Research Article

Journal of MASS SPECTROMETRY

Received: 30 November 2010 Accepted: 7 January 2011 Published online in Wiley Online Library: 2011

(wileyonlinelibrary.com) DOI 10.1002/jms.1889

How to identify and discriminate between the methyl quinates of chlorogenic acids by liquid chromatography–tandem mass spectrometry

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The methyl esters of chlorogenic acids, methyl quinates, are widely distributed in plant materials and frequently appear as extraction artifacts in plant samples. This is the first time when liquid chromatography–tandem mass spectrometry methods have been used for the identification and characterization of the methyl quinates. For this purpose, methyl quinates of mono caffeoylquinic acids and mono feruloylquinic acids were synthesized as authentic standards. The methyl quinates of mono and diacyl chlorogenic acids have shown characteristic fragmentation pattern in their tandem mass spectra. MS^{n+1} spectra of the methyl quinates of diacyl chlorogenic acids were identical to MS^n spectra of mono acyl derivatives. These quinates do not produce any methyl quinate peak at *m/z* 205 if compared with quinic acid peak at *m/z* 191 in negative ion mode. In the MS^n spectra of these quinates, cinnamic acid part or cinnamoyl part was detected as a base peak in negative ion mode. The retention time, order of elution and fragmentation pattern were completely different if compared with LC–MS^n methods developed for chlorogenic acids. These LC–MS^n methods have been applied for the identification and regioisomeric characterization of the methyl quinates of chlorogenic acids in maté tea and woodruff (*Galium odoratum*). Two methyl caffeoylquinates (2 and 4) were identified as methyl 3-caffeoylquinate and methyl 5-caffeoylquinate.

Keywords: chlorogenic acids; methyl feruloylquinate; methyl caffeoylquinate; 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] sinapic acid and dimethoxycinnamic acid are also present in certain plant species.[4–7] Structures are shown in Fig. 1. In the IUPAC system, used throughout this manuscript, (−) -quinic acid is defined as 1L-1(OH),3,4/5-tetrahydroxycyclohexane carboxylic acid but Eliel and Ramirez[8] recommend 1α,3*R*,4α,5*R*-tetrahydroxycyclohexane carboxylic acid. The CGAs show a variety of biological activities like antioxidant, anti-inflammatory, anti-HIV, anti-HBV, radical scavenging, inhibit mutagenesis and carcinogenesis and are considered to be beneficial to human health.[9–13]

Structurally related, but much less common, are similar esters between quinic acid alkyl ester (quinate) and caffeic acid or quinic acid alkyl ester and ferulic acid. These alkyl esters show biological activities like antioxidant, anti-stress, anti-aging and they inhibit collagenase, reverse transcriptase and DNA polymerase.[14–17] On many occasions, methyl caffeoylquinates,[18–20] ethyl caffeoylquinates,[21] butyl caffeoylquinates,[22] methyl feruloylquinates,[23] methyl dicaffeoylquinates,[15,16,24] butyl dicaffeoylquinates,[25] methyl caffeoyl-sinapoylquinates and ethyl caffeoyl-sinapoylquinates[26] are reported in nature by the tedious isolation and purification methods followed by spectroscopic identification. On occasions methyl quinates might as well be the product formed after methanolic extraction and could frequently be artifacts in plant analysis. On no occasion have the alkyl quinates of CGAs in nature or dietary materials have been analyzed by tandem mass spectrometry and characterized to their regioisomeric level.

Plants that synthesize CGAs commonly produce many related compounds (over 70 in the coffee bean, for example) and using liquid chromatography–tandem mass spectrometry (LC–MS^n) it has been possible to discriminate between individual isomers of monoacyl, diacyl and triacyl CGAs, without the need to isolate the pure compounds.[4,6,7,27,28] This paper reports the development and application of LC–MS^n methods to the CGAs methyl esters.

Experimental

Chemicals and materials

All the analytical grade solvents were purchased from Sigma–Aldrich (Bremen, Germany). The CGAs, 3-caffeoylquinic acid, 4-caffeoylquinic acid, 5-caffeoylquinic acid (CGA), 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid were purchased from PhytoLab (Vestenbergsgreuth, Germany). Green maté tea and green Robusta coffee beans were purchased from a supermarket in Bremen (Germany). Woodruff (*Galium odoratum*) leaves were collected from the Rhododendron Botanical Garden, Bremen (Germany).

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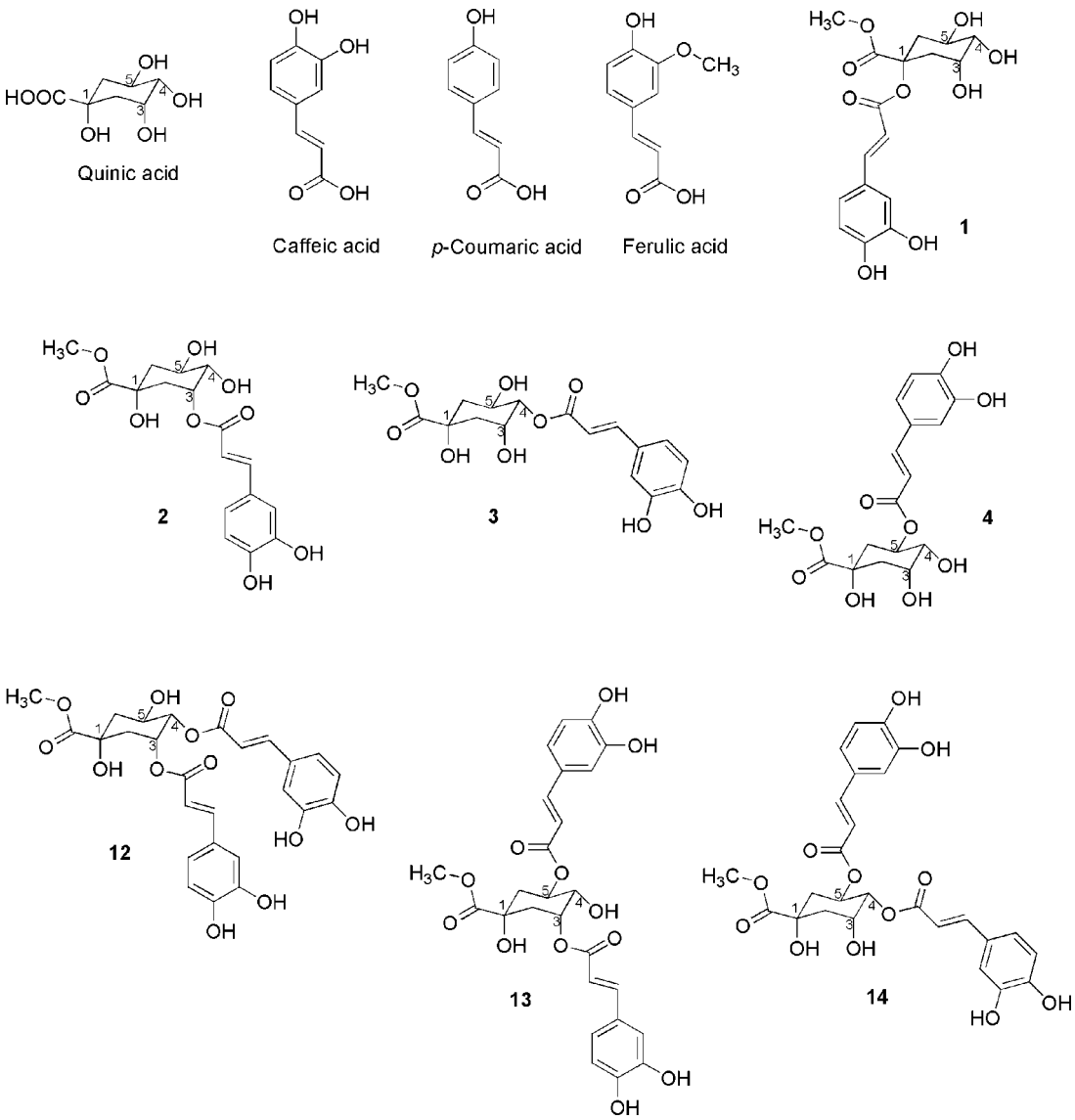

Figure 1. Representative structures of the methyl quinates of chlorogenic acids.

Extracts of green coffee beans, maté tea and woodruff (*G. odoratum*)

The extraction of green Robusta coffee beans, maté tea and woodruff were carried out using the methods reported previously.[6,7] The aqueous extractions were also carried out to confirm that methyl quinates were not only artifacts of methanolic extractions.

Preparation of a mixture of methyl quinates of CGAs from green coffee extract

A green Robusta coffee beans extract of 500 mg was refluxed with methanol (20 ml) and acidic resin (Amberlite IR 120 H^+, 500 mg) for 12 h, and then filtered to remove the resin. The methanol was removed by evaporation *in vacuo* and the extracts were stored at −20 °C until required, thawed at room temperature, dissolved in methanol (60 mg/10 ml of methanol), filtered through a membrane filter and used directly for LC–MS.

Synthesis of methyl 3-, 4- and 5-caffeoylquinate and methyl 3,4-, 3,5- and 4,5-dicaffeoylquinate

The CGAs (0.5 mg of each) were dissolved in methanol (5 ml) and refluxed in the presence of an acid catalyst (Amberlite IR 120 H^+, 2 mg) for 10 h, filtered and used directly for LC–MS^n analysis.

Synthesis of the mixtures of regioisomers of methyl caffeoylquinate (MCQ) (1–4) and methyl feruloylquinate (MFQ) (5–8)

To a solution of methyl quinate (101 mg, 0.49 mmol) and DMAP (16 mg, 0.12 mmol) in CH_2Cl_2 (10 ml) were added triethylamine (4 ml) and 3,4-diacetylcaffeic acid chloride/4-acetylferuloylchloride (0.49 mmol) at room temperature. The reaction mixture was stirred for 4 h and acidified with 2 M HCl (pH ≈ 1) and then stirred for an additional 1 h to remove the acetyl protecting groups. The layers were separated and the aqueous phase was re-extracted with CH_2Cl_2 (1 × 20 ml) and EtOAc (2 × 20 ml). The combined organic layers were dried over Na_2SO_4, filtered and the solvents were removed *in vacuo*. The resulting

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esters were analyzed by high-performance liquid chromatography (HPLC)/MS.

Liquid chromatography–tandem mass spectrometry (LC–MS^n)

The LC equipment (Agilent 1100 series, Karlsruhe, Germany) comprised 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 HCT Ultra, Bremen, Germany) operating in full scan, auto MS^n mode to obtain fragment ion *m/z*. Tandem mass spectra were acquired in 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%. MS operating conditions (negative mode) had been optimized using 5-caffeoylquinic acid 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) fitted with an ESI source and internal calibration was achieved with 10 ml of 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.

High-performance liquid chromatography (HPLC)

Separation was achieved on a 150 mm × 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 : 0.02, 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 80 and 10 min isocratic to re-equilibrate.

Results and Discussion

General LC–MS chromatographic and spectroscopic data

All data for methyl quinates of CGAs presented in this paper use the recommended IUPAC numbering system[1] and specimen structures are presented in Fig. 1. In general, peak identities were consistent both within and between analyses. However, when the mass spectrum for a particular compound included two ions of similar mean intensities, within-analysis experimental error dictated that in some individual MS scans one would be more intense and for other scans the reverse would be true. This phenomenon was encountered primarily when the signal intensity was lower, that is with quantitatively minor components and/or higher order spectra. For example, methyl 3-caffeoyl-5-*p*-coumaroylquinate (M3C,5*p*CoQ) (**19**) produces MS^2 ions at *m/z* 349 and 367 which are essentially coequal in some spectra. However, in this particular case the lower mass ion has been assigned consistently as the base peak. Fragment ions with intensities <10% of the base peak have been reported only when they are needed for comparison.

For the selective syntheses of M3CQ (**2**), M4CQ (**3**), M5CQ (**4**), M3,4-diCQ (**12**), M3,5-diCQ (**13**) and M4,5diCQ (**14**), their respective CGAs were treated with methanol in the presence of an acid catalyst. Mixtures of the regioisomers of mono-acylated CGA methyl esters were synthesized by a simple condensation reaction between a cinnamoyl chloride and methyl quinate (Fig. 2). In these mixtures, their elemental composition was confirmed by high-resolution mass spectrometry with the error being below 5 ppm (Table 1).

It is well known that green coffee beans are rich in CGAs (more than 70 compounds) and therefore the extract of green coffee beans was treated with methanol in the presence of an acid catalyst to generate a series of methyl quinates of CGAs (Table 2).

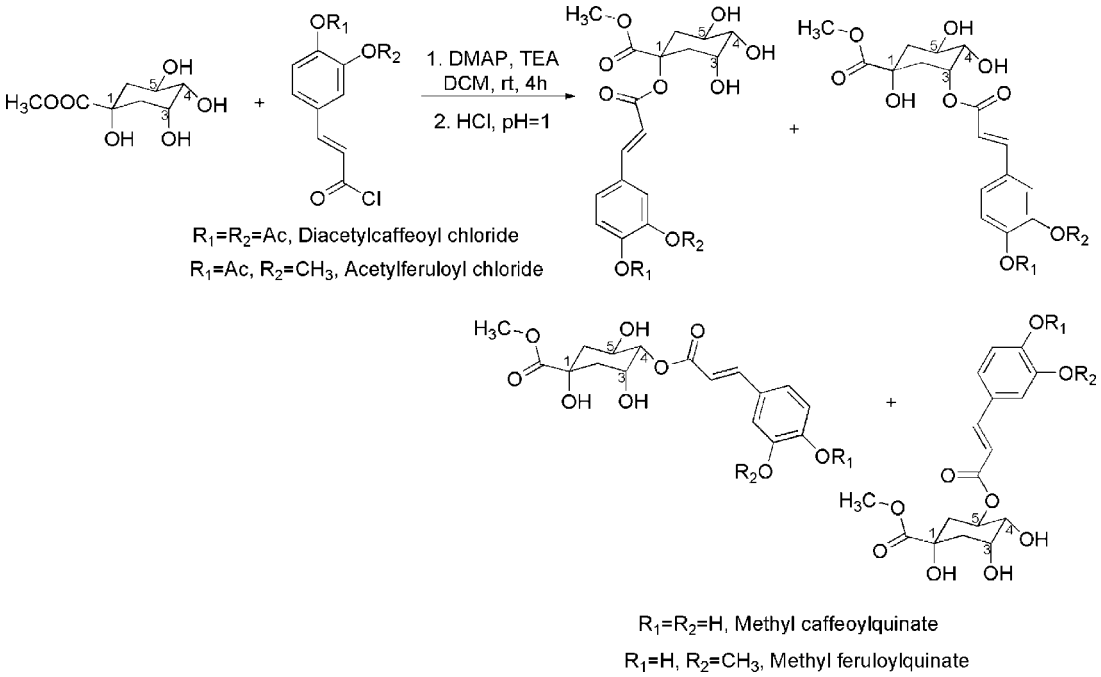

Figure 2. Synthesis of regioisomers of methyl caffeoylquinates and methyl feruloylquinates.

Table 1. High-resolution mass (MS-TOF) data of methyl quinates of chlorogenic acids and their parent ions (M − H)

No.	Methyl quinate	Mol. formula	Theor. *m/z* (M − H)	Exp. *m/z* (M − H)	Error (ppm)	Exp. *m/z* (M − H)[a]	Error (ppm)
1	M1CQ	$C_{17}H_{20}O_9$	367.1035	–		367.1021	3.7
2	M3CQ	$C_{17}H_{20}O_9$	367.1035	367.1038	−1.0	367.1032	0.8
3	M4CQ	$C_{17}H_{20}O_9$	367.1035	367.1038	−1.0	367.1026	2.3
4	M5CQ	$C_{17}H_{20}O_9$	367.1035	367.1044	−2.5	367.1032	0.8
5	M1FQ	$C_{18}H_{22}O_9$	381.1191	–		381.1185	1.5
6	M3FQ	$C_{18}H_{22}O_9$	381.1191	381.1201	−2.7	381.1176	4.0
7	M4FQ	$C_{18}H_{22}O_9$	381.1191	381.1197	−1.6	381.1185	1.5
8	M5FQ	$C_{18}H_{22}O_9$	381.1191	381.1198	−1.8	381.1192	−0.4
9	M3*p*CoQ	$C_{17}H_{20}O_8$	351.1085	351.1098	−3.6	–	
10	M4*p*CoQ	$C_{17}H_{20}O_8$	351.1085	351.1085	0.0	–	
11	M5*p*CoQ	$C_{17}H_{20}O_8$	351.1085	351.1087	−0.3	–	
12	M3,4-diCQ	$C_{26}H_{26}O_{12}$	529.1351	529.1331	3.8	–	
13	M3,5-diCQ	$C_{26}H_{26}O_{12}$	529.1351	529.1357	−1.0	–	
14	M4,5-diCQ	$C_{26}H_{26}O_{12}$	529.1351	529.1373	−4.0	–	
15	M3C-4FQ	$C_{27}H_{28}O_{12}$	543.1508	543.1518	−1.9	–	
16	M3C-5FQ	$C_{27}H_{28}O_{12}$	543.1508	543.1519	−2.0	–	
17	43C-5FQ	$C_{27}H_{28}O_{12}$	543.1508	543.1516	−1.4	–	
18	M3C-4*p*CoQ	$C_{26}H_{26}O_{11}$	513.1402	513.1413	−2.0	–	
19	M3C-5*p*CoQ	$C_{26}H_{26}O_{11}$	513.1402	513.1416	−2.6	–	
20	M4*p*Co-5CQ	$C_{26}H_{26}O_{11}$	513.1402	513.1406	−0.7	–	
21	M3,4-diFQ	$C_{28}H_{30}O_{12}$	557.1664	557.1685	−3.7	–	
22	M3,5-diFQ	$C_{28}H_{30}O_{12}$	557.1664	557.1672	−1.4	–	
23	M4,5-diFQ	$C_{28}H_{30}O_{12}$	557.1664	557.1675	−1.8	–	
24	M3C-5SiQ	$C_{28}H_{30}O_{13}$	573.1614	573.1626	−2.1	–	

[a] Synthetic methyl quinates.

Table 2. Numbering and abbreviations of Chlorogeinic acids of green Robusta coffee beans and their respective methyl quinates (after the treatment with acidic methanol) (* denotes not detected)

No.	Methyl quinate	Abbreviation	Chlorogenic acid	Abbreviation
1	Methyl 1-caffeoylquinate*	M1CQ	1-Caffeoylquinic acid*	1-CQA
2	Methyl 3-caffeoylquinate	M3CQ	3-Caffeoylquinic acid	3-CQA
3	Methyl 4-caffeoylquinate	M4CQ	4-Caffeoylquinic acid	4-CQA
4	Methyl 5-caffeoylquinate	M5CQ	5-Caffeoylquinic acid	5-CQA
5	Methyl 1-feruloylquinate*	M1FQ	1-Feruloylquinic acid*	1-FQA
6	Methyl 3-feruloylquinate	M3FQ	3-Feruloylquinic acid	3-FQA
7	Methyl 4-feruloylquinate	M4FQ	4-Feruloylquinic acid	4-FQA
8	Methyl 5-feruloylquinate	M5FQ	5-Feruloylquinic acid	5-FQA
9	Methyl 3-*p*-coumaroylquate	M3*p*CoQ	3-*p*-Coumaroylquinic acid	3-*p*CoQA
10	Methyl 4-*p*-coumaroylquate	M4*p*CoQ	4-*p*-Coumaroylquinic acid	4-*p*CoQA
11	Methyl 5-*p*-coumaroylquate	M5*p*CoQ	5-*p*-Coumaroylquinic acid	5-*p*CoQA
12	Methyl 3,4-dicaffeoylquinate	M3,4-diCQ	3,4-Dicaffeoylquinic acid	3,4-diCQA
13	Methyl 3,5-dicaffeoylquinate	M3,5-diCQ	3,5-Dicaffeoylquinic acid	3,5-diCQA
14	Methyl 4,5-dicaffeoylquinate	M4,5-diCQ	4,5-Dicaffeoylquinic acid	4,5-diCQA
15	Methyl 3-caffeoyl-4-feruloylquinate	M3C-4FQ	3-Caffeoyl-4-feruloylquinic acid	3C-4FQA
16	Methyl 3-caffeoyl-5-feruloylquinate	M3C-5FQ	3-Caffeoyl-5-feruloylquinic acid	3C-5FQA
17	Methyl 4-caffeoyl-5-feruloylquinate	M4C-5FQ	4-Caffeoyl-5-feruloylquinic acid	4C-5FQA
18	Methyl 3-caffeoyl-4-*p*-coumaroylquinate	M3C-4*p*CoQ	3-Caffeoyl-4-*p*-coumaroylquinic acid	3C-4*p*CoQA
19	Methyl 3-caffeoyl-5-*p*-coumaroylquinate	M3C-5*p*CoQ	3-Caffeoyl-5-*p*-coumaroylquinic acid	3C-5*p*CoQA
20	Methyl 4-*p*-coumaroyl-5-caffeoylquinate	M4*p*Co-5CQ	4-*p*-Coumaroyl-5-caffeoylquinic acid	4*p*Co-5CQA
21	Methyl 3,4-diferuloylquinate	M3,4-diFQ	3,4-Diferuloylquinic acid	3,4-diFQA
22	Methyl 3,5-diferuloylquinate	M3,5-diFQ	3,5-Diferuloylquinic acid	3,5-diFQA
23	Methyl 4,5-diferuloylquinate	M4,5-diFQ	4,5-Diferuloylquinic acid	4,5-diFQA
24	Methyl 3-caffeoyl-5-sinapoylquinate	M3C-5SiQ	3-Caffeoyl-5-sinapoylquinic acid	3C-5SiQA

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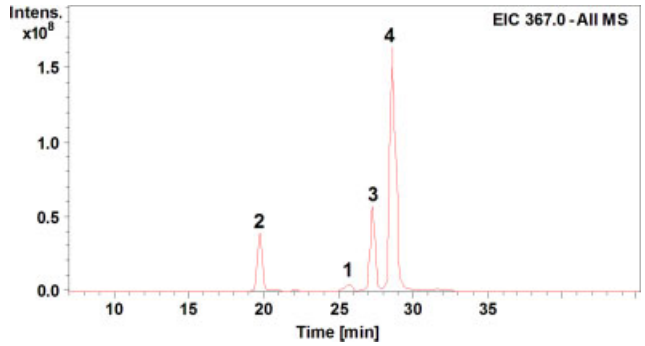

Figure 3. Extracted ion chromatogram (EIC) of methyl-caffeoylquinic acids (**1**–**4**) in negative ion mode at *m/z* 367.

Characterization of methyl caffeoylquinates (*M*, 367)

3-Caffeoylquinic acid, 4-caffeoylquinic acid and 5-caffeoylquinic acid were treated with methanol in the presence of an acid catalyst which resulted in the formation of their respective methyl esters. The retention times and fragmentation patterns were compared with the synthetic mixture of mono caffeoyl quinic acid methyl esters, and the presence of these esters was investigated in the green coffee extract after treatment with methanol. Three of the four regioisomers present in the mixture of caffeoylquinic acid methyl esters could thus be identified by their retention times and fragmentation patterns in mass spectra and the fourth isomer followed automatically (Fig. 3). The order of elution for the four isomers was M3CQ > M1CQ > M4CQ > M5CQ. This order of elution is completely different when compared with the one observed for the caffeoyl quinic acids (1 > 3 > 5 > 4). The first and third eluting isomers M3CQ (**2**) and M4CQ (**3**), respectively, produced both the MS^2 base peak at *m/z* 161 ([caffeic acid-H_2O-H^+]$^-$) and an MS^2 secondary peak at *m/z* 135 ([caffeic acid-CO_2-H^+]$^-$) (Figs 4 and 5). They produced the MS^3 base peak at *m/z* 133 and

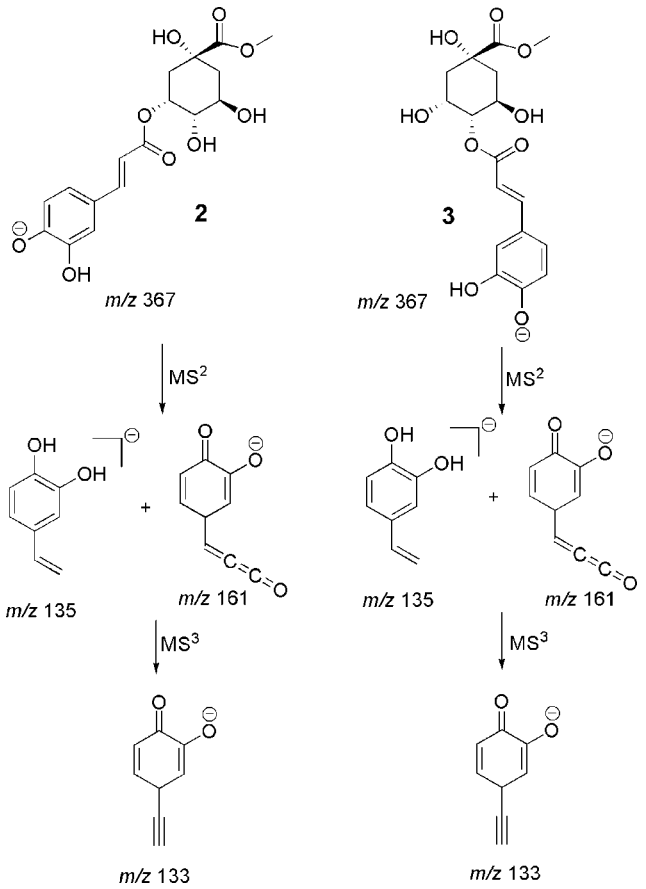

Figure 5. Proposed fragmentation pathway for methyl 3-caffeoylquinate **2** and methyl 4-caffeoylquinate **3**.

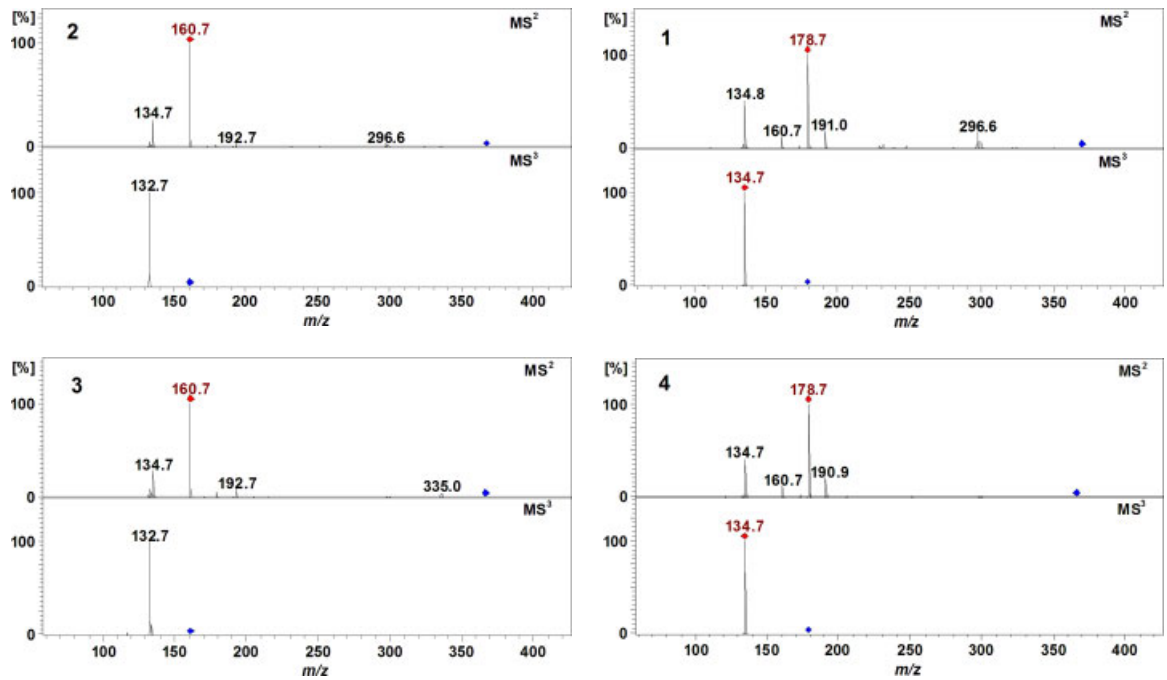

Figure 4. MS^3 spectra of methyl caffeoylquinates (**1**–**4**) (parent ion at *m/z* 367 in negative ion mode).

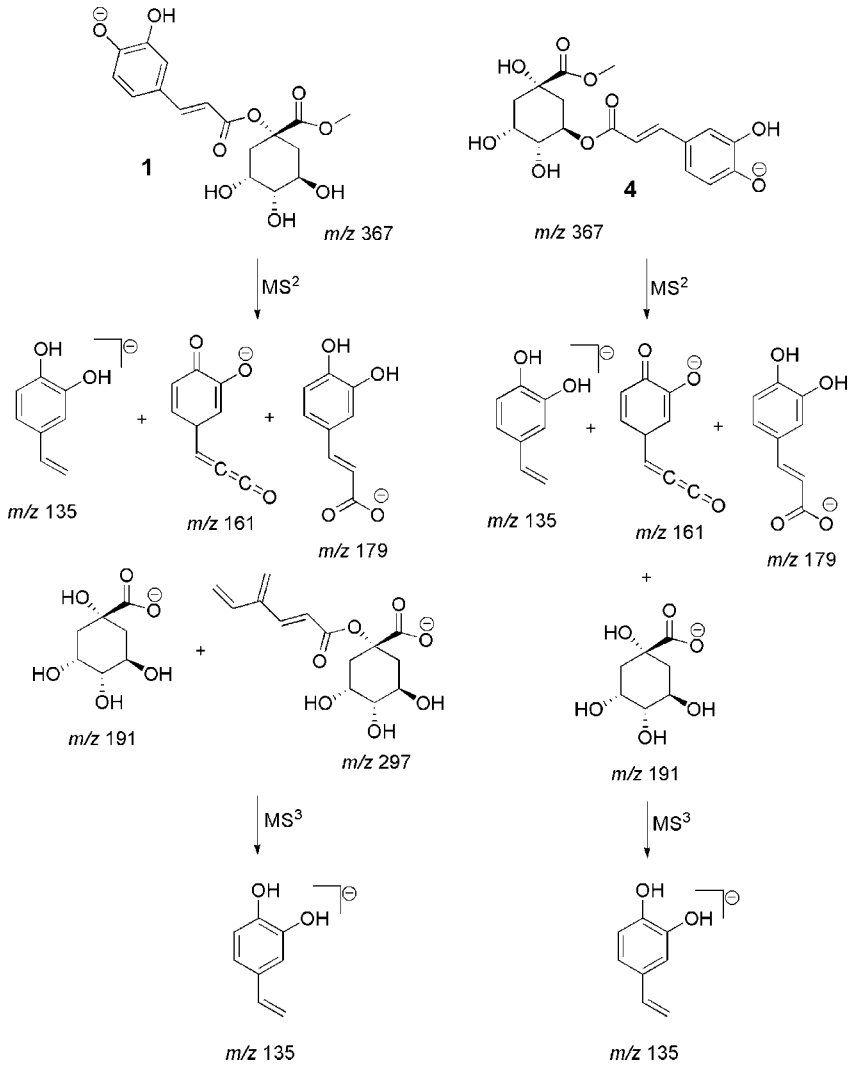

Figure 6. Proposed fragmentation pathway for methyl 1-caffeoylquinate **1** and methyl 5-caffeoylquinate **4**.

no MS^3 secondary peaks were observed. The second and fourth eluting isomers M1CQ (**1**) and M5CQ (**4**) produced the MS^2 base peak at *m/z* 179 and MS^2 secondary peaks at *m/z* 135 ([caffeic acid-CO_2-H^+]$^-$), 161 ([caffeic acid-H_2O-H^+]$^-$) and 191 ([quinic acid-H^+]$^-$) (Fig. 4). They produced the MS^3 base peak at *m/z* 135 ([caffeic acid-CO_2-H^+]$^-$) by the loss of a CO_2 molecule. The isomer **1** produced the MS^2 secondary peak at *m/z* 297 by the loss of 70 amu ($C_3H_2O_2$) from the phenyl ring (Fig. 6) in which peak is completely absent in **4** (Fig. 4).

Two methyl caffeoylquinates were detected in green maté tea and woodruff extracts. Based on the retention times and fragmentation patterns they were assigned as methyl 3-caffeoylquinate **2** and methyl 5-caffeoylquinate **4**, respectively.

Characterization of methyl feruloylquinates (*M*$_r$ 381)

A mixture of regioisomers of methyl feruloylquinate was synthesized (Fig. 2) and analyzed by HPLC–MS^n. The fragmentation behavior and retention times were compared with the methyl feruloylquinates present in green coffee extract (treated with acidic methanol). They follow the order of elution M3FQ > M1FQ > M4FQ > M5FQ which is similar to methyl caffeoylquinates. The most hydrophilic isomer **6** produced the MS^2 base peak at *m/z* 175 ([ferulic acid-H_2O-H^+]$^-$) and secondary peaks at *m/z* 160 ([ferulic acid-CH_3-H_2O-H^+]$^-$), 261 and 313 (Figs 7 and 8). It produced the MS^3 base peak at *m/z* 160 ([ferulic acid-CH_3-H_2O-H^+]$^-$) and the MS^4 base peak at *m/z* 132 ([ferulic acid-CO_2-CH_3-H_2O-H^+]$^-$). Based on the above arguments, this isomer was certainly identified as methyl 3-feruloylquinate.

The third eluting isomer **7** produced the MS^2 base peak at *m/z* 175 ([ferulic acid-H_2O-H^+]$^-$) and the secondary peak at *m/z* 160 ([ferulic acid-CH_3-H_2O-H^+]$^-$) (Figs 7 and 8 and Table 3). For this isomer specifically, there were no MS^2 secondary peaks observed at *m/z* 261 and 313 (Figs 7 and 8), which were present in methyl 3-feruloylquinate **6**. Its MS^3 and MS^4 spectra were similar to methyl 3-feruloylquinate. This specific fragmentation behavior was observed for methyl caffeoylquinates. Consequently, this isomer was assigned as methyl 4-feruloylquinate.

The second and fourth eluting isomers (**5** and **8**, respectively) produced their MS^2 base peak at *m/z* 193 and a secondary peak at *m/z* 134 (Figs 7 and 9). They produced the MS^3 base peak at *m/z* 134 and the secondary peak at *m/z* 149. Isomer **5** produced the MS^2 secondary peak at *m/z* 261 which is completely absent in isomer **8**. These isomers produced the MS^2 base peak at *m/z* 193 (due to the ferulic acid part, which is similar to methyl 1-caffeoylquinate

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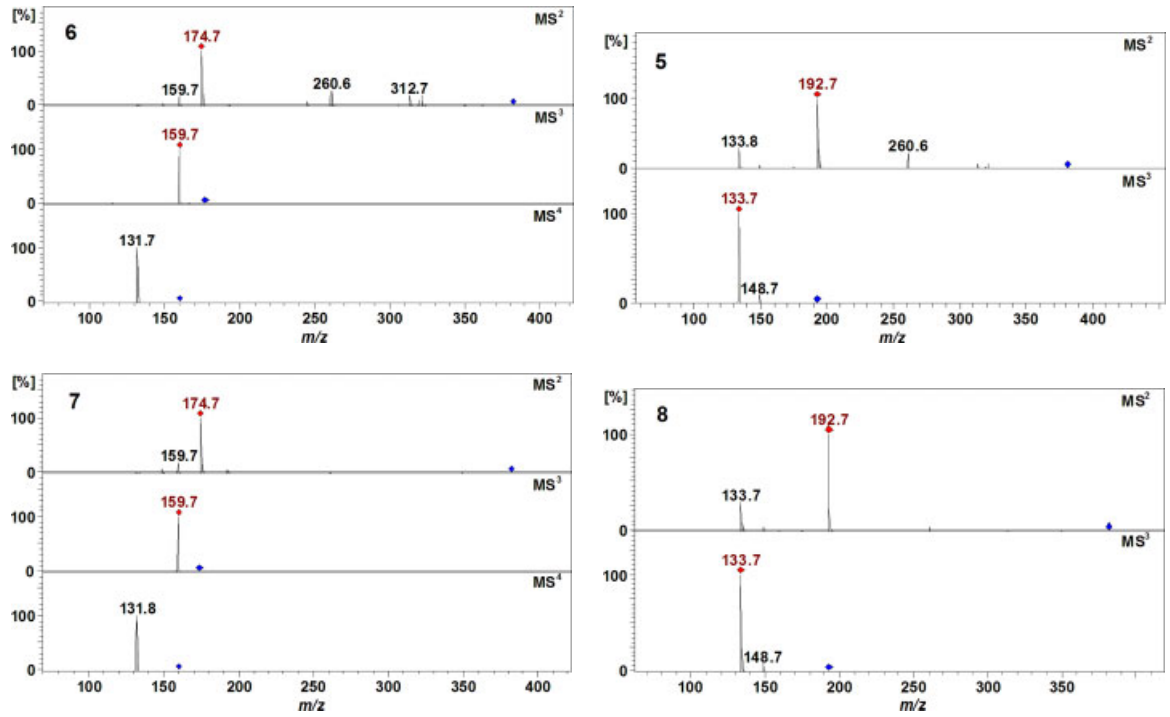

Figure 7. MS^3 spectra of methyl feruloylquinates (**5**–**8**) (parent ion at *m/z* 381 in negative ion mode).

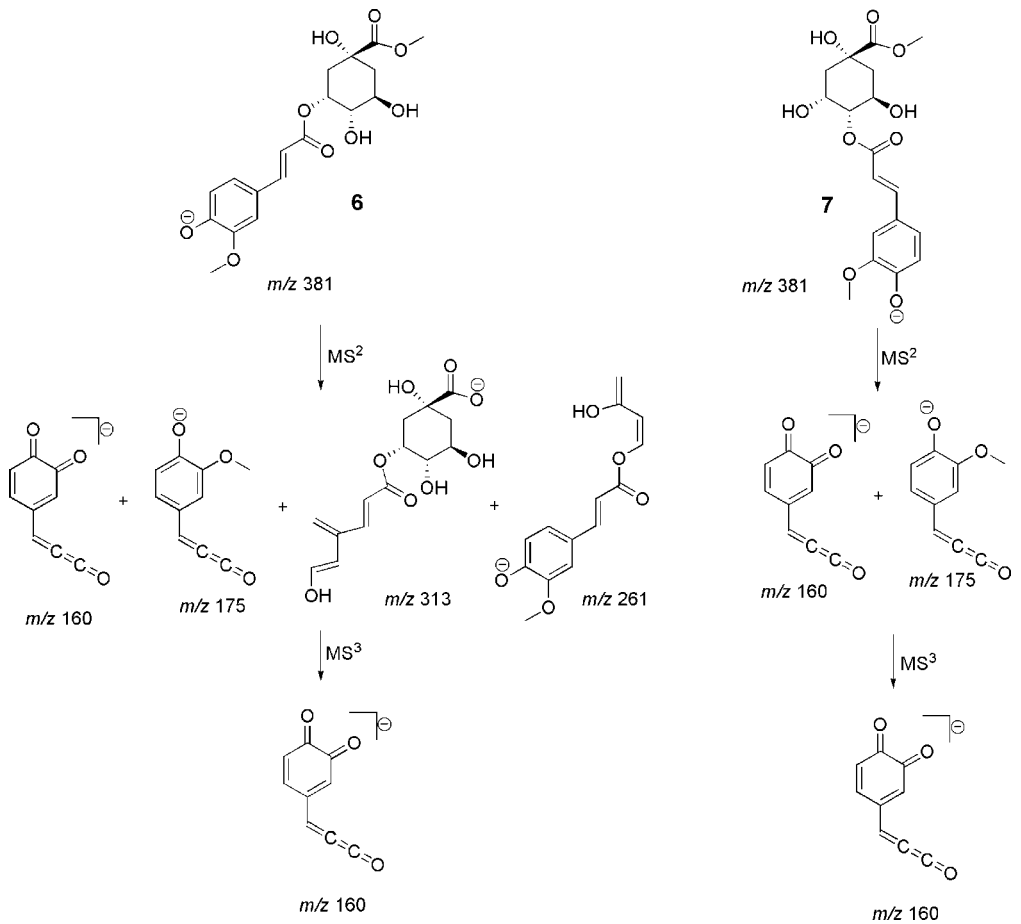

Figure 8. Proposed fragmentation pathway for methyl 3-feruloylquinate **6** and methyl 4-caffeoylquinate **7**.

Table 3. Negative ion MS^2 and MS^3 fragmentation data for the monoacyl methyl quinate of chlorogenic acids

		MS^1						MS^2				MS^3		
			Base peak	Secondary peak								Base peak	Secondary peak	
No.	Compd.	Parent ion	*m/z*	*m/z*	Int	*m/z*	Int	*m/z*	Int	*m/z*	Int	*m/z*	*m/z*	Int
1	M1CQ	367.0	178.7	134.8	50	160.7	10	191.0	20	296.6	20	134.7	–	
2	M3CQ	367.1	160.7	134.7	25	b.p.		192.7	5	296.6	5	132.7	–	
3	M4CQ	367.1	160.7	134.7	30	b.p.		192.7	7	355.0	5	132.7	–	
4	M5CQ	367.1	178.7	134.8	40	160.7	14	191.0	20	–		134.7	–	
5	M1FQ	381.1	192.7	133.8	35	260.6	30	–		–		133.7	148.7	10
6	M3FQ	381.1	174.7	159.7	22	260.6	30	312.7	20	–		159.7	–	
7	M4FQ	381.1	174.7	159.7	25	–		–		–		159.7	–	
8	M5FQ	381.1	192.7	133.7	30	–		–		–		133.7	148.7	10
9	M3*p*CoQ	351.0	144.7	119.0	10	–		–		–		116.8	118.9	32
10	M4*p*CoQ	351.1	144.7	119.0	18	–		–		–		116.8	–	
11	M5*p*CoQ	351.0	162.7	118.7	22	–		–		–		118.8	–	

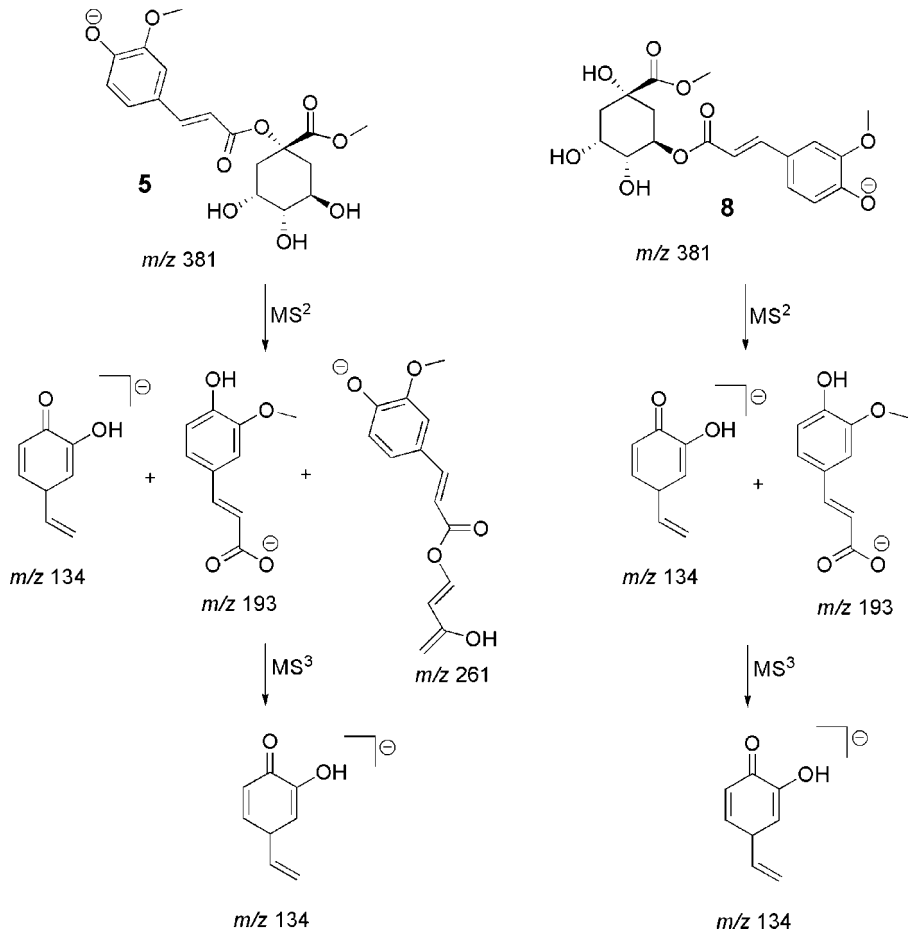

Figure 9. Proposed fragmentation pathway for methyl 1-feruloylquinate **5** and methyl 5-caffeoylquinate **8**.

1 and methyl 5-caffeoylquinate **4**, Table 3). Therefore, these isomers were identified as methyl 1-feruloylquinate **5** and methyl 5-feruloylquinate **8**, respectively.

Characterization of methyl *p*-coumaroylquinates (M_r 351)

Three isomers were detected at *m/z* 351 and they were assigned as methyl *p*-coumaroylquinates. The first and second eluting isomers (**9** and **10**, respectively) produced the MS^2 base peak at *m/z* 145 ([*p*-coumaric acid-H_2O-H^+]$^-$) and identical secondary peaks (Fig. 10 and Table 3). They produced the MS^3 base peak at *m/z* 117 ([*p*-coumaric acid-H_2O-CO-H^+]$^-$) but the MS^3 secondary peak at *m/z* 119 ([*p*-coumaric acid-CO_2-H^+]$^-$) which was absent in the second eluting isomer **10** (Fig. 10 and Table 3). Based on the order of the elution and fragmentation analogy with methyl caffeoylquinates and methyl feruloylquinates, the first and second eluting isomers

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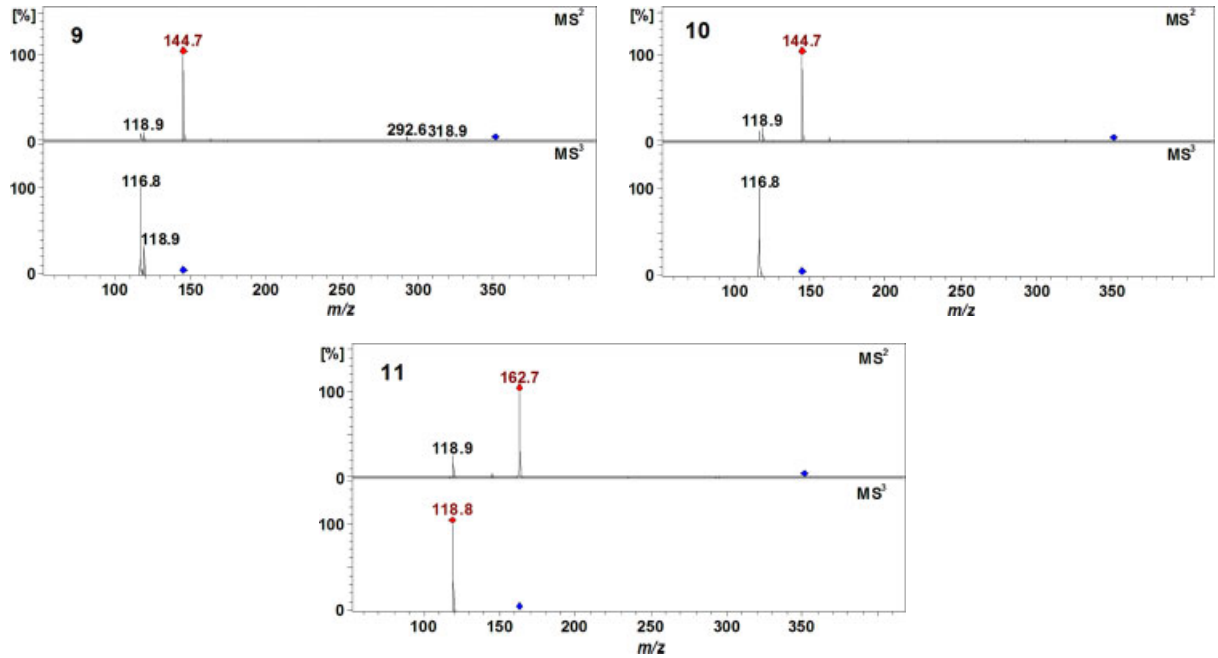

Figure 10. MS^3 spectra of methyl *p*-coumaroylquinates (**9**–**11**) (parent ion at *m/z* 351 in negative ion mode).

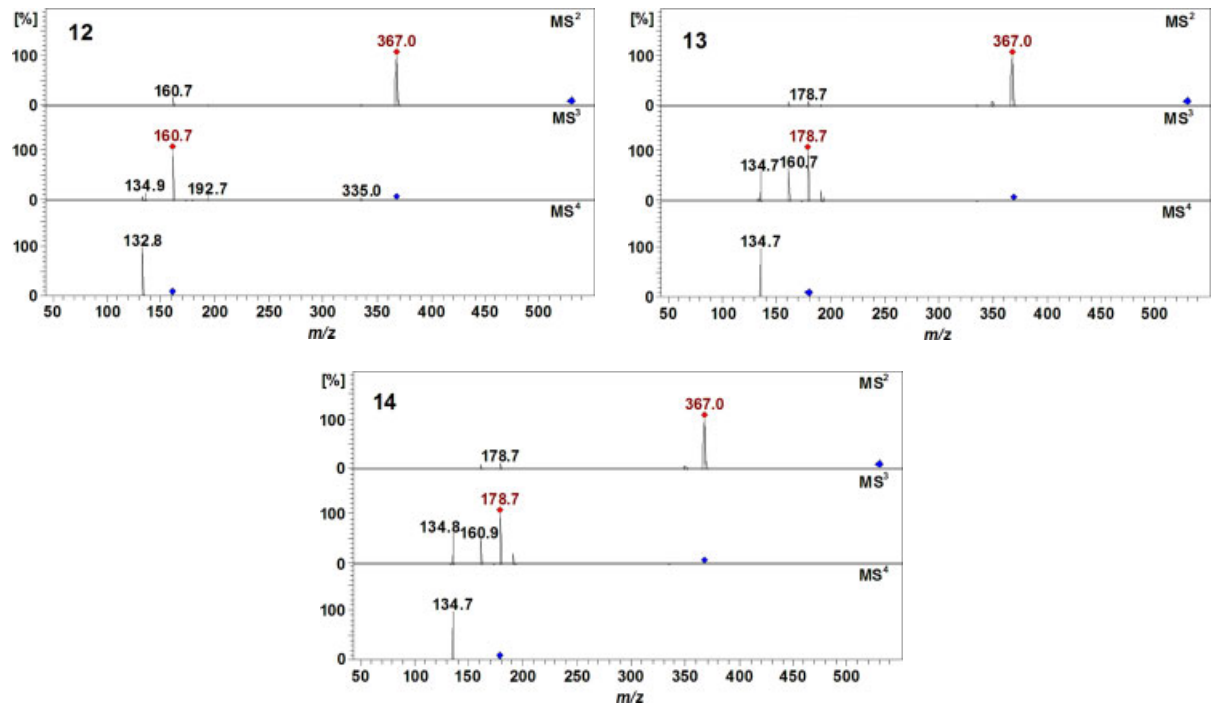

Figure 11. MS^4 spectra of methyl dicaffeoylquinates (**12**–**14**) (parent ion at *m/z* 529 in negative ion mode).

were assigned as methyl 3-*p*-coumaroylquinate (**9**) and methyl 4-*p*-coumaroylquinate (**10**), respectively.

The third eluting isomer **11** produced the MS^2 base peak at *m/z* 163 ([*p*-coumaric acid-H^+]$^-$) by the loss of the quinate part and the secondary peak at *m/z* 119 ([*p*-coumaric acid-CO_2-H^+]$^-$) (Fig. 10 and Table 3). This isomer also gives the MS^2 base peak at *m/z* 163 due to the *p*-coumaric acid part which is consistent with the methyl 5-cinnamoylquinates; therefore, it was assigned as methyl 5-*p*-coumaroylquinate.

Characterization of methyl dicaffeoylquinates (M_r 529)

Methyl 3,4-dicaffeoylquinate **12** eluted first and produced the MS^2 base peak at *m/z* 367 ([methyl caffeoylquinate-H^+]$^-$) by the loss of the caffeoyl residue and the secondary peak at *m/z* 161 ([caffeic acid-H_2O-H^+]$^-$) (Fig. 11 and Table 4). It produced the MS^3 base peak at *m/z* 161 ([caffeic acid-H_2O-H^+]$^-$), MS^3 secondary peaks at *m/z* 135 ([caffeic acid-CO_2-H^+]$^-$) and *m/z* 193 and the MS^4 base peak at *m/z* 133 ([caffeic acid-H_2O-CO-H^+]$^-$) (Fig. 11 and Table 4).

Table 4. Negative ion MS^2, MS^3 and MS^4 fragmentation data for the diacyl methyl quinates of chlorogenic acids

		MS^1	MS^2							MS^3							MS^4		
			Base peak	Secondary peak						Base peak	Secondary peak						Base peak	Secondary peak	
No.	Compd.	Parent ion	*m/z*	*m/z*	Int	*m/z*	Int	*m/z*	Int	*m/z*	*m/z*	Int	*m/z*	Int	*m/z*	Int	*m/z*	*m/z*	Int
12	M3,4-diCQ	529.1	367.0	160.7	15	–		–		160.7	134.9	16	192.7	10	335.0	7	132.8	–	
13	M3,5-diCQ	529.1	367.0	178.7	7	–		–		178.7	134.8	60	160.7	70	–		134.7	–	
14	M4,5-diCQ	529.1	367.0	178.7	10	–		–		178.7	134.8	70	160.7	60	–		134.7	–	
15	M3C-4FQ	543.0	367.0	160.7	30	–		–		160.7	134.9	10	–		–		132.8	–	
16	M3C-5FQ	543.0	349.0	160.7	25	192.7	10	367.0	40	192.8	133.9	20	160.7	90	228.8	16	133.8	–	
17	43C-5FQ	543.1	349.0	160.7	10	192.7	13	367.0	25	160.7	132.9	10	192.7	12	228.8	40	132.7	–	
18	M3C-4*p*CoQ	513.1	349.0	–		–		363.0	18	160.7	132.8	8	–		304.9	5	132.7	–	
19	M3C-5*p*CoQ	513.1	349.0	–		–		–		160.7	132.8	20	–		304.9	10	132.8	118.8	20
20	M4*p*Co-5CQ	513.1	363.1	174.7	15	192.8	30	348.0	40	348.0	174.7	30	–		–		176.7	132.7	80
21	M3,4-diFQ	557.1	367.0	160.7	7	–		–		160.7	134.8	30	–		–		132.7	–	
22	M3,5-diFQ	557.1	349.1	162.8	10	–		367.1	88	160.7	132.8	11	228.8	5	304.9	5	132.8	–	
23	M4,5-diFQ	557.1	349.1	162.8	6	–		367.1	40	160.7	132.8	10	–		–		132.8	89.0	93
24	M3C-5SiQ	573.2	349.1	222.8	25	379.0	20	367.1	40	160.7	132.8	12	–		304.9	5	132.8	–	

The second eluting isomer is methyl 3,5-dicaffeoylquinate **13** which produced the MS^2 base peak at *m/z* 367 ([caffeoylquinic acid methyl ester-H^+]$^-$) (Fig. 11 and Table 4). It produced the MS^3 base peak at *m/z* 179 ([caffeic acid-H^+]$^-$) and the secondary peaks at *m/z* 161 ([caffeic acid-H_2O-H^+]$^-$), *m/z* 135 ([caffeic acid-CO_2-H^+]$^-$) and *m/z* 191 ([quinic acid-H^+]$^-$) (Fig. 11 and Table 4). The MS^3 spectrum of methyl 3,5-dicaffeoylquinate is similar to MS^2 spectrum of methyl 5-caffeoylquiniate. From this it is clear that methyl 3,5-dicaffeoylquinate loses its C3 caffeoyl residue preferentially to the C5 caffeoyl residue in the MS^2 spectrum.

The third eluting isomer is methyl 4,5-dicaffeoylquinate **14** which produced the MS^2 base peak at *m/z* 367 ([methyl 5-caffeoylquinate-H^+]$^-$) by the loss of the C4 caffeoyl residue (Fig. 11). It produced the MS^3 base peak at *m/z* 179 ([caffeic acid-H^+]$^-$) and secondary peaks at *m/z* 161 ([caffeic acid-H_2O-H^+]$^-$), *m/z* 135 ([caffeic acid-CO_2-H^+]$^-$) and *m/z* 191 ([quinic acid-H^+]$^-$) (Fig. 11 and Table 4). It produced the MS^4 base peak at *m/z* 135 ([caffeic acid-CO_2-H^+]$^-$). The MS^3 secondary peak at *m/z* 161 is less intense if compared with the second eluting isomer.

It is interesting to notice that the secondary peak at *m/z* 161 in the MS^3 spectrum of methyl 3,5-dicaffeoylquinate is more intense than in the MS^3 spectrum of methyl 4,5-dicaffeoylquinate (Fig. 11 and Table 4). From the above arguments, it becomes clear that the loss of the C4 residue takes place easier than the C3 residue.

Characterization of methyl caffeoyl-feruloylquinates (M_r 543)

Three isomers produced their parent ion at *m/z* 543 and they were assigned as caffeoyl-feruloylquinic acid methyl esters. The most hydrophilic isomer **15** produced the MS^2 base peak at *m/z* 367 ([methyl 3-caffeoylquinate-H^+]$^-$) by the loss of a feruloyl residue and the secondary peak at *m/z* 161 (Fig. 12 and Table 4). It produced the MS^3 base peak at *m/z* 161 ([caffeic acid-H_2O-H^+]$^-$) and the secondary peak at 135 ([caffeic acid-CO_2-H^+]$^-$). This isomer has MS^2, MS^3 and MS^4 spectra identical to methyl 3,4-dicaffeoylquinate **12** which suggest either methyl 3-caffeoyl-4-feruloylquinate or methyl 4-caffeeoyl-3-feruloylquinate. In the green coffee extract only 3-caffeoyl-4-feruloylquinic acid was present (Table 2) which confirmed the presence of methyl 3-caffeoyl-4-feruloylquinate (**15**). From this, it is also clear that methyl 3,4-diacylquinate loses its C4 acyl residue preferentially to the C3 acyl residue.

The second and third eluting isomers (**16** and **17**, respectively) produced the MS^2 base peak at *m/z* 349 [feruloylquinic acid-H_2O-H^+]$^-$) and a secondary peak at *m/z* 367 ([methyl caffeoylquinate-H^+]$^-$) (Fig. 12 and Table 4). The second eluting isomer **16** produced the MS^3 base peak at *m/z* 193 ([ferulic acid-H_2O-H^+]$^-$) and the secondary peak at *m/z* 161 ([ferulic acid-CH_3OH-H^+]$^-$) while the third eluting isomer produced the MS^3 base peak at *m/z* 161 ([ferulic acid-CH_3OH-H^+]$^-$) and the secondary peak at *m/z* 193 ([ferulic acid-H_2O-H^+]$^-$). These both isomers show fragmentation similarity to methyl 3,5- and 4,5-caffeoylquinate. They lose their C3 and C4 acyl residues preferentially to the C5 residue.

The second eluting isomer **16** loses its C3 caffeoyl residue first and produces a dehydrated base peak in the MS^2 spectrum. Similarly, the third eluting isomer **17** loses its C4 caffeoyl residue first and produces a dehydrated base peak in the MS^2 spectrum (Fig. 12 and Table 4). The second eluting isomer **16** produced the MS^2 secondary peak at *m/z* 367, which is more intense than in the case of the third eluting isomer **17** (Fig. 12). Therefore, it is clear that the loss of the caffeoyl residue takes place easier for the third isomer (Fig. 12 and Table 2). For further evidence MS^3 experiments of *m/z* 543 + 367 for isomers **16** and **17** were performed and these probed the MS^3 spectra similarity to methyl 3-caffeoylquinate and methyl 4-caffeoylquinate. Based on the above arguments, relative hydrophobicity and fragmentation analogy with the methyl dicaffeoylquinates, the second and third eluting isomers were assigned as methyl 3-caffeoyl-5-feruloylquinate (**16**) and methyl 4-caffeoyl-5-feruloylquinate (**17**).

Characterization of methyl diferuloylquinates (M_r 557)

Three isomers were detected at *m/z* 557 and they were assigned as methyl diferuloylquinates (**18**–**20**). From the previous discussion of methyl quinates, it is generalized that the most hydrophilic isomer is methyl 3,4-diferuloylquinate **18** which produced the MS^2 base peak at *m/z* 349 ([feruloylquinic acid methyl ester-CH_3-H_2O-H^+]$^-$) and the secondary peak at *m/z* 363 ([feruloylquinic acid methyl ester-H_2O-H^+]$^-$) (Fig. 13 and Table 4). It produced the MS^3 base peak at *m/z* 161 ([ferulic acid-CH_3OH-H^+]$^-$) and

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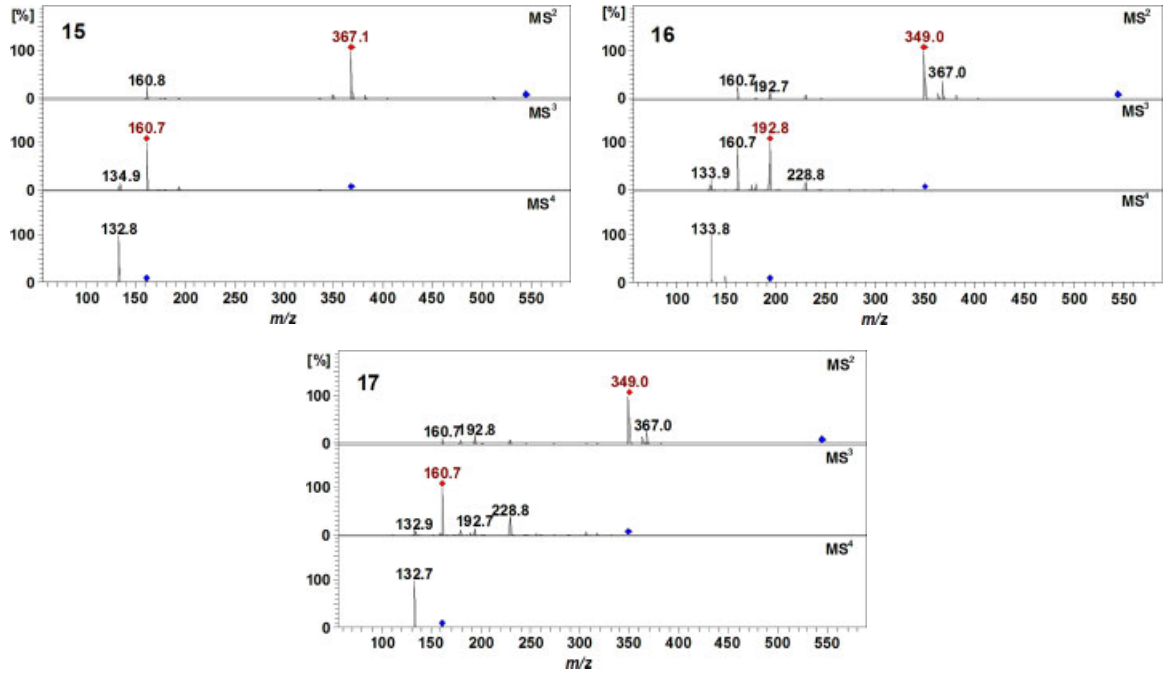

Figure 12. MS^3 spectra of methyl caffeoyl-feruloylquinates (**15**–**17**) (parent ion at *m/z* 543 in negative ion mode).

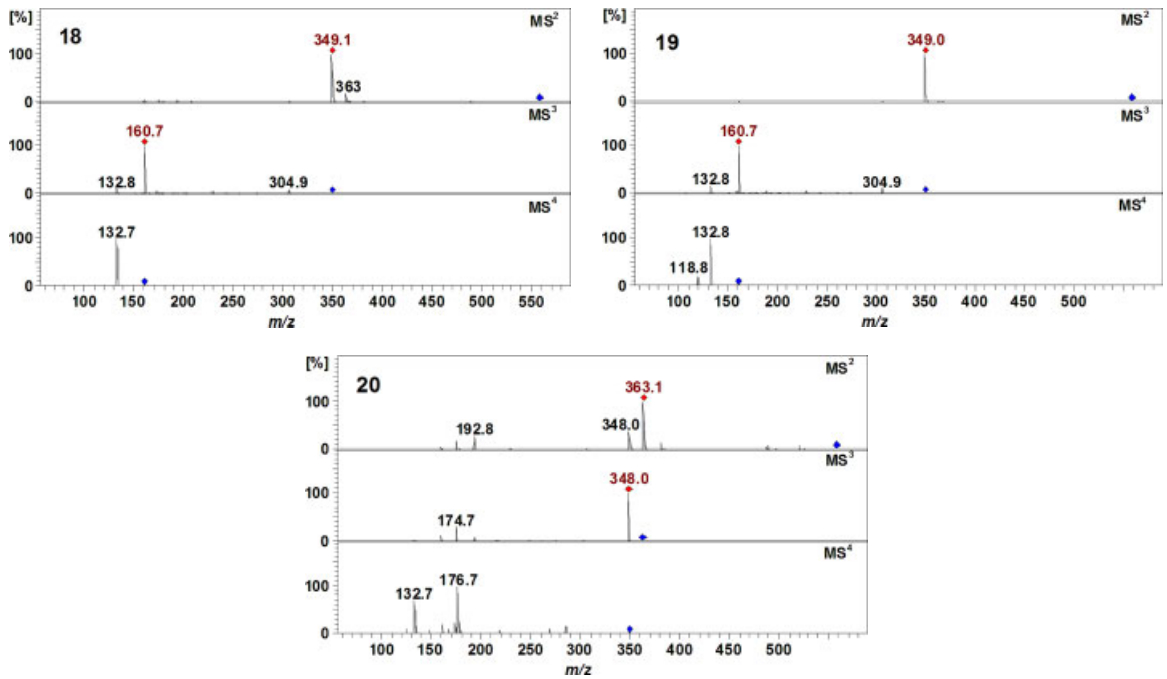

Figure 13. MS^3 spectra of methyl diferuloylquinates (**18**–**20**) (parent ion at *m/z* 557 in negative ion mode).

secondary peaks at *m/z* 133 ([ferulic acid-CO-CH_3OH-H^+]$^-$) and *m/z* 305 ([feruloylquinic acid methyl ester-CO_2-H_2O-H^+]$^-$) and MS^4 base peak at *m/z* 133 ([ferulic acid-CO-CH_3OH-H^+]$^-$) (Fig. 13 and Table 4). The second eluting isomer **19** was assigned as methyl 3,5-diferuloylquinate based on the order of elution and it produced the MS^n spectra similar to methyl 3,4-diferuloylquinate. In the case of the second eluting isomer (**19**), no secondary peak was observed in the MS^2 spectrum and it produced the MS^4 secondary peak at *m/z* 119 which is completely absent in the first eluting isomer (**18**) (Fig. 13 and Table 4).

The most hydrophobic isomer **20** was assigned as methyl 4, 5-diferuloylquinate based on the order of elution. It produced the MS^2 base peak at *m/z* 363 ([feruloylquinic acid methyl ester-H_2O-H^+]$^-$) by the loss of a feruloyl residue and a water molecule and secondary peaks at *m/z* 349 ([feruloylquinic acid methyl ester-CH_3-H_2O-H^+]$^-$), 193 ([ferulic acid-H^+]$^-$) and 175 ([ferulic acid-H_2O-H^+]$^-$) (Fig. 13 and Table 4). It produced the MS^3 base peak at *m/z* 349 and secondary peaks at *m/z* 193, 175 and 160. It showed the MS^4 base peak at *m/z* 177 and secondary peaks at *m/z* 173 ([quinic acid-H_2O-H^+]$^-$), 161 ([ferulic

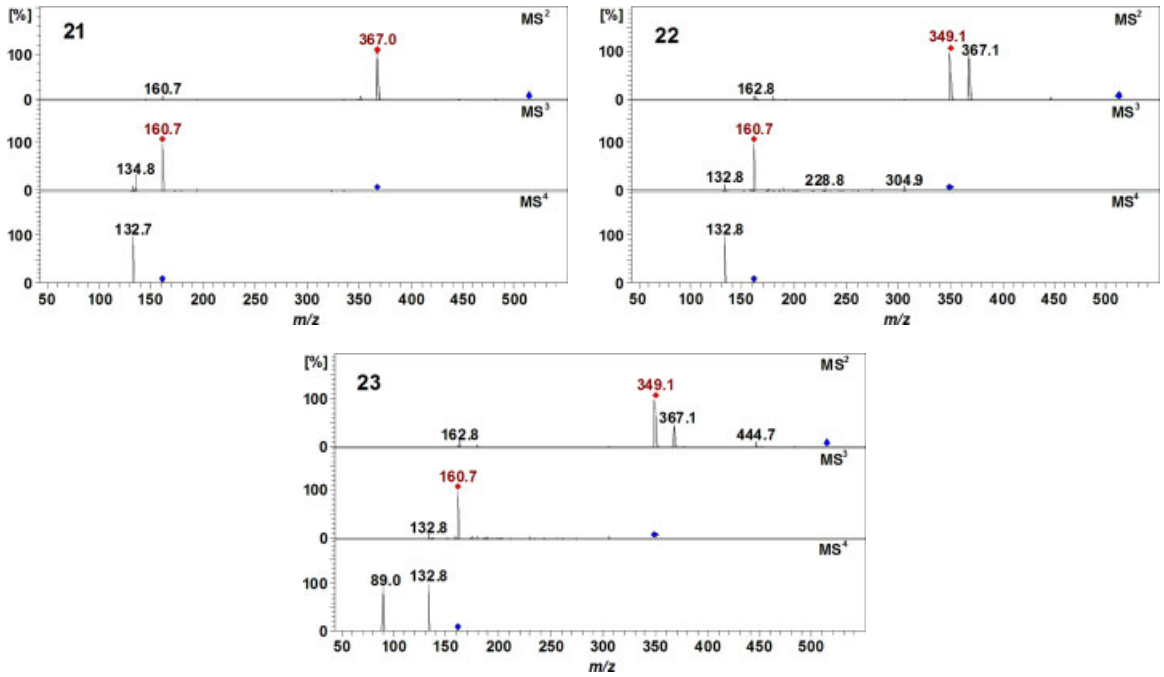

Figure 14. MS^3 spectra of methyl *p*-coumaroyl-caffeoylquinates (**21**–**23**) (parent ion at *m/z* 513 in negative ion mode).

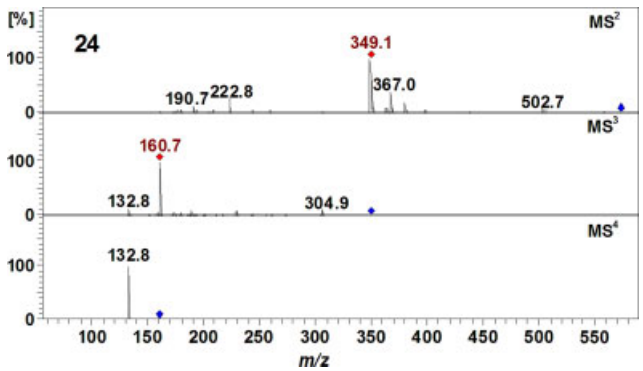

Figure 15. MS^3 spectra of methyl sinapoyl-caffeoylquinate (**24**) (parent ion at *m/z* 573 in negative ion mode).

acid-CH_3OH-H^+]$^-$) and 135 ([ferulic acid-CH_3COO-H^+]$^-$) (Fig. 13 and Table 4).

Characterization of methyl *p*-coumaroyl-caffeoylquinates (*M*$_r$ 513)

Three isomers were detected at *m/z* 513 and assigned as methyl *p*-coumaroyl-caffeoylquinates (**21**–**23**). The first eluting isomer **21** produced the MS^2 base peak at *m/z* 367 ([methyl 4-caffeoylquinate-H^+]$^-$) by the loss of *p*-coumaroyl residue and secondary peaks at *m/z* 351 ([methyl 3-*p*-coumaroylquinate-H^+]$^-$) and 161 ([caffeic acid-H_2O-H^+]$^-$) (Fig. 14 and Table 4). It produced the MS^3 base peak at *m/z* 161 ([caffeic acid-H_2O-H^+]$^-$), the MS^3 secondary peak at *m/z* 135 ([caffeic acid-CO_2-H^+]$^-$) and the MS^4 base peak at *m/z* 133 ([caffeic acid-H_2O-CO-H^+]$^-$) (Fig. 14 and Table 4). This isomer showed MS^{n+1} spectra identical to MS^{n+1} spectra of methyl 3,4-dicaffeoylquinate and methyl 3-caffeoyl-4-feruloylquinate (Table 4). Based on the above arguments and the fragment analogy with 3,4-dicaffeoylquinate and methyl 3-caffeoyl-4-feruloylquinate, this isomer was assigned as 3-caffeoyl-4-*p*-coumaroylquinate **21**.

Similarly, the second and third eluting isomers showed fragmentation analogy with methyl caffeoyl-feruloylquinates (Fig. 14 and Table 2) and they were assigned as methyl 3-caffeoyl-5-*p*-coumaroylquinate (**22**) and methyl 4-*p*-coumaroyl-5-caffeoylquinate (**23**). For further evidence MS^3 experiments of *m/z* 513 + 367 were performed for isomers **22** and **23** and the results were in agreement with the assignments.

Characterization of methyl sinapoyl-caffeoylquinates (*M*$_r$ 513)

One methyl caffeoyl-sinapoylquinate was detected and it showed MS^{n+1} spectra similar to methyl 3-caffeoyl-5-feruloylquimate **17** (Fig. 15 and Table 4). Based on the fragmentation analogy with **17** this isomer was assigned as methyl 3-caffeoyl-5-sinapoylquinate **24**.

Conclusions

In conclusion, we have shown that methyl quinates of CGAs can be resolved chromatographically and readily distinguished by their tandem mass spectra. The fragmentation patterns and fragmentation mechanisms are distinctively different from those observed for CGAs. This observation can be readily explained by taking into account that fragmentation of the acyl moieties in CGAs are induced by intramolecular protonation of the acyl moieties.[27,29,30] The acidic COOH group is absent in methylquinates and hence a different fragmentation pathway is followed.

The LC–MS^n methods introduced here can be applied for the rapid identification of alkyl quinates of CGA from natural sources, food materials, beverages and biological systems.

Acknowledgements

We acknowledge funding from Jacobs University Bremen GmbH and excellent technical support from Ms Anja Müller.

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