Research Article

Phytochemical Analysis

Received: 22 August 2013, Revised: 30 December 2013, Accepted: 14 January 2014 Published online in Wiley Online Library: 5 March 2014

(wileyonlinelibrary.com) DOI 10.1002/pca.2508

Rapid Identification of Polymethoxylated Flavonoids in Traditional Chinese Medicines with a Practical Strategy of Stepwise Mass Defect Filtering Coupled to Diagnostic Product Ions Analysis based on a Hybrid LTQ-Orbitrap Mass Spectrometer

Jia-Yu Zhang,[a] Fang Wang,[b] Hong Zhang,[c] Jian-Qiu Lu[a]* and Yan-Jiang Qiao[b]**

ABSTRACT:

Introduction – The methodology of stepwise mass defect filtering (MDF) approach coupled to diagnostic product ions (DPIs) analysis on a hybrid linear trap quadrupole (LTQ)/orbitrap mass spectrometer was the first to be established to screen and identify structural analogues from complex herbal extracts.

Objective – To develop an analytical methodology that could be adopted to screen and identify structural analogues in traditional Chinese medicines (TCMs) rapidly and accurately.

Methods – Taking polymethoxylated flavonoids (PMFs) in the leaves of *Citrus reticulata* Blanco as an example, high-resolution mass data were acquired by high-performance liquid chromatography (HPLC) coupled with a LTQ/orbitrap mass spectrometer. The stepwise MDF with multiple mass defect windows or mass windows enabled the original data to be analysed much faster and more accurately by reducing the potential interferences of matrix ions. Additionally, analysis of DPIs could provide a criterion to classify the target constituents detected into certain chemical families.

Results – In total, 81 PMFs, including 50 polymethoxyflavones and 31 polymethoxyflavanones or polymethoxychalcones, were screened and identified from the original data and preliminarily identified.

Conclusion – The analytical methodology developed could be used as a rapid, effective technique to screen and identify compounds from TCM extracts and other organic matter mixtures with compounds that can also be classified into families based on the common carbon skeletons. Copyright © 2014 John Wiley & Sons, Ltd.

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Keywords: Diagnostic product ions analysis; HPLC–LTQ/orbitrap mass spectrometer; stepwise mass defect filtering; polymethoxyflavonoids

Introduction

Due to the increasing popularity worldwide of traditional Chinese medicines (TCMs), owing to the increasing prevalence of chronic and systematic diseases and limitations of western medicines, more efforts should be devoted to develop effective and reliable methodologies with the capability of comprehensive structural characterisation to guarantee their consistency and therapeutic efficacy in the process of 'modernisation' and 'globalisation' of TCMs (Normile, 2003; Xue and Roy, 2003). It is well known that TCMs, either in the form of a single herb or a group of herbs in a composite formula, are a complex mixture containing hundreds of different chemical constituents responsible for their therapeutic effects. In this respect, the rapid screening and identification of constituents, particularly microconstituents in TCMs, is an integral part of the drug discovery and development process.

Although the separation and identification of compounds in TCMs using phytochemistry methods have developed, the analytical results demonstrate that numerous compounds have not as yet been investigated (Liu *et al*., 2009; Yang *et al*., 2012). Currently, future performance of large-scale analysis is greatly reliant on the continuous innovation of analytical techniques.

* Correspondence to: Jian-Qiu Lu, Center of Scientific Experiment, Beijing University of Chinese Medicine, Beijing 100029, China.
E-mail: lujq@vip.sina.com

** Correspondence to: Yan-Jiang Qiao, School of Chinese Pharmacy, Beijing University of Chinese Medicine, Beijing 100102, China.
E-mail: yanjiangqiao@sina.com

[a] Center of Scientific Experiment, Beijing University of Chinese Medicine, Beijing 100029, China

[b] School of Chinese Pharmacy, Beijing University of Chinese Medicine, Beijing 100102, China

[c] Nation Center for the Develepment of TCM Technology, Beijing 100027, China

As a result of the excellent ability to analyse multiconstituents in complex matrices, HPLC–ESI/MS^n has become a powerful approach for the rapid identification of constituents in TCM extracts (Zhang *et al.*, 2012a, 2012b, 2013a). In this regard, a new hybrid linear trap quadrupole (LTQ) and orbitrap analytical platform is applied to the analysis of small molecules in biological and TCM samples (Zhu *et al.*, 2007; Li *et al.*, 2012; Xu *et al.*, 2012). It consists of a two-dimensional ion trap coupled with an orbitrap, and allows two different scan types to be acquired simultaneously. The orbitrap mass spectrometer, otherwise defined as an electrostatic Fourier transform mass spectrometer, provides a higher mass resolution and mass accuracy than other mass spectrometers. The ion trap can provide multistage MS^n spectra using data-dependent analysis, and mass accuracies of 5 ppm can be obtained by the orbitrap scan in an external calibration mode. This advantage facilitates the identification of known and novel constituents in TCMs.

In previous reports, the detection of the TCM constituents using LC–MS involves the detection of parent ions and subsequent structural elucidation of constituents based on molecular weights, fragmentation pathways and/or elemental compositions. Obviously, it was quite tedious and error-prone to distinguish the relatively small signals from the complex matrices in full-scan mass chromatograms. Until now, only a few relevant strategies have been reported, such as the energy gradient neutral-loss scan strategy (Qu *et al.*, 2004), 'fragmentation–degradation' strategy for metabolic products (García-Reyes *et al.*, 2007) and '*de novo* identification' (Hao *et al.*, 2008), all of which have been limited to the structural elucidation of only one or several certain categories of compounds. We have recently described a modified strategy of diagnostic fragment-ion based coupled to diagnostic fragment-ion intensity analysis to rapidly screen and identify the serial constituents, especially the chlorogenic acid (CAG) isomers in Flos Lonicerae Japonicae, which greatly improved analysis efficiency (Zhang *et al.*, 2013b). However, this methodology was largely dependent on predefined fragment ions summarised from standards.

Mass-defect filtering (MDF), a data processing technique integrated in the MetWorks Software (Thermo Scientific, Bremen, Germany), presented a novel approach to tackling the problem. Initially, the MDF technique was developed for metabolites detection purposes based on a narrow and well-defined mass-defect range between the parent drug and metabolites. With the mass-defect window set at about ±50 mDa from that of the parent drug, a significant number of background interference ions can be removed and the metabolite profile of the biological sample can be obtained (Zhu *et al.*, 2006; Zhang *et al.*, 2009). Theoretically, compounds in TCMs could be classified into several families, which usually share similar carbon skeleton or substructures. Therefore, MDF also could be applied to detect herbal constituents (Yan *et al.*, 2010; Xie *et al.*, 2012). It was extremely important for certain homologue constituents to define a mass-defect window according to the proper filter reference and corresponding substituents. Such a mass-defect window can be applied to exclude the majority of irrelevant ions automatically and conveniently from the complex matrices. The resulting simplified data could facilitate the identification of structural analogues in TCMs. On the basis of built-in MDF technology, we developed a powerful stepwise MDF method with multiple mass-defect windows or multiple mass windows according to those of the MDF, which could rapidly capture much more characteristic structural analogues in complex herbal extracts.

In this study, the effectiveness of stepwise MDF was evaluated by analysing polymethoxylated flavonoids (PMFs) in the leaves of *Citrus reticulata* Blanco. The PMFs are a kind of the specific flavonoid subclasses with all, or almost all, hydroxyls capped by methylation, and they have high oral bioavailability (Li *et al.*, 2006; Maserejian *et al.*, 2006). Herein, a liquid chromatography coupled with full-scan high-resolution mass spectrometry (LC–HRMS) method on a HPLC–LTQ/orbitrap mass spectrometer was developed first. Then the stepwise MDF technique was used to screen the characteristic constituents in the complex extract. In this process, analysis of the peak selections and diagnostic product ions (DPIs) was applied to characterise the PMFs in the extract. In addition, results from the stepwise MDF were also compared with those from the MDF method on the same LTQ/orbitrap instrument. We have highlighted the practical and reliable qualities of the stepwise MDF approach in the rapid analysis of PMFs. Importantly, this methodology can be envisioned to exhibit a wide application for the identification of complicated compounds from various complex mixtures, including TCM extracts and other organic matter mixtures with compounds that can also be classified into families based on their common carbon skeletons.

Experimental

Reagents and materials

Eight PMF standards were purchased from the Modern Research Center of Traditional Chinese Medicines, Peking University, PR China, and identified in our laboratory for qualitative analysis (Fig. 1). The purities of all ingredients were determined to be no less than 95% according to HPLC/DAD analysis.

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). Deionised water used throughout the experiment was purified by a Milli-Q Gradient A 10 System (Millipore, Billerica,

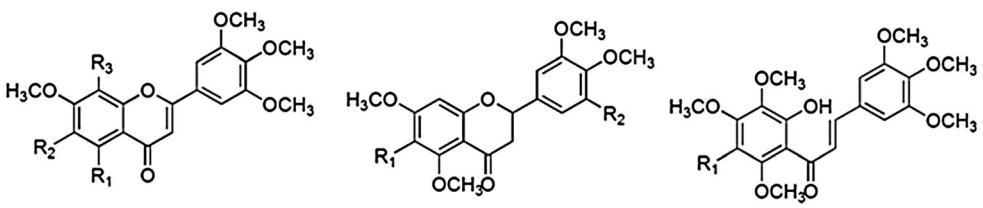


Figure 1. Structures of eight PMF reference standards.

MA, USA). The 0.22 μm microporous membranes were purchased from Xinjinghua Co. (Shanghai, China).

The leaves of *Citrus reticulata* Blanco were collected at random from trees in Tongzhou County, Beijing, China in October 2011. The leaves were deposited in a cool and dry place prior to analysis: their identity was authenticated by Professor Yan-Jiang Qiao, one of the authors, and a voucher specimen was deposited at the Center of Scientific Experiment, Beijing University of Chinese Medicine, Beijing, China.

Sample preparation for analysis

The dried leaves were powdered to a homogeneous size by a mill and sieved through a No. 40 mesh sieve. An amount of 0.5 g was extracted with 25 mL of methanol:water (70:30, v/v) in an ultrasonic bath (Elma Ultrasonics Corp., Singen, Germany) for 30 min at room temperature. The methanol solution was filtered through a 0.22 μm membrane before injection to the HPLC–MS system for analysis.

The HPLC analysis

A Thermo Scientific Accela 600 pump HPLC system was used in the experiment equipped with a binary pump and an autosampler. An Agilent Zorbax Extended C_{18} (250 × 4.6 mm i.d., 5 μm) was used for separation of the PMFs at room temperature. 0.1% formic acid aqueous solution (solvent A) and acetonitrile (solvent B) were used as the mobile phase. The flow rate was 1.0 mL/min and elution conditions at room temperature applied with a linear gradient were as follows: 0–5 min, 20–28% B; 5–70 min, 28–42% B; 70–90 min, 42–64% B.

The ESI/MS/MS analysis

High-resolution MS and MS/MS spectral analysis were performed on an LTQ/orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany). The mass spectrometer was connected to the HPLC instrument via an ESI interface in a post-column splitting ratio of 1:3. Samples were analysed in the positive ion mode with a tune method set as follows: sheath gas (nitrogen) flow rate of 30 AU, auxilary gas (nitrogen) flow rate of 5 AU, spray voltage of 4.0 kV, capillary temperature of 350°C, capillary voltage of 25 V and tube lens voltage of 110 V. Accurate mass analysis were calibrated according to the manufacturer's guidelines using a standard-solution mixture of caffeine, sodium dodecyl sulphate, sodium taurocholate, the tetrapeptide Met-Arg-Phe-Ala (MRFA) acetate salt and Ultramark (Sigma Aldrich). Centroided mass spectra were acquired in the mass range of *m/z* 100–900. The resolution of the orbitrap mass analyser was set at 30000 (full width at half maximum as defined at *m/z* 400). Data-dependent MS/MS scanning was performed to minimise total analysis time as it can trigger fragmentation spectra of target ions. The maximum injection time was 50 ms and there were two microscans. The collision energy for collision-induced dissociation (CID) was adjusted to 35% of maximum, and the isolation width of precursor ions was *m/z* 2.0 Da.

Stepwise MDF approach

The MDF is a new feature in the latest version of MetWorks Software. It can provide more distinct and specific information, which allows users to do faster, more sensitive and more accurate analysis. The stepwise MDF method developed used more mass defect windows or more mass windows in order to expose as many chromatographic peaks as possible from the total ion chromatograms (TIC).

It is well-known that structural analogues in herbs usually shared their similar core substructure with various chemical groups, including hydroxyls, formyls, methoxyls, methyls, glycosylations or a combination of these. Each substituent possesses relatively minor and distinctive variations in the mass of their detected core substructure. So the mass defect profile of the analogues usually changes within a limited range that canbe divided into suitable numbers of windows. Therefore, the determination of the core substructure with different substituent combinations and number of filtering windows were the two essential requirements to realise the stepwise MDF method.

According to the theory outlined above, the first stage of the stepwise MDF was to establish the filtering reference based on all the structures of the published constituents. Then the mass defect and mass ranges according to the substitution of various constitutes were confirmed and divided into proper segments. The respective MDF chromatograms could be obtained according to the setting of filtering windows. Once the filtering setting was applied to the TIC chromatogram, the heterogeneous ions could be removed and characteristic ions remained visible.

Peak selections and data processing

The Thermo Xcaliber 2.1 workstation was used for data acquisition and processing. In order to obtain as many product ions as possible, the peaks detected with intensities over 10000 were selected for identification. The chemical formulae for all parent and product ions of the selected peaks were calculated from the accurate mass using a formula predictor by setting the parameters as follows: C (0–30), H (0–50), O (0–20) and ring double bond (RDB) equivalent value (0–15). Other elements such as N, P, S, Cl and Br were not considered because they are rarely present in the leaves of *Citrus reticulata* Blanco.

Fragmentation mechanisms analysis

Data analysis software (Mass Frontier 7.0, Thermo Scientific) was used to confirm manual elucidation of mechanisms and fragment ion structures. Mass Frontier predicts and displays comprehensive fragmentation pathways on the basis of a set of general ionisation, fragmentation and rearrangement rules, and by automatically extracting a decomposition mechanism for each fragmentation reaction in the fragmentation library that was operated in the positive ion electrospray mode. The general procedures of our strategy and approach are summarised in the diagram shown in Fig. 2.

Results and discussion

Peak detection in the extract by HPLC–LTQ/orbitrap/MS

After the optimisation of extraction procedure and sample preparation, a simple HPLC–LTQ/orbitrap/MS method was developed to detect constituents in the extract. The TIC is shown in Fig. 3A, and most of the constituents were well separated under the gradient elution condition with high resolution and good sensitivity. However, signals derived from matrices made finding characteristic compounds extremely challenging, and only 23 obvious peaks could be distinguished from the background, which meant the $[M+H]^+$ ions of many constituents of low abundance were possibly submerged in the background ions and led to easily missing the compounds of interest during the manual inspection.

Establishment of the stepwise MDF approach to detect the characteristic constituents

In order to reduce the potential interferences of matrix ions and identify the characteristic compounds rapidly and globally, the stepwise MDF was applied to capture the characteristic peaks.

Based on the concept of the stepwise MDF, the first stage was the definition of the filter reference according to the core substructure. The PMFs including polymethoxyflavones, polymethoxyflavanones and polymethoxychalcones have a common skeleton of dimethoxylflavanone with a maximum seven substituents on their A, B and C rings. It should be pointed out that

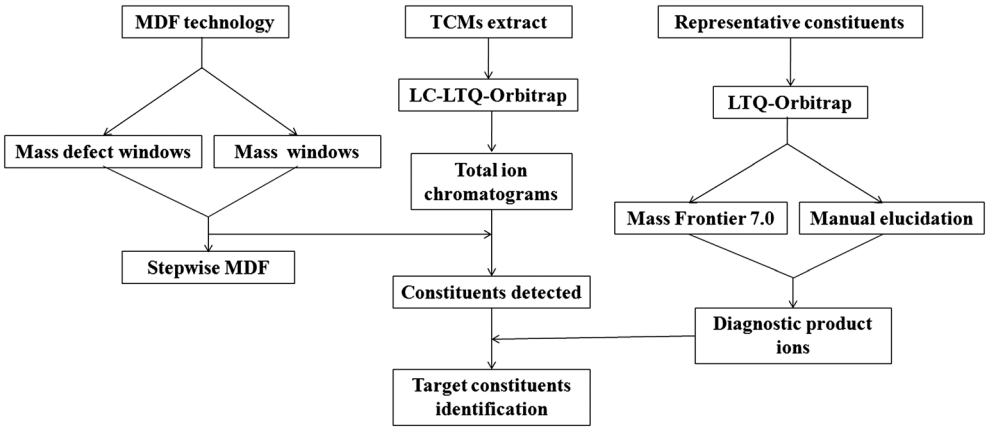


Figure 2. Summary diagram of the developed strategy and methodology.

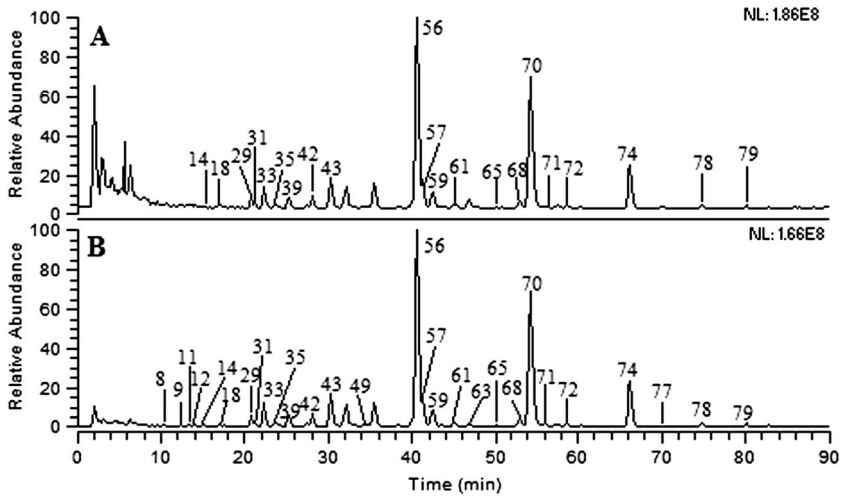
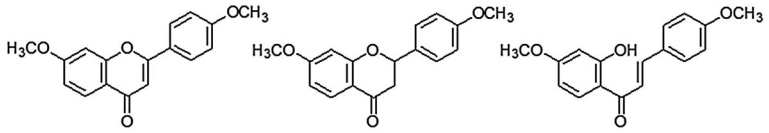


Figure 3. The unfiltered TIC obtained by (A) HPLC–LTQ/orbitrap/MS and (B) the corresponding filtered chromatogram of PMFs obtained by MDF.

chalcones could be considered as flavanones because they have the same molecular weights. Typically, the substituents of PMFs were predominantly found to be hydroxyls, methoxyls and desaturation. According to the summary of the mass defects of the various substituents, methoxyl groups generated the increase of mass defects; whereas hydroxyl groups generated a decrease. Therefore, the maximum number of five hydroxyl groups was assigned to obtain the minimum value of mass defect corresponding to $C_{17}H_{17}O_9$ and five methoxyl groups was assigned to obtain the maximum value corresponding to $C_{22}H_{27}O_9$. The calculated mass defect range was from 0.070 to 0.1660 and the filter setting was determined as dimethoxylflavanone over the mass range of 282–436 Da (Fig. 4). The MDF method when first adopted picked out 30 PMF peaks from the TIC chromatogram (Fig. 3B), which demonstrated that MDF could provide a little more information. In the stepwise MDF method, the mass-defect range or mass range was divided equally into two to five windows to achieve the generated MDF chromatograms, respectively. The results demonstrated that stepwise MDF with five mass defect windows or mass windows within the mass defect range or mass range could expose more PMF peaks than the MDF method alone (see Fig. S1 in the online Supporting information). The noise levels of the filtered chromatograms by the stepwise MDF with five mass-defect windows were between about 2.40×10^5 and 1.38×10^6 counts per seconds (cps, Fig. 5), and were lower than those of the original chromatogram (1.86×10^6 cps) and MDF chromatogram (1.64×10^6 cps). Meanwhile, the noise levels of the filtered chromatogram by the stepwise MDF with five mass windows were between about 1.32×10^4 and 1.20×10^6 cps (Fig. 6). Therefore, stepwise MDF with multiple mass-defect windows or mass windows could decrease the noise level of the matrix more than the traditional MDF method. More importantly, 69 and 66 PMF candidates were, respectively, distinguished by stepwise MDF with five mass-defect windows or mass windows (see Table S1 in the online Supporting information), which were much more than the number of constituents detected in the MDF chromatogram and TIC chromatogram.

Determinations of DPIs and fragmentation patterns analysis for PMFs

In order to perform structural identification of the PMF candidates screened by the stepwise MDF from the extract of the leaves of *C. reticulata* Blanco, eight PMF standards were analysed by HPLC–ESI/LTQ/orbitrap/MS. All the PMF standards, including four polymethoxyflavones, two polymethoxyflavanones and two polymethoxychalcones, exhibited $[M+H]^+$ ions of sufficient intensity that could be subsequently isolated automatically and subjected to CID/MS/MS analysis (Table 1). The DPIs from the



Flavone:$C_{17}H_{15}O_4$ *m/z* 283.09702 mass defect: 97.02 mmu

Flavanone:$C_{17}H_{17}O_4$ *m/z* 285.11267 mass defect: 112.67 mmu

Chalcone:$C_{17}H_{17}O_4$ *m/z* 285.11267 mass defect: 112.67 mmu

Substituent	Number		Flavone			Flavanone/Chalcone		
	min	max	Formula[M+H]$^+$ (max)	*m/z* [M+H]$^+$ max (amu)	Mass defect (mmu)	Formula[M+H]$^+$ (max)	*m/z* [M+H]$^+$ max (amu)	Mass defect (mmu)
Hydroxyl	0	×5	$C_{17}H_{15}O_9$	363.07159	71.06	$C_{17}H_{17}O_9$	365.08724	87.24
Methoxyl	0	×5	$C_{22}H_{25}O_9$	433.14984	149.31	$C_{22}H_{27}O_9$	435.16549	165.49

Figure 4. The proposed MDF approach with predefined filter reference and substituents.

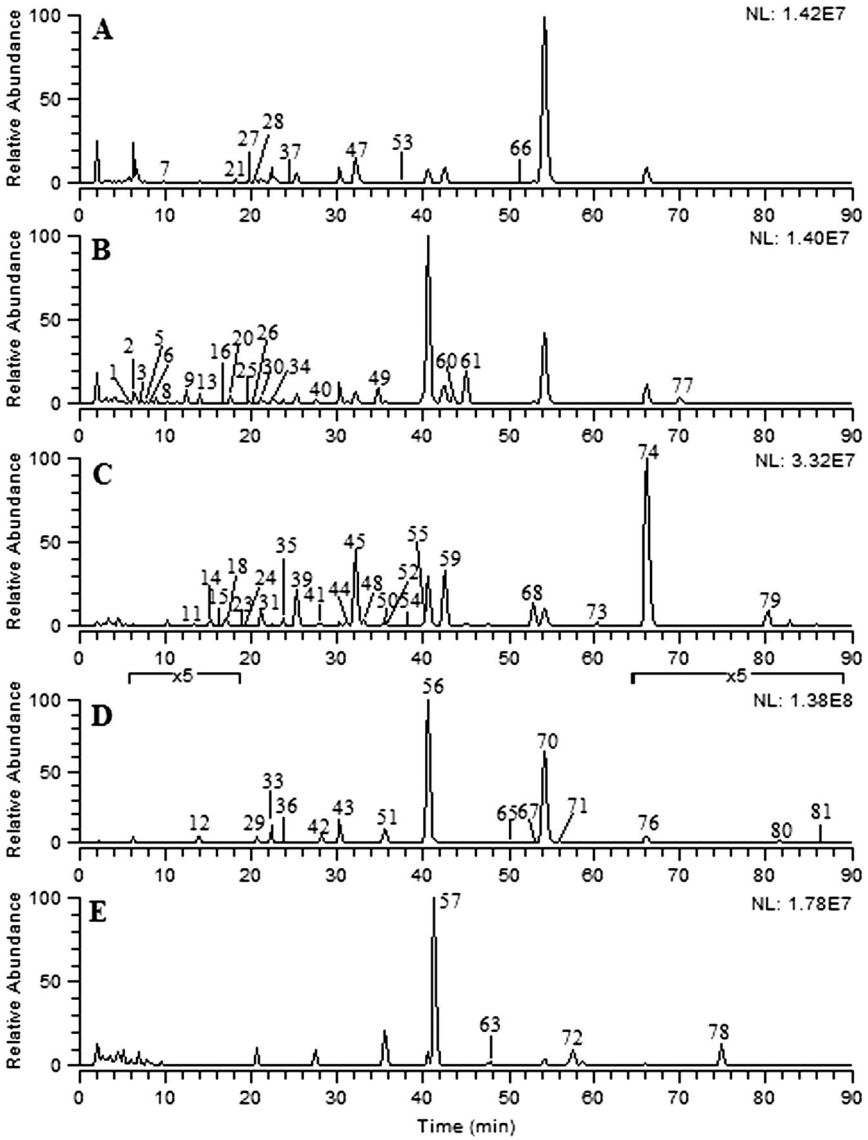


Figure 5. The corresponding filtered chromatograms of PMFs obtained by stepwise MDF with multiple mass-defect windows set as follows: (A) 70.0–89.2 mmu; (B) 89.2–108.4 mmu; (C) 108.4–127.6 mmu; (D) 127.6–146.8 mmu; (E) 146.8–166.0 mmu.

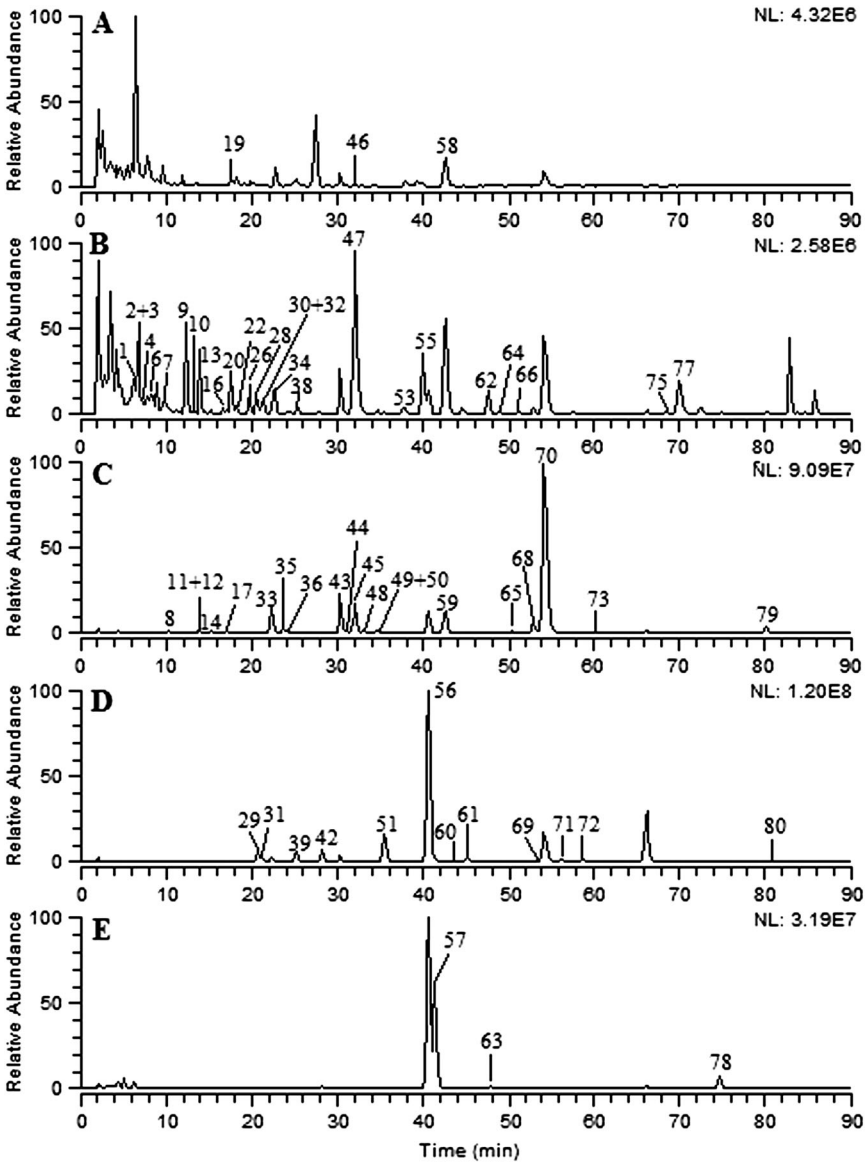


Figure 6. The corresponding filtered chromatograms of PMFs obtained by stepwise MDF with multiple mass windows set as follows: (A) 282–312 amu; (B) 312–343 amu; (C) 343–374 amu; (D) 374–404 amu; (E) 404–436 amu.

proposed fragmentation patterns from the Mass Frontier 7.0 software and manual elucidation made for the structural identification of PMFs in the extracts. The nomenclature commonly used for mass products of flavonoids was adopted in this work (Domon and Costello, 1988).

Four polymethoxyflavone standards were subsequently analysed first in the CID/MS/MS experiment. By comparison of their product ion spectra, some characteristic dissociation pathways could be summarised for further characterisation of the other polymethoxyflavones. First, all of the $[M+H]^+$ ions could lose one or more methyl radicals ($CH_3^{\bullet}$) in their ESI/MS^2 spectra, and formed the base peaks of $[M+H-15]^+$, $[M+H-30]^+$ and $[M+H-45]^+$. Second, the other dissociation pathways of $[M+H]^+$ by loss of 16 (CH_4), 18 (H_2O), 28 (CO), 29 ($HCO^{\bullet}$), 31 ($OCH_3^{\bullet}$), 33 ($H_2O+CH_3^{\bullet}$), 43 ($CH_3^{\bullet}+CO$), 44 ($HCO^{\bullet}+CH_3^{\bullet}$), 46 ($H_2O+CO$), 48 ($2CH_3^{\bullet}+H_2O$), 59 ($2CH_3^{\bullet}$) and 61 ($H_2O+CO+CH_3^{\bullet}$) were detected as DPIs in their MS^n spectra. These main product ions mentioned above could form the ESI/MS^n DPIs of polymethoxyflavones for rapid screening and their identification from various complex TCM extracts.

In the CID/MS/MS experiment, the fragmentation pathways of two polymethoxyflavanone derivatives (**5** and **6**) were similar to each other. For example, compound **6** gave the $[M+H]^+$ ion at *m/z* 375.1442 ($C_{20}H_{23}O_7$) in its ESI/MS spectrum, which further generated the prominent ion at *m/z* 221.0813 ($C_{12}H_{13}O_4$) as the base peak in its MS^2 spectrum. It could be deduced that its dominating fragmentation pathway was Retro-Diels-Alder (RDA) cleavage from the 1,4-position of the C-ring. Meanwhile, the minor ion at *m/z* 181.0501 ($C_9H_9O_4$) was also detected, owing to the RDA fragmentation from the 1,3-position of the C-ring. The loss of 15 ($CH_3^{\bullet}$), 28 (CO), 30 ($2CH_3^{\bullet}$) and 31 ($OCH_3^{\bullet}$) from the base peak could also generate a series of DPIs for polymethoxyflavanones in their MS^n spectra. This kind of fragmentation pathway where the $[M+H]^+$ ions underwent RDA reaction prior to the neutral loss of $CH_3^{\bullet}$, H_2O, CO, etc., was strikingly different from common flavanones. Therefore,

Table 1. Characterisations of eight PMFs standards by CID/MS/MS

Peak	Experimental mass *m/z*	Empirical formula	Mass error ppm	MS^2 (*m/z*)				MS^3 (*m/z*)			
				P-ion (%)[a]		ppm	Radical loss	P-ion (%)[a]		ppm	Radical loss
1	403.1391	$C_{21}H_{23}O_8$	0.9	373.0928* (100)	$C_{19}H_{17}O_8$	2.7	$2CH_3^{\cdot}$	345.0978 (100)	$C_{18}H_{17}O_7$	2.7	CO
				388.1164 (50.8)	$C_{20}H_{20}O_8$	2.9	$CH_3^{\cdot}$	340.0588 (55.5)	$C_{18}H_{12}O_7$	3.1	$H_2O + CH_3^{\cdot}$
				342.1112 (40.1)	$C_{19}H_{18}O_6$	4.1	$H_2O + CO + CH_3^{\cdot}$	358.0695 (25.9)	$C_{18}H_{14}O_8$	3.3	$CH_3^{\cdot}$
								312.0638 (23.1)	$C_{17}H_{12}O_6$	3.1	$H_2O + CO + CH_3^{\cdot}$
								343.0457 (18.6)	$C_{17}H_{11}O_8$	2.5	$2CH_3^{\cdot}$
2	403.1391	$C_{21}H_{23}O_8$	0.9	342.1108* (100)	$C_{19}H_{18}O_6$	2.9	$H_2O + CO + CH_3^{\cdot}$	327.0867 (100)	$C_{18}H_{15}O_6$	1.2	$CH_3^{\cdot}$
				388.1162 (45.9)	$C_{20}H_{20}O_8$	2.4	$CH_3^{\cdot}$	281.0812 (54.8)	$C_{17}H_{13}O_4$	1.3	$H_2O + CO + CH_3^{\cdot}$
				373.0928 (28.3)	$C_{19}H_{17}O_8$	2.7	$2CH_3^{\cdot}$	309.0762 (27.7)	$C_{18}H_{13}O_5$	1.5	$H_2O + CH_3^{\cdot}$
				359.1135 (25.3)	$C_{19}H_{19}O_7$	2.7	$HCO^{\cdot} + CH_3^{\cdot}$	312.0633 (13.8)	$C_{17}H_{12}O_6$	1.5	$2CH_3^{\cdot}$
3	359.1129	$C_{19}H_{19}O_7$	1.0	344.0895* (100)	$C_{18}H_{16}O_7$	1.3	$CH_3^{\cdot}$	315.0873 (100)	$C_{17}H_{15}O_6$	3.1	$HCO^{\cdot}$
				315.0871 (16.9)	$C_{17}H_{15}O_6$	2.5	$HCO^{\cdot} + CH_3^{\cdot}$	328.0585 (64.6)	$C_{17}H_{12}O_7$	2.3	CH_4
								326.0794 (26.8)	$C_{18}H_{14}O_6$	2.8	H_2O
								299.0559 (19.8)	$C_{16}H_{11}O_6$	3.0	$H_2O + CH_3^{\cdot}$
4	389.1237	$C_{20}H_{21}O_8$	1.6	374.1004* (100)	$C_{19}H_{18}O_8$	2.1	$CH_3^{\cdot}$	345.0974 (100)	$C_{18}H_{17}O_7$	1.5	$HCO^{\cdot}$
				328.0950 (95.2)	$C_{18}H_{16}O_6$	2.6	$H_2O + CO + CH_3^{\cdot}$	312.0633 (78.2)	$C_{17}H_{12}O_6$	1.5	$HCO^{\cdot} + CH_3^{\cdot} + H_2O$
				359.0772 (25.1)	$C_{18}H_{15}O_8$	3.0	$2CH_3^{\cdot}$	358.0689 (29.3)	$C_{18}H_{14}O_8$	1.6	CH_4
				345.0976 (15.2)	$C_{18}H_{17}O_7$	2.1	$HCO^{\cdot} + CH_3^{\cdot}$	356.0895 (27.6)	$C_{19}H_{16}O_7$	1.3	H_2O
				356.0901 (14.8)	$C_{19}H_{16}O_7$	2.9	$H_2O + CH_3^{\cdot}$	341.0662 (26.9)	C18H13O7	1.8	$H_2O + CH_3^{\cdot}$
								359.0766 (26.1)	$C_{18}H_{15}O_8$	1.3	$CH_3^{\cdot}$
5	375.1442	$C_{20}H_{23}O_7$	1.0	211.0609* (100)	$C_{10}H_{11}O_5$	3.8	$^{1,3}A^{+}$ [b]	196.0372 (100)	$C_9H_8O_5$	2.9	$CH_3^{\cdot}$
				191.0710 (42.9)	$C_{11}H_{11}O_3$	3.8	$^{1,4}B^{+}$ [b]	178.0267 (24)	$C_9H_6O_4$	3.6	$H_2O + CH_3^{\cdot}$
				357.1348 (19.8)	$C_{20}H_{21}O_6$	4.3	H_2O	183.0659 (8.6)	$C_9H_{11}O_4$	3.9	CO
6	375.1442	$C_{20}H_{23}O_7$	1.0	221.0813* (100)	$C_{12}H_{13}O_4$	2.1	$^{1,4}B^{+}$ [b]	193.0862 (100)	$C_{11}H_{13}O_3$	1.5	CO
				181.0501 (16.9)	$C_9H_9O_4$	3.1	$^{1,3}A^{+}$ [b]	190.0628 (54.5)	$C_{11}H_{10}O_3$	1.9	$OCH_3^{\cdot}$
				357.1343 (8.1)	$C_{20}H_{21}O_6$	2.9	H_2O	206.0578 (45.9)	$C_{11}H_{10}O_4$	2.1	$CH_3^{\cdot}$
								191.0344 (37.5)	$C_{10}H_7O_4$	2.7	$2\ CH_3^{\cdot}$
7	405.1543	$C_{21}H_{25}O_8$	−0.2	221.0812* (100)	$C_{12}H_{13}O_4$	1.7	$^{X}B^{+}$ [c]	193.0862 (100)	$C_{11}H_{13}O_3$	1.5	CO
				387.1447 (32.1)	$C_{21}H_{23}O_7$	2.3	H_2O	190.0628 (52.1)	$C_{11}H_{10}O_3$	1.9	$OCH_3^{\cdot}$
				211.0608 (37.9)	$C_{10}H_{11}O_5$	3.3	$^{Y}A^{+}$ [c]	206.0578 (42.3)	$C_{11}H_{10}O_4$	2.1	$CH_3^{\cdot}$
								191.0344 (39.2)	$C_{10}H_7O_4$	2.7	$2CH_3^{\cdot}$
8	405.1543	$C_{21}H_{25}O_8$	−0.2	221.0815* (100)	$C_{12}H_{13}O_4$	3.0	$^{X}B^{+}$ [c]	193.0865 (100)	$C_{11}H_{13}O_3$	3.0	CO
				387.1451 (32.5)	$C_{21}H_{23}O_8$	3.3	H_2O	190.0631 (53.6)	C11H10O3	3.4	$OCH_3^{\cdot}$
				211.0610 (25.3)	$C_{10}H_{11}O_5$	4.3	$^{Y}A^{+}$ [c]	206.0581 (48.3)	$C_{11}H_{10}O4$	3.6	$CH_3^{\cdot}$
								191.0347 (39.1)	$C_{10}H_7O_4$	4.3	$2CH_3^{\cdot}$

[a]P-ion (%), the product ions and the relative intensity.
[b]$^{1,3}A^{+}$, $^{1,4}B^{+}$ stand for the fragment ions from the RDA cleavage from 1,3-position on the C-ring of flavanones.
[c]$^{Y}A^{+}$, $^{X}B^{+}$ the fragment ions from the RDA cleavage from the C-ring of chalcones.
* Precursor-ion for next stage MS.

Table 2. The structural identification of 81 PMFs detected in the leaves of *Citrus reticulata* Blanco

Categories	Peaks	Amounts	PMFs
1	19, 46, 58	3	Monohydroxy-dimethoxyflavone
2	55	1	Trimethoxyflavone
3	3, 7, 47, 53, 66	5	Dihydroxy-dimethoxyflavone
4	62	1	Trimethoxychalcone **or** Trimethoxyflavanone
5	64	1	Dihydroxy-dimethoxychalcone **or** Dihydroxy-dimethoxyflavanone
6	9, 13, 16, 20, 34, 38, 77	7	Monohydroxy-trimethoxyflavone
7	21, 22, 27, 28, 37	5	Trihydroxy-dimethoxyflavone
8	10, 32, 75	3	Monohydroxy-trimethoxychalcone **or** Monohydroxy-trimethoxyflavanone
9	1, 2, 4, 6, 26, 30	6	Trihydroxy-dimethoxychalcone **or** Trihydroxy-dimethoxyflavanone
10	41, 45, 59	3	Tetramethoxyflavone
11	5, 49	2	Dihydroxy-trimethoxyflavone
12	67	1	Tetramethoxyflavanone **or** Tetramethoxychalcone
13	48, 50, 52	3	Dihydroxy-trimethoxyflavanone **or** Dihydroxy-trimethoxychalcone
14	8, 11, 14, 18, 23, 24, 35, 44, 68, 73, 79	11	Monohydroxy-tetramethoxyflavone
15	25, 40	2	Trihydroxy-trimethoxyflavone
16	12, 17, 36, 65	4	Monohydroxy-tetramethoxyflavanone **or** Monohydroxy-tetramethoxychalcone
17	33, 43, 70	3	Pentamethoxyflavone
18	29, 51, 72, 76, 80, 81	6	Pentamethoxyflavanone **or** pentamethoxychalcone
19	60, 61	2	Dihydroxy-tetramethoxyflavone
20	15, 54	2	Dihydroxy-Tetramethoxychalcone **or** Dihydroxy-Tetramethoxyflavanone
21	31, 39, 74	3	Monohydroxy-pentamethoxyflavone
22	42,71	2	Monohydroxy-pentamethoxyflavanone **or** Monohydroxy-pentamethoxychalcone
23	56	1	Hexamethoxyflavone
24	57,78	2	Hexamethoxychalcone **or** Hexamethoxyflavanone
25	69	1	Monohydroxy-hexamethoxyflavone
26	63	1	Heptamethoxyflavone

this particular pathway and DPIs could be adopted as a shortcut to rapidly distinguish polymethoxyflavanones from common flavanones.

Compounds **7** and **8** attributed to polymethoxychalcones were also analysed by the CID/MS/MS experiments. Their dissociation pathways were similar on the whole. Taking compound **7**, for example, the RDA cleavage at bond X of its $[M+H]^+$ ion (405.1543, $C_{21}H_{25}O_8$) to yield the base peak ion $^{X}B^+$ at *m/z* 221.0812 ($C_{12}H_{13}O_4$) and at bond Y to yield the minor ion $^{Y}A^+$ at *m/z* 211.0608 ($C_{10}H_{11}O_5$) could also be simultaneously detected in its positive MS^2 spectrum. This kind of fragmentation pathway was very similar to what happened to polymethoxyflavanones. This is reasonable because cyclisation of 6′-hydroxychalcones to flavanones has been reported in many studies demonstrating an intramolecular equilibrium being present between a flavanone-type and a chalcone-type molecular ion. Meanwhile, the product ions detected from the loss of 15 ($CH_3^{\bullet}$), 16 (CH_4), 18 (H_2O), 28 (CO), 30 ($2CH_3^{\bullet}$) and 31 ($OCH_3^{\bullet}$) also could be adopted as DPIs for polymethoxychalcones.

HPLC–LTQ/orbitrap/MS analysis of the PMFs in the herbal extract

The capability of MS^n fragmentation and mass accuracy of each MS^n stage made HPLC–LTQ/orbitrap/MS an attractive tool for structural identification. The effectiveness of the stepwise MDF approach using the core substructure as the filter reference was well demonstrated for screening PMFs in the leaves of *C. reticulata* Blanco. Sixty-nine PMF candidates were distinguished after filtering by the stepwise MDF with multiple mass-defect windows, and 66 candidates were distinguished by the stepwise MDF with multiple mass windows. Accurate mass (< 5 ppm) and DPI analyses were then applied to validate the PMF candidates. It was anticipated that a total of 81 peaks attributed to PMFs would be found in a 90 min chromatographic run from the large quantity of information data available beforehand by the stepwise MDF (Table S1). Based on the results of structural inference, the coverage of PMFs from the stepwise MDF with multiple mass-defect windows was 85.19%, while the coverage of the stepwise MDF with multiple mass windows was 81.48%. In contrast, the coverage of the original TIC and MDF methods were just 28.40% and 37.04%, respectively.

Meanwhile, 81 PMF candidates were tentatively identified as 50 polymethoxyflavones and 31 polymethoxyflavanones or polymethoxychalcones according to their respective fragmentation pathways and DPIs (Table 2). It is possible to go into a deep discussion of the results with regard to the possible substitution patterns of detected PMFs. However, for polymethoxylated flavones, the chances of RDA cleavage taking place are rare during the MS/MS fragmentations. As for polymethoxylated flavanones and chalcones, because it was difficult to define the

substituents on ring C, the number of methoxyls and hydroxyls on ring A and ring B could not be defined. Therefore, they were preliminarily identified as M-hydroxy-N-methoxyflavone, flavanone or chalcone (where M and N stand for mono, di, tri, tetra, penta, hexa or hepta).

Furthermore, hydroxylated polymethoxyflavonoids (OH-PMFs) have drawn more and more attention in recent years because accumulating evidence has suggested that they have much stronger health-promoting biological activities compared with their permethoxylated counterparts (Pan *et al*., 2007; Xiao *et al*., 2009). Additionally, OH-PMFs are even more rare in the medicinal plants. In this study, using the stepwise MDF method, 63 hydroxylated polymethoxyflavonoids have been screened out and identified from the leaves of *C. reticulata* Blanco, including 42 hydroxylated polymethoxyflavones, 21 hydroxylated polymethoxyflavanones or hydroxylated polymethoxychalcones, which indicated that the leaves of *C. reticulata* Blanco could be adopted as one kind of forward-looking anticancer medicine.

Conclusions

In conclusion, a highly sensitive and effective strategy for rapid screening and identification of structural analogues from complex herbal extract has been developed using a stepwise MDF approach coupled to DPI analysis on a hybrid LTQ/Orbitrap mass spectrometer. This is the first report on the chemical analysis of TCMs using such a strategy. Compared with the conventional manual inspection and fragmentation-based method, the stepwise MDF approach with multiple mass-defect windows or mass windows enabled the original data to be analysed much faster and more accurately by reducing the potential interferences of matrix ions. Therefore, it can search for a greater number of potential active compounds from the original data, thereby increasing the coverage of constituent screening. Moreover, this method could provide much more information about target constituents than the traditional MDF method. Additionally, DPI analysis can provide a criterion to classify the target compounds detected into certain chemical families. In the study, eight PMF standards were analysed by CID/MS/MS to obtain the respective fragmentation pathways and DPIs for polymethoxyflavones, polymethoxyflavanones and polymethoxychalcones, which could be taken as the basis for further analysis of the PMFs in the extract. As a result, a total of 81 PMFs including 50 polymethoxyflavones and 31 polymethoxyflavanones or polymethoxychalcones were screened and identified from the leaves of *C. reticulata* Blanco after the stepwise MDF filtering, *m/z* value screening and DPI analysis. More importantly, it is possible for the stepwise MDF methodology to be extended to elucidating compounds from other organic matter mixtures, such as substance analysis in vegetables, water-quality analysis, natural organic-matter analysis in soil, pesticide multiresidue analysis in food, and so on, with the view that the compounds contained in such matrices also can be classified into families based on the common carbon skeletons.

Acknowledgements

The authors greatly appreciate the financial support from National S & T Major Project and Created Major New Drugs Project (No. 2010ZX09502-002) and China Postdoctoral Science Foundation (No. 2013M530563).

REFERENCES

Domon B, Costello CE. 1988. A systematic nomenclature of carbohydrate fragmentation in FAB-MS/MS spectra of glycoconjugates. *Glycoconj J* **5**: 397–409.

García-Reyes JF, Molina-Díaz A, Fernandez-Alba AR. 2007. Identification of pesticide transformation products in food by liquid chromatography/time-of-flight mass spectrometry via 'fragmentation-degradation' relationships. *Anal Chem* **79**: 307–321.

Hao HP, Cui N, Wang GJ, Xiang BR, Liang Y, Xu XY, Zhang H, Yang J, Zheng CN, Wu L, Gong P, Wang W. 2008. Global detection and identification of nontarget components from herbal preparations by liquid chromatography hybrid ion trap time-of-flight mass spectrometry and a strategy. *Anal Chem* **80**: 8187–8194.

Li RW, Theriault AG, Au K, Douglas TD, Casaschi A, Kurowska EM, Mukherjee R. 2006. Citrus polymethoxylated flavones improve lipid and glucose homeostasis and modulate adipocytokines in fructose-induced insulin resistant hamsters. *Life Sci* **79**: 365–373.

Li X, Zhang YF, Yang L, Feng Y, Deng YH, Liu YM, Zeng X. 2012. Chemical profiling of constituents of *Smilacis glabrae* using ultra-high pressure liquid chromatography coupled with LTQ Orbitrap mass spectrometry. *Nat Prod Commun* **7**: 181–184.

Liu YY, Li JB, He JM, Zeper A, Qu J, Yu SS, Ma SG, Liu J, Du D. 2009. Identification of new trace triterpenoid saponins from the roots of *Panax notoginseng* by high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry. *Rapid Commun Mass Spectrom* **23**: 667–679.

Maserejian NN, Giovannucci E, Rosner B, Zavras A, Joshipura K. 2006. Prospective study of fruits and vegetables and risk of oral premalignant lesions in men. *Am J Epidemiol* **164**: 556–566.

Normile D. 2003. The new face of Chinese medicine. *Science* **299**: 188–190.

Pan MH, Lai YS, Lai CS, Wang YJ, Li SM, Lo CY, Dushenkov S, Ho CT. 2007. 5-Hydroxy-3, 6, 7, 8, 3′, 4′-hexamethoxyflavone induces apoptosis through reactive oxygen species production, growth arrest and DNA damage-inducible gene 153 expression, and caspase activation in human leukemia cells. *J Agric Food Chem* **55**: 5081–5091.

Qu J, Liang QL, Luo GA, Wang YM. 2004. Screening and identification of glycosides in biological samples using energy-gradient neutral loss scan and liquid chromatography tandem mass spectrometry. *Anal Chem* **76**: 2239–2247.

Xiao H, Yang CS, Li SM, Jin HY, Ho CT, Patel T. 2009. Monodemethylated polymethoxyflavones from sweet orange (*Citrus sinensis*) peel inhibit growth of human lung cancer cells by apoptosis. *Mol Nutr Food Res* **53**: 398–406.

Xie T, Liang Y, Hao H, A J, Xie L, Gong P, Dai C, Liu L, Kang A, Zheng X, Wang G. 2012. Rapid identification of ophiopogonins and ophiopogonones in *Ophiopogon japonicus* extract with a practical technique of mass defect filtering based on high resolution mass spectrometry. *J Chromatogr A* **1227**: 234–244.

Xu W, Zhang J, Huang ZH, Qiu XH. 2012. Identification of new dianthrone glycosides from *Polygonum multiflorum* Thunb. using high-performance liquid chromatography coupled with LTQ-Orbitrap mass spectrometry detection: a strategy for the rapid detection of new low abundant metabolites from traditional Chinese medicines. *Anal Methods* **4**: 1806–1812.

Xue TH, Roy R. 2003. Studying Traditional Chinese Medicine. *Science* **300**: 740–741.

Yan G, Sun H, Sun W, Zhao L, Meng X, Wang X. 2010. Rapid and global detection and characterization of aconitum alkaloids in Yin Chen Si Ni Tang, a traditional Chinese medical formula, by ultra performance liquid chromatography-high resolution mass spectrometry and automated data analysis. *J Pharm Biomed Anal* **53**: 421–431.

Yang WZ, Ye M, Qiao X, Liu CF, Miao WJ, Bo T, Tao HY, Guo DA. 2012. A strategy for efficient discovery of new natural compounds by integrating orthogonal column chromatography and liquid chromatography/mass spectrometry analysis: its application in *Panax ginseng*, *Panax quinquefolium* and *Panax notoginseng* to characterize 437 potential new ginsenosides. *Anal Chim Acta* **739**: 56–66.

Zhang HY, Zhang DL, Ray KL, Zhu MS. 2009. Mass defect filter technique and its applications to drug metabolite identification by high-resolution mass spectrometry. *J Mass Spectrom* **44**: 999–1016.

Zhang JY, Li N, Zhou Y, Jiang Y, Tu PF. 2012a. Simultaneous qualitative and quantitative determination of major polymethoxylated flavonoid in the leaves of *Murraya paniculata* by RRLC-DAD-ESI-MS^n. *Anal Methods* **4**: 3399–3406.

Zhang JY, Zhang Q, Zhang HX, Ma Q, Lu JQ, Qiao YJ. 2012b. Characterization of polymethoxylated flavonoids (PMFs) in the peels of 'Shatangju' mandarin (*Citrus reticulata* Blanco) by online high-performance liquid chromatography coupled to photodiode array detection and electrospray tandem mass spectrometry. *J Agric Food Chem* **60**: 9023–9034.

Zhang JY, Lu JQ, Gao XY, Zhang Q, Ling N, Tu PF. 2013a. Characterization of thirty-nine polymethoxylated flavonoids (PMFs) in the branches of *Murraya paniculata* by HPLC-DAD-ESI-MS/MS. *Chin J Nat Med* **11**: 63–70.

Zhang JY, Zhang Q, Ling N, Wang ZJ, Lu JQ, Qiao YJ. 2013b. 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. *Talanta* **104**: 1–9.

Zhu MS, Ma L, Zhang DL, Ray KL, Zhao WP, Humphreys WG, Sanders M, Zhang HY. 2006. Detection and characterization of metabolites in biological matrices using mass defect filtering of liquid chromatography/high resolution mass spectrometry data. *Drug Metab Dispos* **34**: 1722–1733.

Zhu MS, Ma L, Zhang HY, Humphreys WG. 2007. Detection and structural characterization of glutathione-trapped reactive metabolites using liquid chromatography-high-resolution mass spectrometry and mass defect filtering. *Anal Chem* **79**: 8333–8341.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site.