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Diagnostic fragment-ion-based and extension strategy coupled to DFIs intensity analysis for identification of chlorogenic acids isomers in Flos Lonicerae Japonicae by HPLC-ESI-MS^n

Jia-Yu Zhang [a,b], Qian Zhang [a], Ning Li [c], Zi-Jian Wang [b], Jian-Qiu Lu [b,**], Yan-Jiang Qiao [a,*]

[a] School of Chinese Pharmacy, Beijing University of Chinese Medicine, Beijing 100102, China
[b] Center of Scientific Experiment, Beijing University of Chinese Medicine, Beijing 100029, China
[c] State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University Health Science Center, Beijing 100191, China

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ABSTRACT

A method of modified diagnostic fragment-ion-based extension strategy (DFIBES) coupled to DFIs (diagnostic fragmentation ions) intensity analysis was successfully established to simultaneously screen and identify the chlorogenic acids (CGAs) in Flos Lonicerae Japonicae (FLJ) by HPLC-ESI-MS^n. DFIs, such as m/z 191 [quinic acid-H]$^-$, m/z 179 [caffeic acid-H]$^-$ and m/z 173 [quinic acid–H–H_2O]$^-$ were determined or proposed from the fragmentation patterns analysis of corresponding reference substances for every chemical family of CGAs. A "structure extension" method was then proposed based on the well-demonstrated fragmentation patterns and was successively applied into the rapid screening of CGAs in FLJ. Considering that substitution isomerism is a common phenomenon, a full ESI-MS^n fragmentation analysis according to the intensity of DFIs has been performed to identify the CGA isomers. Based on the DFIs and intensity analysis, 41 peaks attributed to CGAs including 4 caffeoylquinic acids (CQA), 7 CQA glycosides, 6 dicaffeoylquinic acids (DiCQA), 10 DiCQA glycosides, 1 tricaffeoylquinic acids (TriCQA), 4*p*-coumaroylquinic acids (*p*CoQA), 3 feruloylquinic acids (FQA) and 6 caffeoylferuloylquinic acids (CFQA) were identified preliminarily in a 65-min chromatographic run. It was the first time to systematically report the presence of CGAs in FLJ, especially for CQA glycosides, DiCQA glycosides, TriCQA, *p*CoQA and CFQA. All the results indicated that the method of developed DFIBES coupled to DFIs analysis was feasible, reliable and universal for screening and identifying the constituents with the same carbon skeletons especially the isomeric compounds from the complex extract of TCMs.

1. Introduction

Nowadays, the traditional Chinese medicines (TCMs) have been gained increasing popularity worldwide, owing to the changes in the types of disease, especially the prevalence of chronic and systematic diseases and limitations of western medicines [1–4]. However, because of the complexity of the chemical compositions and unclear mechanisms of action, it is difficult to guarantee the consistency of quality and therapeutic efficacy of TCMs. It is well known that TCMs, either formed as a single herb or a group of herbs in composite formula, are a complex mixture containing hundreds of different chemical constituents responsible for their therapeutic effects [5–7]. In this respect, comprehensive analytical methods for the characterization of their chemical constituents and quality evaluation of a complex chemical system are urgently required for better address the inherent holistic nature of TCMs.

In the past ten years, HPLC-ESI-MS and HPLC-ESI-MS/MS have been becoming a very powerful approach for the rapid identification of constituents in botanic extracts and crude material of TCMs [8–16]. Undoubtedly, the combined application of tandem mass spectrometry for identifying the complicated compounds in TCMs would generate a large quantity of information data, such as the elemental compositions, the fragmentation patterns information of multiple-stage, and so on. The said information data is of great helpful for the structural elucidation of constituents in TCMs. However, a new challenge of information processing appears. For example, a compound could give rise to several

Abbreviations: FLJ, Flos Lonicerae Japonicae; CGAs, Chlorogenic acids; CQA, Caffeoylquinic acid; DiCQA, Dicaffeoylquinic acid; TriCQA, Tricaffeoylquinic acid; FQA, Feruloylquinic acid; *p*CoQA, *p*-coumaroylquinic acid; CFQA, Caffeoylferuloylquinic acid; TCMs, Traditional Chinese medicines; DFIBES, Diagnostic fragment-ion-based extension strategy; DFIs, Diagnostic fragmentation ions; CID, Collision induced dissociation

* Corresponding author. Tel./fax: +86 10 84738621.
** Corresponding author. Tel./fax: +86 10 64286203.
E-mail addresses: lujq@vip.sina.com (J.-Q. Lu), yanjiangqiao@sina.com (Y.-J. Qiao).

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quasi-molecular ions, each of which could further generate a number of fragment ions in collision induced dissociation (CID) mode. Moreover, there are usually hundreds or thousands of compounds contained in the TCMs, which makes it a quite difficult and tedious task to deal with the extremely large information data.

Therefore, a strategy for efficient mass spectra analysis is highly demanded for rapid characterization of the naturally occurring substances in TCMs. To date, only a few relevant strategies have been reported, such as energy gradient neutral loss scan strategy (EGNLS) [17], "Fragmentation-Degradation" strategy for metabolic products [18] and "de novo identification" [19], all of which have been limited to the structural elucidation of only one or several certain categories of compounds. A universal strategy of diagnostic fragment-ion-based extension strategy (DFIBES) for rapid structural identification has been raised recently [20]. It was originally proposed from the universal fact that the compounds contained in TCMs could usually be structurally classified into several families with the same carbon skeletons or substructures, from which the same fragment ions (diagnostic fragmentation ions, DFIs) could be determined by the tandem mass spectrometry. The modified and universally applicable strategy DFIBES could be applied into the rapid detection and identification of the complicated compounds in TCMs. However, it has failed to differentiate the isomeric compounds with slight differences in the linkage positions of structural units and with the similar fragmentation behaviors. Meanwhile, inadequate attention was focused on the relative intensities of DFIs, which could be adopted as an important foundation to distinguish isomeric compounds from each other. Therefore, in this paper, a method of modified strategy of DFIBES coupled to DFIs intensity analysis was established to screen and identify the isomeric compounds rapidly based on the use of high performance chromatography–electrospray ionization source in combination with tandem ion trap (HPLC-ESI-IT-MS/MS), which integrates the capabilities of IT-MS/MS with LC separation in a single instrument.

Chlorogenic acids (CGAs) are a large family of esters formed between quinic acid and one to four residues of certain cinnamic acids, most commonly caffeic, *p*-coumaric and ferulic [21–23]. The distinctive characteristic of CGAs is that they usually have many isomers owing to the different substituted positions of cinnamic acids on quinic acid. Because of the deficiency of reference standards and the great structural similarity, it is of great difficulty to screen and discriminate them from positional isomers. In order to examine the feasibility, reliability and universality of the developed method, CGAs in Flos Lonicerae Japonicae (FLJ, named Jinyinhua in Chinese) was taken as a TCM example. FLJ possesses many biological functions, including antimicrobial, antioxidative, antiviral and anti-inflammatory activities, in which CGAs have been regarded as one kind of important effective constituents. However, there has been no systematical report about CGAs in FLJ so far as we are aware [24–26]. Therefore, we adopted an established methodology of modified DFIBES strategy coupled to DFIs intensity analysis to rapidly screen and identify CGA isomers in FLJ.

2. Experimental

2.1. Chemicals and materials

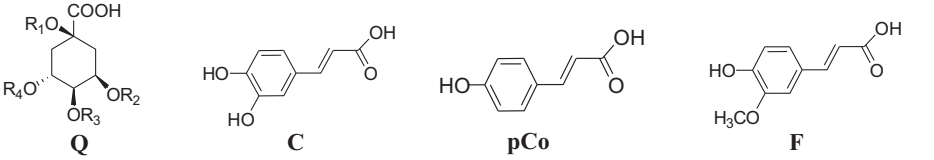
Nine CGA reference substances were purchased from Chengdu Biopurify Phtochemicals Ltd (Chengdu, China). Their structures (shown in Fig. 1) were fully elucidated by the comparison of their spectra data (ESI-MS and ^{1}H, ^{13}C NMR) with those published literature values [27,28]. The purities of the nine compounds were determined to be no less than 95% by HPLC-UV.

HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid was purchased from Sigma Aldrich (St. Louis, MO, USA). Deionized water used throughout the experiment was purified by a Milli-Q Gradient A 10 System (Millipore, Billerica, MA, USA). The 0.22 μm membranes were purchased from Xinjinghua Co. (Shanghai, China).

Material of FLJ was purchased from Yabao Pharmaceutical Group (Beijing, China), and was authenticated by Professor Yan-Jiang Qiao. The voucher specimen was deposited at Center of Scientific Experiment, Beijing University of Chinese Medicine, China.

2.2. Sample preparation for analysis

Dried powders of FLJ were weighed accurately (1.0 g) and placed into a 50 mL flask containing 25 mL of methanol/water (90:10, v/v). Then the mixture was extracted in ultrasonic bath (Eima Ultrasonics Corp., Germany) at room temperature for 1.0 h. The resulting mixture was filtered through a 0.22 μm membrane,



Q **C** **pCo** **F**

Peak	Compounds	R_1	R_2	R_3	R_4
1	3-CQA	H	C	H	H
2	5-CQA	H	H	H	C
3	4-CQA	H	H	C	H
4	1-CQA	C	H	H	H
12	1,3-diCQA	C	C	H	H
13	3,4-diCQA	H	C	C	H
14	3,5-diCQA	H	C	H	C
15	1,5-diCQA	C	H	H	C
16	4,5-diCQA	H	H	C	C

Peak	Compounds	R_1	R_2	R_3	R_4
17	1,4-diCQA	C	H	C	H
28	3,4,5-triCQA	H	C	C	C
29	3-*p*CoQA	H	*p*Co	H	H
30	5-*p*CoQA	H	H	H	*p*Co
31	4-*p*CoQA	H	H	*p*Co	H
32	1-*p*CoQA	*p*Co	H	H	H
33	5-FQA	H	H	H	F
34	4-FQA	H	H	F	H
35	3-FQA	H	F	H	H

Fig. 1. Structures of selected CGA identified from Flos Lonicerae Japonicae. Q, quinic acid; C, caffeic acid; *p*Co, *p*-coumaric acid; F, ferulic acid.

and an aliquot of 10 μL of the filtrate was injected into the HPLC–MS system for analysis.

2.3. HPLC-DAD-ESI-MS/MS analysis

The HPLC-DAD analysis was carried out on an Agilent 1100 Series liquid chromatograph system (Agilent Technologies, USA), equipped with a binary pump, an auto sampler, a photo-diode array detector and a column temperature controller. The analytical column was an Agilent Zorbax SB C_{18} (5 μm, 250 × 4.6 mm i.d.) with the oven temperature maintained at 25 °C. A mobile phase composed of eluent A (0.1% formic acid in water, v/v) and B (0.1% formic acid in acetonitrile-methanol 3:1, v/v) with a gradient elution was employed for the separation. The elution conditions applied with a linear gradient as follows: 0–5 min, 2–8% B; 5–10 min, 8–12% B; 10–25 min, 12–15% B; 25–30 min, 15–21% B; 30–41 min, 21–24% B; 41–47 min, 24% B; 47–61 min, 24–36% B; 61–65 min, 36–100% B. The flow rate was at 1.0 mL/min and peaks were detected at 327 nm.

For ESI-MS/MS analysis, an MSD Trap XCT Plus Mass spectrometer (Santa Clara, CA, USA) was connected to the same Agilent 1100 HPLC instrument via an electrospray ionization (ESI) interface. The HPLC effluent was introduced into the ESI source in a post-column splitting ratio of 1:4. The ESI-MS operating conditions (negative ion) had been optimised using 5-CQA as follows: nebulizer gas pressure of 40.00 psi; dry gas flow rate of 11.00 L/min; electrospray voltage of the ion source of 3500 V; capillary temperature of 350 °C; capillary exit of 121.0 V; skimmer of 40.0 V; compound stability of 50%; trap drive level of 100%; target mass of *m*/*z* 400; scan range of *m*/*z* 100–800; AutoMS(4) operation mode; collision energy of 1 V; SmartFrag start ampl of 30%, SmartFrag end ampl of 200%. As required, more sensitive targeted MS^n experiments were also used to seek compounds with a particular molecular ion that might otherwise have been overlooked, e.g., *m*/*z* 337 to seek *p*-coumaroylquinic acids (*p*CoQA), *m*/*z* 353 to seek caffeoylquinic acids (CQA), *m*/*z* 367 to seek feruloylquinic acids (FQA), *m*/*z* 515 to seek dicaffeoylquinic acids (DiCQA), *m*/*z* 529 to seek caffeoylferuloylquinic acids (CFQA), and *m*/*z* 677 to seek tricaffeoylquinic acids (TriCQA). A data-dependent program was used in the HPLC-ESI-MS^n analysis so that the protonated or deprotonated ions could be selected for further MS^n analysis. Nitrogen (> 99.99%) and He (> 99.99%) were used as sheath and damping gas, respectively. The Agilent 6300 Series Trap Control workstation (Version 6.1) was used for the data processing.

2.4. The establishment of DFIBES coupled to DFIs intensity analysis strategy

A strategy of DFIBES for screening of nontargeted compounds was adopted to facilitate the mass spectra analysis for structure characterization in this study [20,29]. Compounds in TCMs are typically sorted into several classes based on their carbon skeletons. It is easily understood that the compounds with same carbon skeletons will undergo the similar fragmentation pathway in CID mode and thus generate similar DFIs from the common carbon skeletons. Therefore, a series of DFIs representing a certain parent nucleus or substitution groups can be used as the characteristic peaks to select out the corresponding chemical family. A further "structure extension" approach which is similar to "formula extension" approach proposed by Kujawinski and Behn [30] can then be applied for the detailed structure characterizations of the compounds detected.

The critical step of DFIBES is to determine the DFIs that were valuable in screening and deducing nontargeted compounds of the same class. For this purpose, 9 representative reference compounds were subsequently analyzed by ESI-IT-MS/MS to determine the common DFIs of CGAs. Once the carbon skeletons were determined by the recognition of DFIs, the chemical group of a certain compound could be easily deduced from the quasi-molecular ions and the corresponding MS/MS fragment ions. Then the structurally characterized DFIs could be used as a useful screening standard for rapidly locating the exact candidates containing such a substitution group and/or substructure. Then the most possible structure could be determined from these candidates by fragmentation comparisons. In the end, the difference among DFIs intensity would be analyzed and adopted as a significant modification of DFIBES strategy to differentiate the isomeric compounds in TCMs.

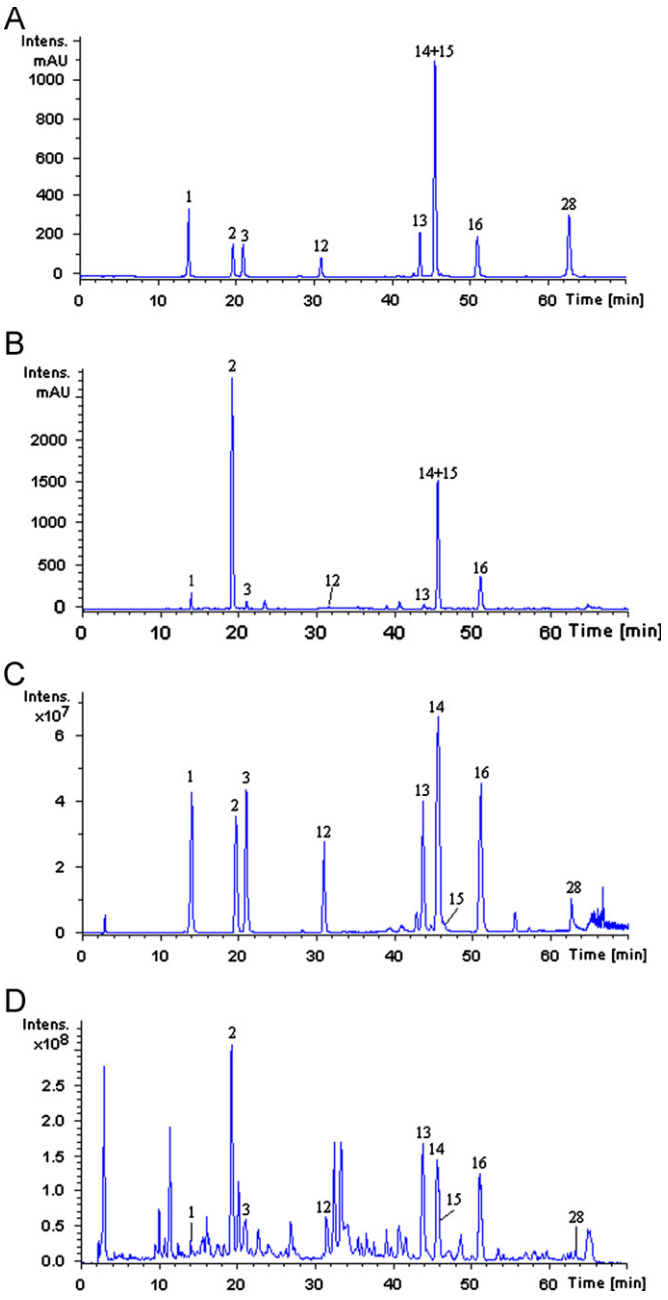


Fig. 2. HPLC-DAD-MS/MS analysis of extract of Flos Lonicerae Japonicae. (A) HPLC-DAD chromatogram of reference standards at 327 nm; (B) HPLC-DAD chromatogram of the extract at 327 nm; (C) the ESI-MS total ion chromatogram (TIC) of reference standards in negative mode. (D) the ESI-MS total ion chromatogram (TIC) of the extract in negative mode.

3. Results and discussion

3.1. Optimization of HPLC conditions

In order to obtain satisfactory extraction efficiency for all the CGAs, the extraction conditions, including extraction methods (ultrasonication, refluxing and standing overnight), extraction solvents (70, 90, 100% methanol) and extraction time (30, 45 and 60 min) were assessed based on orthogonal experiments. The best extraction efficiency was obtained by ultrasonication extraction with 90% methanol for 60 min. The different HPLC parameters including mobile phases (methanol/water, acetonitrile/water and acetonitrile/methanol/water), the concentration of formic acid in water (0.05, 0.1 and 0.3%), category of RP-ODS columns (Agilent Zorbax SB C_{18} column, 250 × 4.6 mm i.d., 5 μm and Agilent Zorbax Extended C_{18}, 250 × 4.6 mm i.d., 5 μm), column temperature (20, 25 and 30 °C), flow rate (0.8, 1.0 and 1.2 mL/min) and gradient elution methods were examined. The addition of formic acid was advantageous to obtain the best resolution of adjacent peaks during chromatographic separation (shown in Fig. 2).

3.2. Optimization of ESI-MS/MS conditions

In order to achieve the optimum conditions to identify CGAs in FLJ, all factors related to MS performance including ionization mode, nebulizer gas pressure, electrospray voltage of the ion source and collision energy have been experimented. The results showed that the ESI in negative ion mode was more sensitive to CGAs than in positive ion mode. The major CGAs were well detected (shown in Fig. 2), and most of the investigated compounds exhibited quasi-molecular ions $[M-H]^-$ and product-ions with rich structural information in the negative mode of CID-MS/MS.

Table 1
Characterization of CGA in Flos Lonicerae Japonicae and DFIs determined by HPLC-DAD-ESI-MS/MS.

No.	No.	t_R[a]	$[M-H]^-$ (m/z)	MS^2(m/z) P-ion (%)[b]	MS^3(m/z) P-ion (%)[b]	MS^4(m/z) P-ion (%)[b]	Identification
Type A	1[c]	14.10	353	191*(100),179(39.8)	173*(100),127(94.7),111(30.1)	155(100), 111(79.6)	3-CQA
	2[c]	19.41	353	191*(100),179(7.7)	173*(100),127(95.8),111(35.1)	111(100), 155(79.6)	5-CQA
	3[c]	21.29	353	173*(100),179(46.8),191(19.3)	111(100),155(49.5),135(14.4)	–	4-CQA
	4	26.11	353	191*(100),179(6.2)	127(100),173(49.5),111(28.2),109(16.7)	–	1-CQA
Type B1	5	10.40	515	353*(100), 191(33.9)	191*(100),179(7.6)	173(100),127(86.7)	1-CQA glycoside or 5-CQA glycoside
	6	12.74	515	353*(100), 191(76.2)	191*(100),179(11.1)	111(100),127(32.1),173(10.3)	1-CQA glycoside or 5-CQA glycoside
	7	13.59	515	353*(100)	191*(100),179(12.6),173(11.7),	173(100)	1-CQA glycoside or 5-CQA glycoside
	8	14.53	515	353*(100)	173(100),179(38.4)	–	4-CQA glycoside
	9	15.71	515	353*(100),191(14.5)	191*(100),173(7.3)	155(100),127(24.6)	1-CQA glycoside or 5-CQA glycoside
	10	16.31	515	353*(100)	191*(100),179(41.9)	173(100),127(84.9)	3-CQA glycoside
	11	18.11	515	353*(100)	191(100),179(36.4),173(69.5),	–	3-CQA glycoside
Type B2	12[c]	31.61	515	353*(100)	191*(100),179(14.4)	173(100)	1,3-DiCQA
	13[c]	43.66	515	353*(100),173(18.2),179(11.4)	173*(100),179(61.3),191(36.9)	111(100),155(54.8),109(43.1)	3,4-DiCQA
	14[c]	45.81	515	353*(100),179(46.8),135(7.7)	191*(100),179(46.8),135(7.7)	127(100),173(88.5),111(30.2)	3,5-DiCQA
	15[c]	46.98	515	353*(100)	191*(100),179(52.7),173(13.5)	127(100),173(93.6),111(29.4)	1,5-DiCQA
	16[c]	50.73	515	353*(100)	173*(100),179(50.6),191(21.7)	111(100),155(39.9),109(12.1)	4,5-DiCQA
	17	59.79	515	353*(100)	191*(100),173(85.4),179(69.1)	173(100),127(51.4),111(10.9)	1,4-DiCQA
Type C1	18	34.92	677	515*(100),353(88.8)	353*(100),191(18.1)	191(100),179(18.9)	DiCQA glycoside-1
	19	35.70	677	515*(100),353(79.1)	353*(100),179(94.7)	191(100),179(67.3),173(7.5)	DiCQA glycoside-2
	20	36.31	677	515*(100),353(89.0)	353*(100),	191 (100), 179(18.8)	DiCQA glycoside-3
	21	37.13	677	515*(100),353(19.4)	353*(100),	173(100),179(91.6),191(27.4)	DiCQA glycoside-4
	22	38.87	677	515*(100),353(8.4)	353*(100),341(55.4),179(24.3)	173(100),191(68.0),179(50.2)	DiCQA glycoside-5
	23	40.55	677	515*(100),353(90.5)	353*(100),	191(100),179(54.5),135(14.5)	DiCQA glycoside-6
	24	41.67	677	515*(100),353(63.3)	353*(100),	191(100),179(86.2),173(4.9)	DiCQA glycoside-7
	25	44.29	677	515*(100),353(6.7)	353*(100),341(49.7),179(28.1)	173(100),179(93.9),191(11.4)	DiCQA glycoside-8
	26	56.58	677	515*(100),497(12.9)	341*(100),281(23.0),297(14.4)	–	DiCQA glycoside-9
	27	58.41	677	515*(100),497(19.6)	341*(100),281(65.1),297(39.1),353(26.6)	–	DiCQA glycoside-10
Type C2	28[c]	62.57	677	515*(100),353(9.9)	353*(100),173(15.8)	173(100),179(45),191(28.7)	3,4,5-TriCQA
Type D	29	17.95	337	163*(100),191(8.5)	119(100)	–	3-pCoQA
	30	27.51	337	191*(100),163(6.2)	127*(100),173(90.2),111(28.3)	109(100)	5-pCoQA
	31	28.64	337	173*(100),191(43.9),163(8.2)	111(100)	–	4-pCoQA
	32	33.70	337	191*(100)	173(100),127(82.1)	–	1-pCoQA
Type E	33	31.61	367	191* (100),193(5.6)	127(100),173(81.6),111(29.6)	–	5-FQA
	34	33.64	367	173*(100), 191(17.2)	155(100),111(14.2)	–	4-FQA
	35	35.70	367	193* (100),191(7.2)	149(100),178(72.6)	–	3-FQA
Type F	36	56.31	529	367* (100),353(6.1)	193*(100),161(29.4),173(17.1)	134(100),149(49.2)	CFQA-1
	37	57.03	529	353*(100),367(46.3)	191*(100),179(49.6),135(7.4)	173(100),127(52.7),109(40.1)	CFQA-2
	38	58.90	529	367* (100),353(10.9)	179*(100),173(75.0),191(48.4),193(42)	135(100)	CFQA-3
	39	59.41	529	367* (100)	179*(100),161(85.3),191(23.1),193(21.8)	135(100)	CFQA-4
	40	60.55	529	367* (100),353(61.7)	161(100),179(94.6),191(66.8),193(37.6)	–	CFQA-5
	41	62.64	529	367* (100)	179*(100),161(57.7),191(24.8),193(10.8)	135(100)	CFQA-6

–Too low to be detected.
_DFIs for CGA.

* Precursor-ion for next stage MS.
[a] t_R, retention time.
[b] P-ion (%), the product ions (the relative intensity).
[c] Compounds identified by comparison with reference standards.

3.3. DFIs determinations and fragmentation patterns analysis for CGAs

CGAs could be roughly classified into six types: CQA (type A), DiCQA (type B), TriCQA (type C), *p*CoQA (type D), FQA (type E) and CFQA (type F) based on their structures. Negative ion mode was operated to investigate the DFIs and the fragmentation patterns of CGAs with 9 standards (representing type A–C).

The DFIs were firstly determined from the fragment ions of CGA standards (Table 1). For type A–C compounds, the common DFIs were determined as *m/z* 191($C_7H_{11}O_6^-$) corresponding to [quinic acid-H]$^-$ (DFI 1), *m/z* 179 ($C_9H_7O_4^-$) corresponding to [caffeic acid-H]$^-$ (DFI 2), *m/z* 173 ($C_7H_9O_5^-$) corresponding to [quinic acid–H–H_2O]$^-$ (DFI 3), all of which could be regarded as the DFIs for CGAs. Meanwhile, for type A compounds, *m/z* 353 ($C_{16}H_{17}O_9^-$) corresponding to [CQA–H]$^-$ (DFI-A1) was determined as their additional DFI; for type B compounds, *m/z* 515 ($C_{25}H_{23}O_{12}^-$) corresponding to [DiCQA–H]$^-$ (DFI-B1) and *m/z* 353 ($C_{16}H_{17}O_9^-$) corresponding to [CQA–H]$^-$ (DFI-B2) were determined as their additional DFIs; and for type C compounds, *m/z* 677 ($C_{34}H_{29}O_{15}^-$) corresponding to [TriCQA–H]$^-$ (DFI-C1), *m/z* 515 ($C_{25}H_{23}O_{12}^-$) corresponding to [DiCQA–H]$^-$ (DFI-C2) and 353($C_{16}H_{17}O_9^-$) corresponding to [CQA–H]$^-$ (DFI-C3) were determined as their additional DFIs, respectively. According to the common and additional DFIs of the CGAs, three categories of CGAs could be screened and identified rapidly from the extract of FLJ.

The structures of DFIs and the proposed fragmentation patterns of each class of CGAs were summarized in Fig. 3 by taking 3,5-DiCQA as one representative example. The nomenclature proposed by Domon and Costello with some adaptation was adopted to denote the fragment ions [31]. Its [M–H]$^-$ ion (*m/z* 515) produced fragment ions at *m/z* 353, 191, 179, 173, 135, 129, 127 and 111 in CID-MS/MS experiment corresponding to [CQA–H]$^-$, [quinic acid-H]$^-$, [caffeic acid-H]$^-$, [191–H_2O]$^-$, [179–CO_2]$^-$, [173–CO_2]$^-$, [173–CO–H_2O]$^-$ and [191–CO_2–H_2O]$^-$, respectively. The mass difference between the parent ion (*m/z* 515) and the fragment ion (*m/z* 353) was 162 Da, indicating the loss of a caffeoyl moiety in the MS^2 experiment. The intensity of ion at *m/z* 191 produced by the loss of a caffeoyl residue was much greater than that of *m/z* 179, indicating that the loss of a caffeoyl preferentially happened in the MS^3 experiment.

3.4. DFIBES-directed rapid screening for CGAs

DFIBES was then used for the rapid screening and identification of CGAs contained in FLJ. The DFIs were used as markers for screening and classifying CGAs detected into known categories, followed by a "structure extension" approach based on the serial fragment ions analysis for screening the other categories of CGAs.

Here we took type B identification for example to describe the strategy in detail. According to the fragment behaviors of CGAs, cinnamic acid units, quinic acid unit, H_2O and CO were common chemical groups that could be easily eliminated under CID mode. The mass differences existed between DFIs and other fragment ions were associated with cinnamic acid units [e.g., caffeic acid ($C_9H_6O_3$, 162), *p*-coumaric acid ($C_9H_6O_2$, 146) and ferulic acid ($C_{10}H_8O_3$, 176)], water (H_2O, 18) or carbon monoxide (CO, 28). For peak 17, the mass difference between [CQA–H]$^-$ ion (DFI-B2) and quasi-molecular ion was 162 Da, which indicated the loss of a caffeoyl unit. The mass difference between the [CQA–H]$^-$ ion (*m/z* 353) and the fragment ion *m/z* 191 was 162 Da, which corresponded to the loss of another caffeoyl unit, while the mass difference between the [CQA–H]$^-$ ion and the fragment ion *m/z* 179 was 174 Da, which corresponded to a quinic unit, indicating that their structures contained two caffeoyl units and one quinic unit. The difference between the ions at *m/z* 191 and *m/z* 173 was 18 Da, indicating the presence of a quinic unit, too. Then peak 17 was primarily screened to be type B CGAs.

Meanwhile, other 3 categories of CGAs including *p*CoQA, FQA and CFQA were screened out from the FLJ extract by a "structure extension" approach. Similarly, from the respective fragmentation patterns, the additional DFIs for type D, E and F compounds were proposed as follows: *m/z* 337 [*p*CoQA–H]$^-$ (DFI-D1) and *m/z* 163 [cinnamic acid-H]$^-$ (DFI-D2) for type D, *m/z* 367 [FQA–H]$^-$ (DFI-E1) and *m/z* 193 [ferulic acid-H]$^-$ (DFI-E2) for type E, *m/z* 529 [CFQA–H]$^-$ (DFI-F1), *m/z* 367 [FQA–H]$^-$ (DFI-F2), *m/z* 353

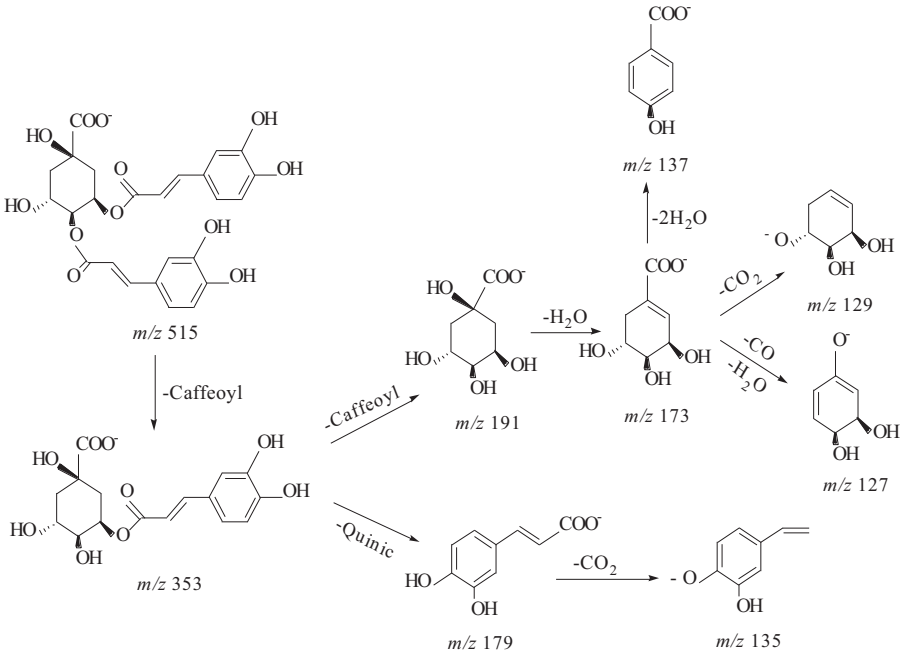


Fig. 3. The proposed fragmentation pathways and common DFIs for CGA (taking 3,5-DiCQA for example).

[CQA–H]$^-$ (DFI-F3) and m/z 193 [ferulic acid-H]$^-$ (DFI-F4) for type F, which were concluded based on the comparison of the structure similarities with type A, B and C compounds as well as the referencing to the previous reports (shown in Table 1).

As a result, peaks 1–4, 5–17 and 18–28 were, respectively classified as type A, B and C, while peaks 29–32, 33–35 and 36–41 were successively designated as type D, E and F of CGAs (shown in Fig. 4).

3.5. Rapid ESI-MS/MS identification for CGA isomers

Unfortunately, it was almost impossible to differentiate isomeric CGAs only by DFIBES due to their minor differences in the substitutional positions of cinnamic acid units. For example, the compounds of peaks 1–4 could generate identical DFIs, such as m/z 353, 191, 179, 173, et al., so it was difficult to tell the peaks apart. However, the differences of DFIs intensity could be adopted to identify their accurate structures.

Type A: Four parent ions at m/z 353 were easily located in the chromatogram of the extract of FLJ (shown in Fig. 4A). It was reported that the linkage position of caffeoyl groups on quinic acid could be determined based on the MS^2 fragmentation [32]. Typically, when the caffeoyl group was linked to quinic acid at 3-OH or 5-OH, the [quinic acid-H]$^-$ ion at m/z 191 was the base peak, and the [caffeic acid-H]$^-$ ion at m/z 179 was more significant for 3-CQA. While the [quinic acid–H_2O–H]$^-$ ion at m/z 173 was the prominent peak, the caffeoyl group was linked at 4-OH. The patterns of fragmentation observed were compared with those of the reference substances and the compounds 1–3 were identified to be 3-CQA, 5-CQA and 4-CQA, respectively. As for 1-CQA, it was nearly impossible to reliably distinguish it from 5-CQA only according to their fragmentations. Fortunately, the available 5-CQA standard enabled 1-CQA to be identified easily in practice. Their MS^2 spectra were shown in Fig. 5.

Type B: Targeted MS^2 experiments (m/z 515) could detect at least 13 signals that were initially interpreted as DiCQA by DFIBES (shown in Fig. 4B). The compounds 5–11 eluted during 10–19 min

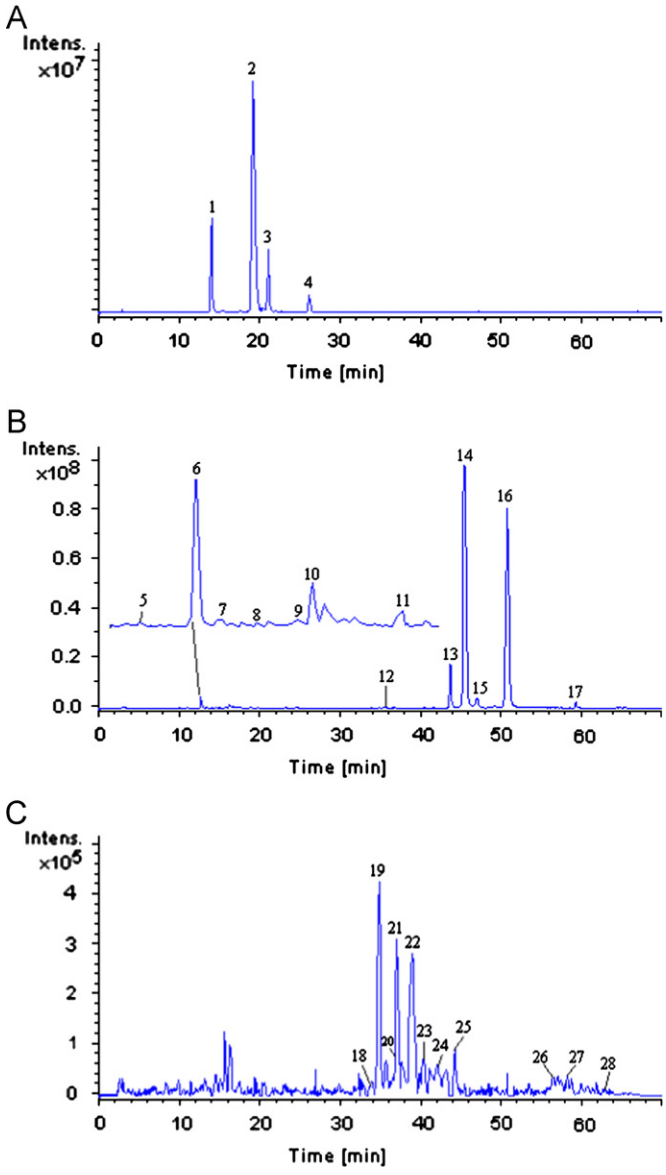


Fig. 4. The EIC-MS peaks of all possible CGA in the extract of Flos Lonicerae Japonicae. (A) m/z 353; (B) m/z 515; (C) m/z 677; (D) m/z 337; (E) m/z 367; (F) m/z 529.

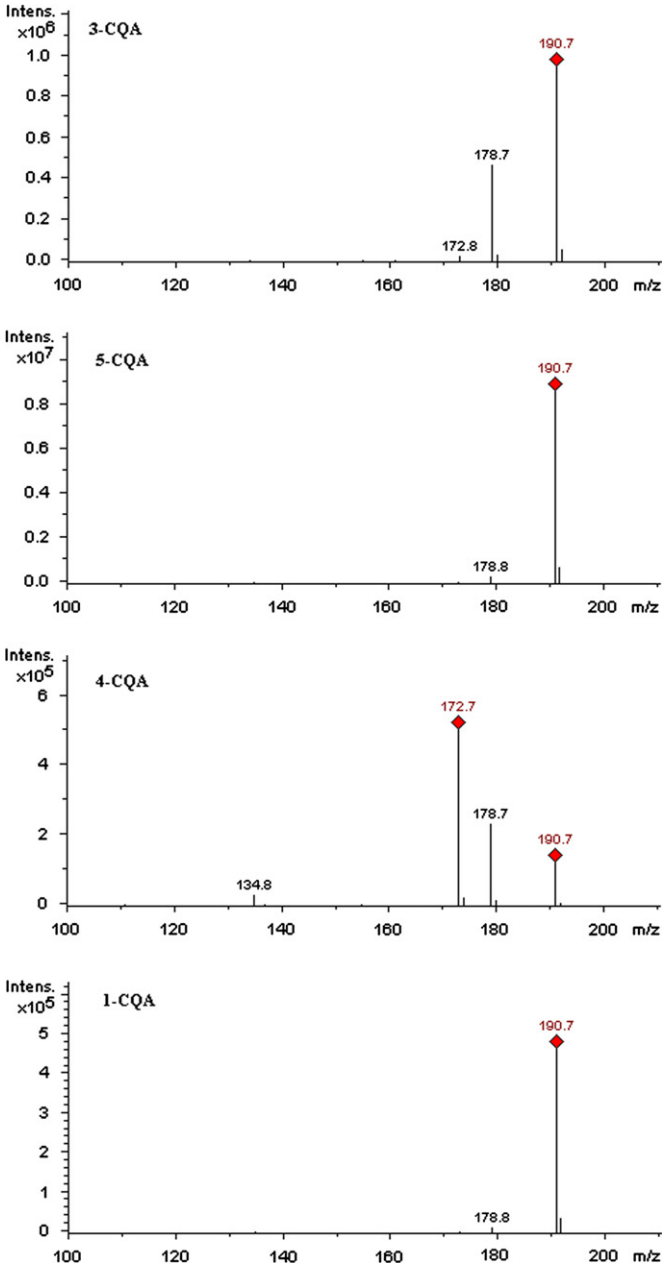


Fig. 5. Negative MS^2 spectra for isomeric CQA in Flos Lonicerae Japonicae.

were remarkably in advance of 1,3-DiCQA eluted at about 30 min, which is the most hydrophilic DiCQA so far characterized by LC-MS^n [33]. So the compounds 5–11 (Type B1) and 12–17 (Type B2) could be characterized separately. Compounds 12–17 all gave the $[M–H]^-$ ion at *m/z* 515 and the $[M–H–162]^-$ ion at *m/z* 353. However, their MS^3 spectra were significantly different. Both compounds 12 and 14 produced base peak ion at *m/z* 191 and secondary peak at *m/z* 179. As reviewed above, they were identified as 3-substituted quinic acids. By comparing with reference substances, the former was assigned as 3,5-DiCQA, while the latter was identified as 1,3-DiCQA. Compounds 13 and 16 both produced base peak at *m/z* 173, indicating that they could be identified as 4-substituted quinic acids. According to the literature [34], 3,5-dicaffeoylquinic acid was eluted from the reverse-phase column easier than 4,5-dicaffeoylquinic acid. Thus, they were identified as 3,4-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid, respectively, which was consistent with the result from comparison with standards. Compound 15 was identified as 1,5-DiCQA according to the presence of base peak at *m/z* 191 and minor peak at *m/z* 179. Meanwhile, compound 17 was tentatively identified as 1,4-DiCQA, which was consistent with the fact of secondary peak at *m/z* 173 in its MS^3 spectrum. Their MS^3 spectra were shown in Fig. 6.

Compounds 5–11 (Type B1) eluted earlier than DiCQA were tentatively characterized as CQA glycosides, since the sugar residue could make CQA glycosides much more hydrophilic than the second caffeic acid residue. Their distinctive fragment ions at *m/z* 353 (due to the loss of sugar residue) also suggested they were CQA glycosides. The targeted fragmentation of the MS^2 fragment ion at *m/z* 353 could identify the link position of the caffeic acid and the quinic acid. For example, the intensity of the *m/z* 191 fragment ion of peaks 5–7 and 9 relative to the intensity of the *m/z* 179 and 173 suggested that they might to be glycosides of either 1-CQA or 5-CQA. Similarly for peaks 10–11, the intensity of the *m/z* 179 fragment ion relative to *m/z* 191 suggested that they might be glycosides of 3-CQA. The presence of base peak at *m/z* 173 indicated that peak 8 was 4-CQA glycoside. For each caffeoylquinic acid, there are two possible glycosides (for any given sugar) depending on whether the sugar attached at the 3-OH or the 4-OH of the caffeic acid moiety (Fig. 1). However, these features cannot be distinguished by MS/MS spectra. So far as we are aware, no CQA glycosides have been reported in FLJ previously.

Type C: A search for TriCQA coupled to DFIBES located 9 signals at *m/z* 677 in the extract of FLJ (shown in Fig. 4C). Eight of them were eluted before 4,5-dicaffeoylquinic acid, suggesting that they were too hydrophilic to be TriCQA, so their characterizations were carried out separately. Compound 28 (Type C2), the hydrophobic compounds that was eluted after 4,5-dicaffeoylquinic acid, lost two caffeoyl residues progressively and thus produced a [4-caffeoylquinic acid-H]$^-$ ion in its MS^3 spectrum. By comparing with the reference substance, compound 28 was unambiguously identified as 3,4,5-TriCQA.

The hydrophilic compounds 18–27 (Type C1), eluted between 34 and 44 min, produced MS^4 fragment ions characteristics of a quinic acid residue and a caffeic acid residue. They all produced *m/z* 353 base peak in MS^3 spectra. In four cases they gave predominant ion at *m/z* 341 at the same time. The targeted MS^4 experiment (*m/z* 677 + 515 + 353) established that three produced [4-caffeoylquinic acid-H]$^-$, three produced [3-caffeoylquinic acid-H]$^-$, two produced [5-caffeoylquinic acid-H]$^-$ or [1-caffeoylquinic-H]$^-$, and two remained equivocal due to the absence of *m/z* 353 in their MS^3 spectra. So far as we know, such compounds have not previously been characterized unequivocally, though it has been suggested that artichoke might contain a DiCQA glycoside [35]. Theoretically, for any given sugar, one might expect 24 DiCQA glycosides, and these on fragmentation would be expected at MS^4 to yield [4-caffeoylquinic acid-H]$^-$ on 12 occasions,

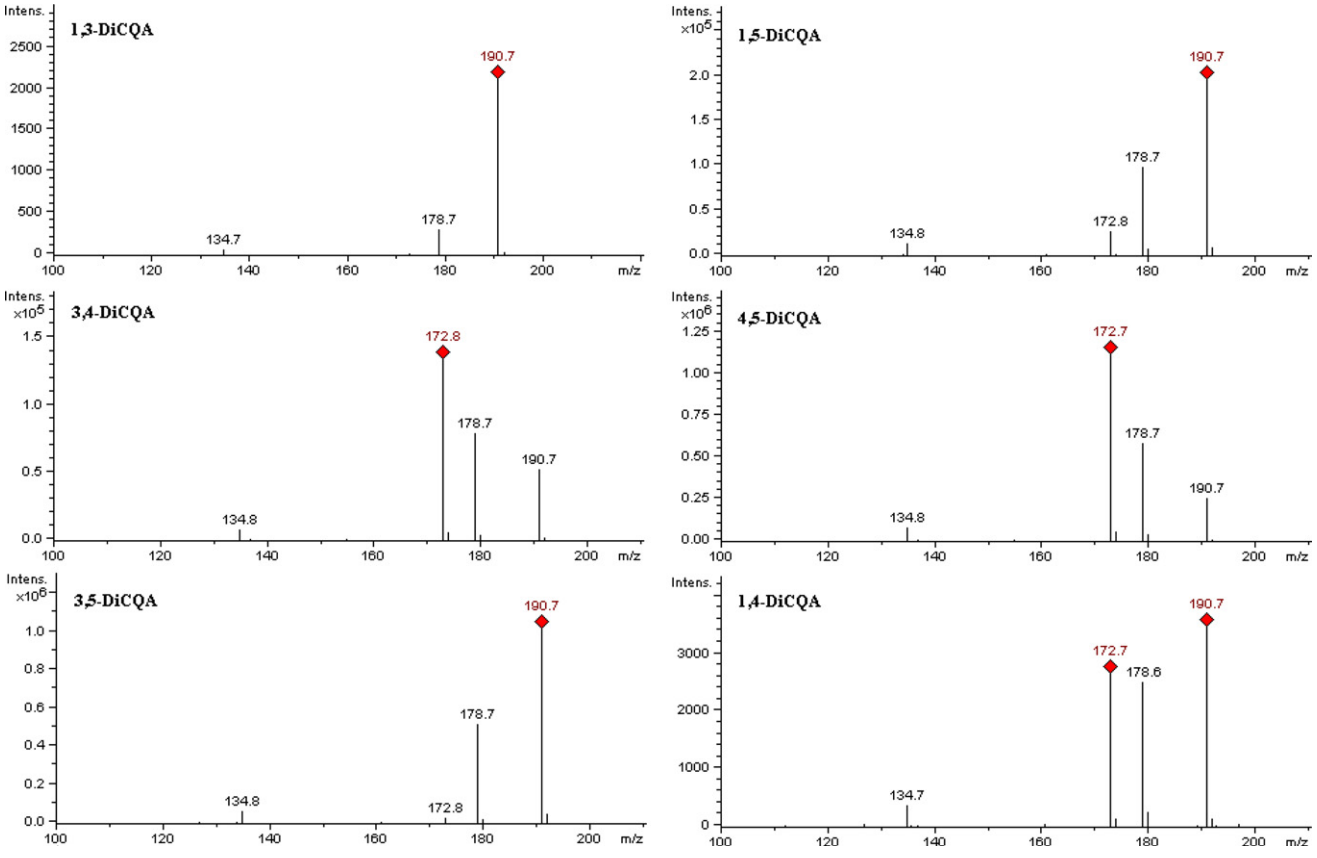


Fig. 6. Negative MS^3 spectra for isomeric DiCQA in Flos Lonicerae Japonicae.

[3-caffeoylquinic acid-H]$^-$ on eight occasions, and [1-caffeoylquinic acid-H]$^-$ and/or [5-caffeoylquinic acid-H]$^-$ on four occasions. It is possible that these DiCQA glycosides are present below the limit of detection, or they are not produced by the plants. Whatever the explanation, our observations provided the evidence for the occurrence of 10 isomeric DiCQA glycosides in FLJ.

Type D: Targeted MS^2 experiment located four *p*CoQA in the extract of FLJ (shown in Fig. 4D). According to the patterns of fragmentation in their MS^2 spectra and the previously published structure-diagnostic hierarchical, the four *p*CoQA were identified to be as follows: 3-*p*CoQA (29) produced MS^2 base peak at *m/z* 163 [coumaric acid-H]$^-$, 5-*p*CoQA (30) produced MS^2 base peak at *m/z* 191 [quinic acid-H]$^-$, while 4-*p*CoQA (31) generated MS^2 base peak at 173 [quinic acid–H–H_2O]$^-$ [36]. Peak 32 gave MS^2 base peak at *m/z* 191 similar to 5-*p*CoQA. According to the eluted sequence of CQA on RP–ODS columns, peak 32 was tentatively identified to be 1-*p*CoQA. Their MS^2 spectra were shown in Fig. 7.

Type E: The same experiment detected three FQA, respectively from FLJ (shown in Fig. 4E). In the previously report, 5-FQA

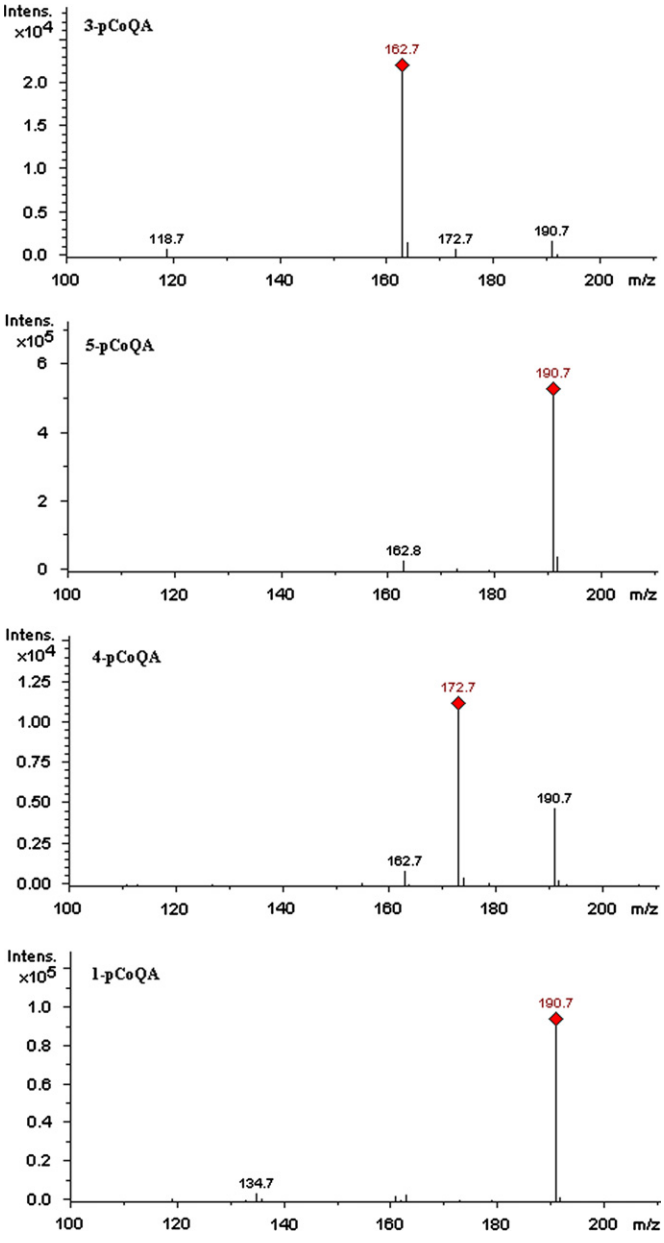


Fig. 7. Negative MS^2 spectra for isomeric *p*CoQA in Flos Lonicerae Japonicae.

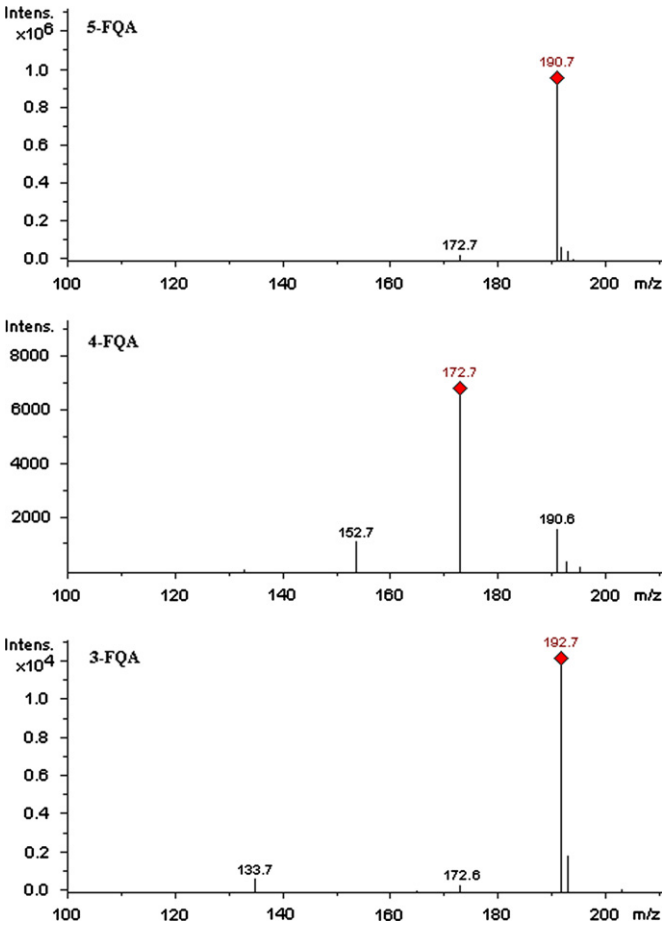


Fig. 8. Negative MS^2 spectra for isomeric FQA in Flos Lonicerae Japonicae.

produced MS^2 base peak at *m/z* 191 [quinic acid-H]$^-$ accompanied by a weak ion at *m/z* 173 [quinic acid–H–H_2O]$^-$, while 4-FQA and 3-FQA generated MS^2 base peak at *m/z* 173 [quinic acid–H–H_2O] and 193 [ferulic acid-H]$^-$, respectively [37]. Their MS^2 spectra were shown in Fig. 8.

Type F: Targeted MS^2 experiments (*m/z* 529) could detect at least 6 signals interpreted as CFQA by DFIBES during 55–63 min (shown in Fig. 4F). Five of them produced MS^2 base peak at *m/z* 367, and the rest one gave the base peak at *m/z* 353. All the six compounds showed MS^2 base peaks of either *m/z* 367 [FQA–H]$^-$ or *m/z* 353 [CQA–H]$^-$and MS^3 significant yields of *m/z* 193 [ferulic acid-H]$^-$, *m/z* 191 [quinic acid-H]$^-$ or *m/z* 179 [caffeic acid-H]$^-$, which were consistent with CFQA isomers. However, there is great difficulty to tell them apart due to the lack of reference substances and published reports.

4. Conclusion

A modified strategy, DFIBES coupled to DFIs intensity analysis method, was successfully established to simultaneously screen and identify the CGAs in FLJ by HPLC-ESI-MS^n. First, DFIs for every chemical family of CGAs were determined or proposed from fragmentation patterns analysis of the corresponding reference substances. The "structure extension" method was then proposed based on the well-demonstrated fragmentation patterns. The presently developed method and strategy were successfully applied into the rapid screening and identification of CGAs in FLJ. Six categories of CGAs could be rapidly screened by the DFIBES strategy. As the structures of the substitution isomerisms

of CGAs are too similar to differentiate them from one another only by DFIBES, a full ESI-MS^n fragmentation analysis according to the intensity of DFIs was performed to identify them. In the end, 41 peaks attributed to CGAs, including 4 CQA, 7 CQA glycosides, 6 DiCQA, 10 DiCQA glycosides, 1 TriCQA, 4 *p*CoQA, 3 FQA and 6 CFQA, were detected in a 65-min chromatographic run and identified preliminarily, wherein 9 of them could be unambiguously identified by comparison with reference substances. It was the first time to systematically report the CGAs presenting in FLJ, especially the CQA glycosides, DiCQA glycosides, CFQA and TriCQA. All the results indicated that the analytical method of developed DFIBES coupled to DFIs intensity could be employed as a feasible, reliable and universal technique to screen and identify the constituents with same carbon skeletons especially the isomeric compounds from complex extract of TCMs. Moreover, it is possible for the methodology to be extended to the fields of elucidating compounds from other organic matter mixtures such as substances analysis in vegetables, water quality analysis, natural organic matter analysis in soil, pesticide multi-residue analysis in food, and so on, in the view of that the compounds contained in such matrix can also be classified into families based on the common carbon skeletons.

Acknowledgments

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