 4,5-dicaffeoylquinic acids. *J. Nat. Prod.* **1983**, *46*, 365−368.
(31) Buta, J. G.; Spaulding, D. W. Endogenous levels of phenolics in tomato fruit during growth and maturation. *J. Plant Growth Regul.* **1997**, *16*, 43−46.
(32) Lim, E. K.; Higgins, G. S.; Li, Y.; Bowles, D. J. Regioselectivity of glucosylation of caffeic acid by a UDP-glucose:glucosyltransferase is maintained in planta. *Biochem. J.* **2003**, *373*, 987−992.
(33) Sumner, L. W.; Mendes, P.; Dixon, R. A. Plant metabolomics: large-scale phytochemistry in the functional genomics era. *Phytochemistry* **2003**, *62*, 817−836.
(34) Harborne, J. B.; Corner, J. J. Plant polyphenols. 4. Hydroxycinnamic acid-sugar derivatives. *Biochem. J.* **1961**, *81*, 242−250.
(35) Carini, M.; Facino, R. M.; Aldini, G.; Calloni, M.; Colombo, L. Characterization of phenolic antioxidants from maté (*Ilex paraguayensis*) by liquid chromatography mass spectrometry and liquid chromatography tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **1998**, *12*, 1813−1819.
(36) Braham, H.; Mighri, Z.; Jannet, H. B.; Matthew, S.; Abreu, P. M. Antioxidant phenolic glycosides from *Moricandia arvensis*. *J. Nat. Prod.* **2005**, *68*, 517−522.
(37) Hussein, S. A. M.; Ayoub, N. A.; Nawwar, M. A. M. Caffeoyl sugar esters and an ellagitannin from *Rubus sanctus*. *Phytochemistry* **2003**, *63*, 905−911.
(38) Jiang, Z. H.; Hirose, Y.; Iwata, H.; Sakamoto, S.; Tanaka, T.; Kouno, I. Caffeoyl, coumaroyl, galloyl, and hexahydroxydiphenoyl glucoses from *Balanophora japonica*. *Chem. Pharm. Bull.* **2001**, *49*, 887−892.
(39) Reschke, A.; Herrmann, K. Vorkommen von 1-*O*-Hydroxycinnamyl-*β*-D-glucosen im Obst. *Z. Lebensm.-Unters.-Forsch.* **1981**, *173*, 458−463.
(40) Naito, K.; Umemura, Y.; Mori, M.; Sumida, T.; Okada, T.; Takamatsu, N.; Okawa, Y.; Hayashi, K.; Saito, N.; Honda, T. Acylated pelargonidin glycosides from a red potato. *Phytochemistry* **1998**, *47*, 109−112.
(41) McNamara, C. E.; Perry, N. B.; Follett, J. M.; Parmenter, G. A.; Douglas, J. A. A new glucosyl feruloyl quinic acid as a potential marker for roots and rhizomes of goldenseal, *Hydrastis canadensis*. *J. Nat. Prod.* **2004**, *67*, 1818−1822.
(42) Sánchez-Rabaneda, F.; Jáuregui, O.; Lamuela-Raventós, R. M.; Bastida, J.; Viladomat, F.; Codina, C. Identification of phenolic compounds in artichoke waste by high-performance liquid chromatography−tandem mass spectrometry. *J. Chromatogr., A* **2003**, *1008*, 57−72.

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