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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

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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

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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

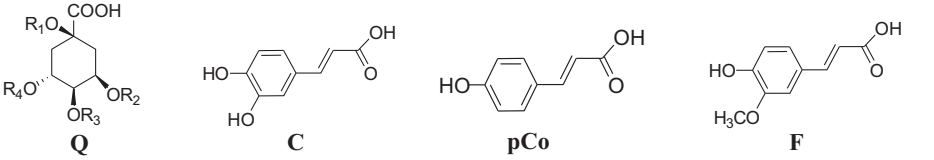
Nine CGA reference substances were purchased from Chengdu Biopurify Phtochemicals Ltd (Chengdu, China). Their structures (shown in Fig. 1) were fully elucidated by the comparison of their spectra data (ESI-MS and ^{1}H, ^{13}C NMR) with those published literature values [27,28]. The purities of the nine 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.