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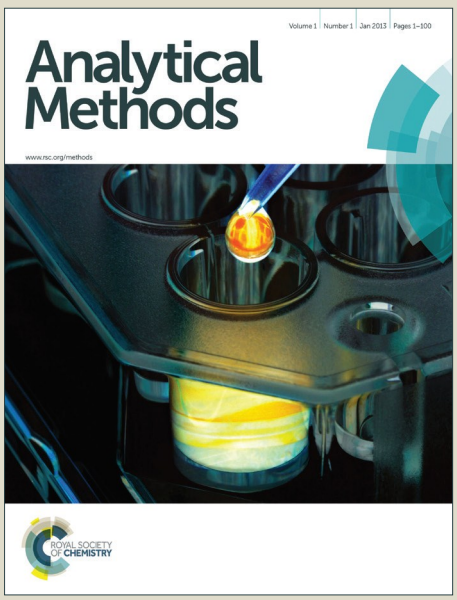

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A practical method for the rapid detection and structural characterization of major constituents from traditional Chinese medical formulas: Analysis Multiple Constituents in Yinchenhao Decoction

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Abstract:

The components of traditional Chinese medical formulas (TCMFs) are usually extremely complex, belonging to hundreds of compound classes with diverse chemical and physical properties. Rapid and effective characterization of TCMFs is imperative in order to provide a first step toward determining which components are responsible for the therapeutic effects of a particular medical plant. A practical method based on Find by Formula (FBF) algorithms is described which provides the rapid detection and structural characterization of major constituents from TCMFs. Results are presented by analysis a classical TCMFs of Yinchenhao Decoction (YCHD). The data of the YCHD extract was acquired using high performance liquid chromatography/quadrupole time-of-flight mass spectrometry (HPLC-Q/TOF-MS/MS) system which features high resolution, mass accuracy, and sensitivity. Based on a prepared simple database file and requisite filter template, the FBF approach was developed and employed to rapidly pick out the peaks of target compounds from mass chromatograms and the particular FBF spectrum with its MS/MS information were listed simultaneously. By this approach, 77 compounds from YCHD were rapidly characterized. The present results demonstrate that this strategy possesses the enormous advantage. This approach is adaptable to analyze complex components from TCMFs and even available to investigate all potential metabolites of one compound with specific molecular formula.

Keywords: Structural characterization; Yinchenhao Decoction; FBF approach; HPLC-Q/TOF-MS/MS

Introduction

The rapid detection and structural characterization of the major constituents have become an integral part of quality control and research on active principles of traditional Chinese medical formulas (TCMFs) in order to promote the modernization that move toward internationalization [1-3]. TCMFs consisting of multiple herbal medicines have complicated chemical combinations, making the detection and identification a huge challenge, due to significant interference from other ingredients. Although we have acquired sufficient compounds information after decades of phytochemical efforts, we currently need a rapid and effective instrument or method to characterizing of these compounds.

Up to now, a large number of detection and technique methods have been reported for characterizing the chemical profiling of TCMFs, including the liquid chromatography-diode array detection (LC-DAD), gas chromatography-mass spectrometry (GC-MS), liquid chromatography-nuclear magnetic resonance (LC–NMR), electrophoresis chromatography-mass spectrometry (EC-MS), and LC-MS [4-7]. The LC-MS technique, especially high performance liquid chromatography-electrospray ionization-quadrupole-time of-flight mass spectrometry (HPLC-ESI-Q-TOF-MS), offering rapid and efficient separation and detection methods with accurate mass measurement and tandem mass spectrometry (MS/MS), has been the most widely applied [8, 9]. However, in most previous reports, the inspection of the MS or MS/MS chromatograms was usually performed manually one by one to screen the components from TCMFs. Obviously, the manual action is labor-intensive, low-effective and error-prone. Moreover, it was often difficult to distinguish the relatively small signals from the complex chemical background in full-scan mass chromatograms.

Find by Formula (FBF) is a targeted feature finding algorithm using a simple imported database, enabling lower detection levels with higher accuracy. It can find compounds by extracting ions that are

calculated from a formula in a database of compounds, including user-specified charge carriers (such as $[M+Na]^+$, $[M+Cl]^-$ and $[M+HCOO]^-$ *etc*) and multimers (such as $[2M-H]^-$). Furthermore, the corresponding MS/MS spectrums can be shown simultaneously so that we confirm the structural rationality of compounds. Following this idea, a strategy involving the establishment of a database of analogous TCMFs constituents was developed in the present study. The resulting simplified data could facilitate the identification of structural analogues in TCMFs.

Therefore, the main objective of the present study was to apply the FBF algorithms to the detection and characterization of major constituents from TCMFs. The effectiveness, rapidity and rationality of the FBF approach was evaluated by analyzing a typical TCMF, Yinchenhao Decoction (YCHD). As described in the *Shanghanlun* [10], a classic traditional Chinese medicine publication, YCHD is a famous prescription which consists of Artemisia capillaries, Fructus Gardeniae, and Rhizoma et Radix Rhei, and it is used clinically in China and Japan for the treatment of dampness-heat and jaundice [11, 12]. We highlighted the practical and reliable qualities of the FBF approach in the rapid analysis of major constituents from YCHD. Importantly, we supposed that this methodology could be envisioned to exhibit a wide application for the identification of complicated components from various complex mixtures and even screening the possible metabolites of a compound with specific molecular formula *in vivo* or *in vitro*.

Experimental

Chemicals and materials

HPLC-MS grade acetonitrile was purchased from Dikma Technologies Inc. (Dikma, CA, USA) and MS grade formic acid was supplied by Sigma-Aldrich (Steinheim, Germany). All other solvents and

chemicals were of analytical grade and were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Ultra-pure water was prepared using a Milli-Q system (Millipore, Billerica, MA, USA).

The reference compounds geniposidic acid (no. 111828-201102), caffeic acid (no. 110885-200102), chlorogenic acid (no. 110753-201314), geniposide (no. 110749-201115), quercetin (100081-200907), rutin (100080-201408), crocin-II (no. 121009-200502), rhein (no. 110757-200206) and emodin (no. 110756-200110) were purchased from the National Institutes for Food and Drug Control (Beijing, China). The genipin gentiobioside was isolated previously from Fructus Gardeniae in our laboratory. Their structures were confirmed by HRMS, 1D and 2D NMR comparison of these data with those reported in the literature [13]. The purity of all reference compounds were determined to be over 98% by HPLC analysis.

The crude herbs of Artemisia capillaries, Fructus Gardeniae and Rhizoma et Radix Rhei were purchased from Shanghai Kangqiao Traditional Chinese Medicine Co. Ltd. (Shanghai, China), and identified by Professor Jingui Shen, Shanghai Institute of Meteria Medica, Chinese Academy of Sciences.

HPLC conditions

HPLC analysis was performed on a Agilent 1260 Series (Agilent, Santa Clara, CA, USA) LC system, equipped with a binary pump, an online degasser, an auto plate-sampler, a thermostatically controlled column compartment and a DAD. Samples were eluted on an Agilent poroshell 120 EC-C_{18} column (100 mm × 2.1 mm, 2.7 μm; Agilent, CA, USA). The column and auto-sampler temperature were maintained at 35 °C and 4 °C, respectively. A mobile phase consisting of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) was applied with the optimized gradient

program as follows: 3-15%B (0–12 min), 15–18% B (12–15 min), 18–20% B (15–18 min), 20–40% B (18–28 min) and 40–90% B (28–45 min). The flow rate was set at 0.35 mL·min^{-1}.

Mass spectrometry conditions

Mass spectrometry was performed on an Agilent 6530 Q-TOF mass spectrometer (Agilent, Santa Clara, CA, USA) equipped with an electrospray ionization (ESI) interface, and was operated in negative ion mode with parameters set as follows: capillary voltage, 3500 V; fragmentor, 150 V; skimmer, 65 V; OCT 1 RF Vpp, 750 V; pressure of nebulizer, 35 psi; drying gas temperature, 300 °C; sheath gas temperature, 350 °C. Nitrogen was used as sheath and drying gas at a flow rate of 8.0 L·min^{-1} and 11.0 L·min^{-1}, respectively. The collision energy (CE) set values of 12 V and 26 V. An external calibration solution (Agilent calibration solution A) was continuously sprayed in the ESI source of the Q-TOF system, employing the ions with *m/z* 112.9855 (TFA anion) and 1033.9881 [HP-0921(TFA adduct)] to recalibrate the mass axis ensuring mass accuracy and reproducibility throughout the chromatographic run.

Sample preparation

Reference compound solutions

Known amounts of reference compounds were dissolved in methanol to obtain ten stock solutions (about 1.0 mg/mL) and stored at 4 °C. All the working solutions were prepared by serial dilution of the stock solutions with methanol. A mixture containing all ten references compounds was also prepared in methanol.

YCHD samples

Artemisia capillaries (18 g) was boiled in 1.2 L distilled water until the volume of water reduced to

about 600 mL, then 15 g Fructus Gardeniae and 6 g Rhizoma et Radix Rhei were added, and kept it boiling for concentrating to 300 mL. The extracted solution was centrifuged at 12,000 rpm for 10 min at 4 °C and the supernatant was concentrated under reduced pressure to afford 30 mL residue, and 1 mL of the residue was filtered through a 0.22 μm microporous membrane before use. An aliquot of 1.0 μL sample was injected into the LC-MS instrument for analysis.

Establishment of FBF approach to detect the characteristic components

Before processing, the following limits were applied to collect the data: the acquisition type set as Auto MS/MS and the mass range was *m/z* 50-1200 for both MS and MS/MS while the acquisition rates were 2 spectra per second and 1 spectra per second, respectively. Besides, the reference ions of *m/z* 112.9855 and 1033.9881 as well as the inherent interference ions in mobile phase were excluded in the acquisition lists all the time.

The strategy for complex constituents identification was implemented using the following three steps:

Step 1. Establish a specialized *.csv file (See *YCHD.csv* in the supplemental data) as the database of chemical constituents of Artemisia capillaries, Fructus Gardeniae and Rhizoma et Radix Rhei according to the previous reported literatures. The reported compounds that were isolated from the plants in the formulae were searched for in the following databases: PubMed of the U.S. National Library of Medicine, SciFinder Scholar of the American Chemical Society and the Chinese National Knowledge Infrastructure (CNKI) of Tsinghua University. The database should contain the molecular formula, the exact molecular weight, and the compound name. Without doubt, it would be better if we could acquire the retention time.

Step 2. Open the acquired mass spectrometry data file of YCHD on the software of Agilent MassHunter

Qualitative Analysis (Version B 06.01), and import the database of YCHD.csv file in the **Method Editor**. Then set up the important requisite filter templates: a. match tolerance, masses for ±5 ppm and retention time for ±0.1 min (if available); b. charge carriers, $[M–H]^-$/ $[M+Cl]^-$/ $[M–H+HCOOH]^-$ and activate the aggregates of $[2M–H]^-$; .c. results, automatically extract EIC, FBF spectrum and separate MS/MS spectrum per CE.

Step 3. Click the **Run** button in the toolbar, and then we can get all the candidates which met the set conditions in the compound list. Importantly, the MS/MS information of candidates and the processed extract ion chromatography (p-EIC) could be acquired simultaneously.

Results and discussion

Optimization of LC and MS conditions

Although MS can distinguish overlapping peaks through extracted ion chromatography (EIC), the isomers have to be identified by their retention behaviors if the co-eluting components have the same *m/z* values. Considering the complex constituents of the sample, a Poroshell 120-EC18 column was selected here to get better separation. Its packing has a solid core of 1.7 μm in size with a 0.5 μm thick porous outer layer and a total particle size of 2.7 μm providing high efficiency at lower column pressures [14]. In addition, appropriate concentration of formic acid (0.1%) was introduced into the mobile phase during HPLC to alleviate the peak tailing and increase the ionization effect. In order to obtain the most sensitive ionization method for analytes, positive and negative ion modes were investigated with the same HPLC mobile phase at a flow rate of 0.35 $mL \cdot min^{-1}$. As can be seen in Supplement Information of **Fig. S1**, more representative fragmentation ions information of the structures were obtained in the negative ion mode.

On-line characterization of major constituents from YCHD

The optimal HPLC-Q/TOF-MS/MS method was applied to analysis the YCHD. The total ion chromatograms (TIC) in negative ESI mode was shown in Fig. 1 (a). After being optimized, FBF approach was used to analysis the full-scan chromatogram of YCHD, the p-EIC was shown in Fig. 1 (b). In order to detect the low abundance peak, the p-EIC was scaled to largest for each chromatogram as shown in Fig. 1(c). It was used for clear labeling and it exhibited that the FBF approach could perform rapid and effective analysis of major constituents in YCHD in comparison with manual inspection. As a result, a total of 77 peaks with information on accurate molecular weight were extracted from the full-scan chromatogram and assigned rapidly to the major constituents in YCHD.

In the p-EIC of YCHD samples, the peaks of compounds 5, 19, 20, 23, 25, 32, 51, 53, 71 and 73 could be unambiguously identified as geniposidic acid, chlorogenic acid, caffeic acid, genipin gentiobioside, geniposide, rutin, quercetin, crocin-II, rhein and emodin by comparing their retention times, molecular weights and fragment ions with standards. The retention times, molecular weights, fragment ions of the 77 compounds plausibly identified are presented in Table 1.

The constituents of YCHD could be mainly classified into five types: organic acid, anthraquinones, iridoid glycosides, crocin glycosides and flavonoids. Some representative characteristic fragmentation behaviors of these five categories of compounds were discussed below. These results would be helpful to give chemical information for later research of any combination of these herbs or other herbs which contain these constituents.

Characterization of organic acid

Peaks 8, 10 and 21 in the first-order mass spectrum yielded the same $[M-H]^-$ ions at *m/z* 353.0878 ($C_{16}H_{17}O_9^-$) with the reference compound of 3-O-caffeoylquinic acid (3-CQA), revealing that these

three compounds were isomers of 3-CQA. However, it was possible to distinguish them according to the clear differences of their fragmentation ions. As described in the literatures [15, 16], peak 21 afforded the product ion of *m/z* 173, a unique fragmentation ion to 4-CQA, as its base peak, thus peak 21 could be identified as 4-CQA. In addition, unlike peak 19, the relatively intense product ions at *m/z* 179 (51.1% base peak) and 135 (62.5% base peak) of peak 10 could indicate it was a 5-CQA (Incidentally, as shown in SciFinder Scholar, some serial numbers in literature were incorrect, authors treated 3-CQA and 5-CQA opposite). The MS/MS information were not possible to reliably distinguish between peaks 8 and 19. Fortunately, peak 19 of 3-CQA, namely chlorogenic acid, was readily available from commercial source, so, in practice, the discrimination was straightforward. The related MS/MS spectra were shown in Fig. 2.

Similarly, peaks 35, 36, 37 and 42 showed the same $[M-H]^-$ ion at *m/z* 515.1207 in line with the $C_{25}H_{24}O_{12}$ formula, indicating that these four compounds were isomers of dicaffeoylquinic acid (di-CQA). ESI-MS/MS spectra provided considerably more structural information; the MS/MS spectra data of these four peaks are displayed in Table 1. Based on these fragmentation behaviors and the literature, peaks 35, 36, 37 and 42 were tentatively identified as 1,4-diCQA, 4,5-diCQA, 3,5-diCQA and 3,4-diCQA, respectively [15, 16]. First, the characteristic diagnostic product ion at *m/z* 173, presented in the MS/MS ion spectra, is indicative of acylation at 4-position. Thus, peak 37 was tentatively identified as 3,5-diCQA. Besides, 1,4-diCQA was also easily distinguished by its unique MS/MS fragments including comparatively intense ions at *m/z* 203 (57.5% base peak) and 299 (21.1% base peak) supported by less intense fragments at *m/z* 255 and 317. As for 3,4-diCQA and 4,5-diCQA, 4,5-diCQA had an intense product ion at *m/z* 335 (25.3% base peak) while it was almost not seen in MS/MS spectrum of 3,4-diCQA. The related MS/MS spectra were shown in Fig. 3.

Characterization of anthraquinones

Anthraquinones, as the major bioactive constituents of rhubarb, include free anthraquinones, anthraquinone glycosides, and sennosides. The MS/MS spectra of five representative free anthraquinones were shown in Fig. 4. In the MS/MS spectrum of chrysophnol, a product ion at *m/z* 225.0556 was observed as the base peak, which generating from the direct neutral loss of CO (28 Da) from *m/z* 253. Due to the carbonyl group at C-9 position was prone to produce the intramolecular hydrogen bonding with the α-OH at C-1 and C-8, we believed the CO elimination might occur at C-10 firstly [17]. Next, the ion at *m/z* 225 further lost a $CH_3\cdot$ (15 Da) and a CO (28 Da), affording the ions at *m/z* 210.0365 and 182.0393, respectively.

Emodin and aloe-emodin, along with rhein and physcion, could be differentiated by their high resolution mass data and MS/MS information. In particularly, rhein and physcion, with the distinct precursor ions of *m/z* 283.0247 and 283.0610, respectively, thus, were facile to identify. In addition, rhein gave the product ions at *m/z* 239, 211 and 183 in its MS/MS spectrum, obviously indicating the existence of carboxyl group, while physcion afforded the ions at *m/z* 240 and 212. As for emodin and aloe-emodin, emodin was initiated by the elimination a carbonyl group to produce the ion at *m/z* 241 and followed by loss of a hydroxyl group to give the ion at *m/z* 225. However, the $[M–H]^-$ ion of aloe-emodin, produced the ions at *m/z* 240.0416, 211.0401, and 183.0437 [17]. Therefore, the distinct difference of MS/MS information between these isomers sufficed their differentiation, and even were valuable for subsequent identification of their corresponding glycosides.

Characterization of iridoid glycosides

Iridoid glycosides are the bioactive components in Fructus Gardeniae which usually give $[M+Cl]^-$ ion as their precursor ions in negative ESI mode. As described in our group previous study, the

fragment patterns were shown that the glycosidic bond of iridoid glycosides was prone to break with neutral losses of 162 Da and always further lost a H_2O. Subsequently, it yielded the characteristic fragment ion at *m/z* 123.0452 ($C_7H_7O_2^-$) by the retro-Diels–Alder (RDA) reaction. When the C-4 position of iridoid glycosides was connected with the carboxyl group, it was easy to lose a molecule of carbon dioxide (CO_2) with neutral losses of 44 Da following deglycosylation, while the C-4 position was connected with the carbomethoxy, it always produced the characteristic fragment ion at *m/z* 101.0244 ($C_4H_5O_3^-$) via the bond cleavage between C_1-O_2 and C_4-C_5. Furthermore, if any groups were substituted at chains of glucose, it was prone to obtain $[M_s+glucosyl–H–H_2O]^-$ and $[M_s–H]^-$ of the substituent [18].

For instance, as shown in Fig. 5, peak 44 at the retention time of 20.784 min (exact molecular weight 696.2265) gave the precursor ion of $[M+Cl]^-$ at *m/z* 731.1947 and afforded the typical product ions at *m/z* 469.1347 ($[coumaroyl+gentiobiosyl–H–H_2O]^-$), 225.0769 ($C_{11}H_{13}O_5^-$), 163.0397 ($[coumaroyl–H]^-$), 145.0294 ($[coumaroyl–H–H_2O]^-$), 123.0451 ($C_7H_7O_2^-$) and 101.0246 ($C_4H_5O_3^-$) , which was confirmed as 6"-O-*trans*-coumaroylgenipin gentiobioside [19].

Characterization of crocin glycosides

Crocin glycosides was the diester formed from the disaccharide gentiobiose and the dicarboxylic acid crocetin. Therefore, they showed the product ions of $[M–162*n]^-$, $[crocetin–H]^-$ and $[crocetin–H–CO_2]^-$ in the MS/MS spectra according to the successive losses of glycosides and carboxyl group. Besides, the crocin glycosides in YCHD seemed to have a *cis-trans* isomer of 13-position. They exhibited the identical MS/MS data except for distinct retention time. Based on the conclusions summarized, it would be possible to consider that the 13-*cis* isomers took a longer retention time on the reverse phase packing. For crocin-I (retention time of 22.892 min) and

13-*cis*-crocin I (retention time of 28.035 min), the $[M+Cl]^-$ ion at *m/z* 1011.3479 with the formula $[(C_{38}H_{54}O_{19})+Cl]^-$ was selected as the precursor ion in the MS/MS experiment to give fragmentation information (Fig. 6). The ions at *m/z* 651.2650 ($C_{32}H_{43}O_{14}^-$) and 327.1596 ($C_{20}H_{23}O_4^-$) corresponded to the successive losses of 324 Da (disaccharide) from the deprotonated molecule. In addition, for the ion at *m/z* 283.1696 ($C_{19}H_{23}O_2^-$), the loss of 44Da could be assigned as CO_2 originating from *m/z* 327 [20].

Characterization of flavonoids

Flavonoids, The mass spectrum of quercetin (peak 51), which was detected at retention time 23.566 min, gave an $[M-H]^-$ ion at *m/z* 301.0347 ($C_{15}H_9O_7^-$). As shown in Table 1, its fragmentation patterns were quite clear as a flavonoid. Characteristic product ions at *m/z* 151.0028 ($C_7H_3O_4^-$) and 107.0135 ($C_6H_3O_2^-$), originating from two pathways of the RDA reaction, could be found in MS/MS spectra. In addition, ions at *m/z* 83.0144 ($C_4H_3O_2^-$), 65.0039 (C_4HO^-) and 178.9974 ($C_8H_3O_5^-$) were also observed.

Peaks 31 and 34 both exhibited $[M-H]^-$ ions at *m/z* 463.0876 ($C_{21}H_{19}O_{12}^-$). Moreover, they had the similar fragment ions of *m/z* 301 and 151 in MS/MS spectra, but different retention times even without regular retention behavior. They could be identified as hyperoside or isoquercitrin, but not precisely identified by the method above, and need isolation and identification by NMR in the future.

The discussion of FBF algorithms

Although the limitations of mass spectrum for identifying exact structures, it is a convenient way to rapidly analyze mass spectrum character to speculate tentative structures. And it is very imperative to apply to TCMFs without isolation and purification each compound. The HPLC-Q/TOF-MS/MS method combine with FBF algorithms requires only a simple *csv* file and the rapid and effective characterization will be performed facilely. However, the deficiency of FBF algorithms should be noted,

we can just characterize the known compounds reported previously using FBF algorithms. As a famous traditional Chinese medicinal materials, the previous phytochemical isolations have been accomplished maturely and the related compounds information are also acquired sufficiently, hence, the compounds in *csv* file will be counted comprehensively.

More importantly, based on the principles of FBF approach, we can not only apply it conveniently to analyze the major constituents in TCMFs like YCHD in this work, but also utilize it as important tools to investigate all potential metabolites of one compound with specific molecular formula. We can list all possible forms of metabolites, their molecular formula and exact molecular weight in the *csv* file. Once the database file is established, it will be a major breakthrough to avoid the matrix interference when we performed a qualitative analysis metabolic process in biological sample.

Conclusions

A primary challenge in herbal analysis was to enable global qualitative performance rapidly and accurately. In the present study, a practical method based on the FBF algorithms was developed for rapid detection and structural characterization of major constituents from YCHD. As a result, a total of 77 compounds were structural characterized. Compared with the conventional manual inspection and fragmentation-based method, the FBF approach enabled the original data to be analyzed much faster and more accurately by reducing the potential interferences of matrix ions. More importantly, according to the principle of FBF, this method can also be used for the rapid analysis the possible metabolites of a compound with specific molecular formula. Meanwhile, it should be noted that the major limitation of this study was that only the known chemical composition was used to validate the characterizing results.

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Figure captions

Fig. 1. The extract ion chromatogram (EIC) of YCHD in negative ion mode (a); the processed extract ion chromatogram (p-EIC) of YCHD in negative ion mode (b); the scaled to largest for each chromatogram of p-EIC (c).

Fig. 2. The MS/MS spectra of the $[M–H]^-$ ions at *m/z* 353.0878 for 1-CQA (a); 5-CQA (b); 3-CQA (c) and 4-CQA (d).

Fig. 3. The MS/MS spectra of the $[M–H]^-$ ions at *m/z* 515.1207 for 1,4-dicaffeoylquinic acid (a); 4,5-dicaffeoylquinic acid (b); 3,5-dicaffeoylquinic acid (c) and 3,4-dicaffeoylquinic acid (d).

Fig. 4. The MS/MS spectra of the $[M–H]^-$ ions at *m/z* 253.0500 for chrysophanol (a); *m/z* 283.0610 for physcion (b); *m/z* 283.0247 for rhein (c); *m/z* 269.0450 for emodin (d) and *m/z* 269.0450 for aloe-emodin (e).

Fig. 5. The FBF spectrum for 6''-O-*trans*-coumaroyl genipin gentiobioside (a) and its MS/MS spectrum of the $[M+Cl]^-$ ion at *m/z* 731.1947 (b).

Fig. 6. The EIC of *m/z* 1011 in negative ion mode (a), the FBF spectrum for crocin-I (b); the MS/MS spectra of the $[M+Cl]^-$ ion at *m/z* 1011.3468 for crocin-I (c) and *m/z* 1011.3475 for 13-*cis*-crocin-I (d).

Table 1. Chemical constituents identified in an extract of YCHD by HPLC-Q/TOF-MS/MS in negative ion mode

Peak No.	t_R (min)	Molecular formula	Theoretical $[M-H]^-$	Measured $[M-H]^-$	Error[a] (ppm)	Characteristic MS/MS fragmentation ions (% base peak) (*m/z*)	Proposed compounds
1	0.803	$C_{12}H_{22}O_{11}$	377.0856[b]	377.0857	0.27	341 (100), 215 (11.1), 179 (20.5), 119 (18.1), 89 (49.6), 59 (34.1)	gentiobiose
2	0.972	$C_5H_7NO_3$	128.0353	128.0364	8.59	101.0257 (17.8), 88.0426 (54.1), 85.0308 (100)	pyroglutamic acid
3	1.309	$C_7H_6O_5$	169.0137	169.0136	-0.59	125.0238 (100), 107.0133 (2.2), 79.0193 (12.2)	gallic acid
4	1.646	$C_{19}H_{26}O_{15}$	493.1199	493.1189	-2.03	313.0564 (63.2), 271.0455 (66.2), 211.0240 (21.9), 169.0137 (100), 125.0237 (30.7)	6-O-Galloylsucrose
5	1.815	$C_{16}H_{22}O_{10}$	373.1140	373.1146	1.61	211.0612 (27.4), 193.0502 (5.8), 167.0712 (13.6), 149.0604 (78.9), 123.0453 (100)	geniposidic acid
6	2.068	$C_{13}H_{16}O_9$	315.0722	315.0722	0.00	153.0190 (32.4), 152.0115 (47.9), 109.0291 (34.4), 108.0227 (100)	protocatechuic acid-3-glucoside
7	2.237	$C_{16}H_{26}O_9$	397.1271[b]	397.1265	-1.51	361.1496 (100), 317.1589 (1.9), 181.0862 (31.7), 137.0964 (11.2), 89.0245 (12.4), 59.0144 (20.6)	villosolside
8	2.405	$C_{16}H_{18}O_9$	353.0878	353.0878	0.00	191.0538 (100), 85.0284 (7.9)	1-CQA
9	2.405	$C_{16}H_{24}O_{11}$	391.1246	391.1250	1.02	229.0713 (24.5), 211.0611 (17.1), 185.0823 (75.2), 167.0712 (93.1), 149.0616 (100), 141.0549 (42.5), 123.0449 (57.9)	shanzhiside
10	3.401	$C_{16}H_{18}O_9$	353.0878	353.0878	0.00	191.0538 (100), 179.0326 (51.1), 135.0429 (62.5)	neochlorogenic acid (5-COA)
11	3.67	$C_{17}H_{24}O_{11}$	439.1013[b]	439.1007	-1.37	403.1247 (8.9), 241.0719 (100), 223.0610 (12.3), 139.0397 (43.7), 101.0239 (31.8)	scandoside methyl ester
12	3.754	$C_{16}H_{26}O_8$	381.1322[b]	381.1319	-0.79	345.1548 (50.6), 179.0547 (15.9), 165.0913 (100), 121.1017 (12.9), 119.0345 (19.0), 89.0245 (56.9)	picrocrocininc acid
13	3.754	$C_{16}H_{22}O_{11}$	389.1089	389.1088	-0.26	227.0566 (24.3), 209.0450 (48.9), 183.0667 (51.1),	deacetylasperulosidic acid

14	4.344	$C_{16}H_{24}O_9$	395.1114[b]	395.1111	-0.76	165.0551 (100), 139.0395 (53.9), 359.1346 (95.4), 197.0813 (100), 125.0612 (62.4), 107.0492 (28.9)	ixoroside
15	4.85	$C_{17}H_{26}O_{11}$	405.1402	405.1395	-1.73	225.0766 (29.3), 179.0566 (57.6), 141.0555 (82.2), 123.0447 (48.5), 119.0349 (30.4), 101.0239 (100)	shanzhiside methyl ester
16	4.934	$C_{17}H_{24}O_{11}$	439.1013[b]	439.1015	0.46	403.1249 (30.5), 241.0714 (100), 223.0615 (14.1), 139.0399 (87.3), 121.0299 (46.8), 101.0242 (24.1)	gardenoside
17	5.778	$C_{17}H_{24}O_{11}$	439.1013[b]	439.1005	-1.82	403.1246 (12.6), 241.0710 (100), 223.0617 (40.8), 139.0394 (71.9), 101.0248 (52.6)	deacetylasperulosidic acid methyl ester
18	6.368	$C_{15}H_{14}O_6$	289.0718	289.0710	-2.77	245.0816 (45.1), 203.0706 (35.3), 137.0242 (30.25), 123.0450 (78.6), 109.0294 (100),	catechin
19	6.436	$C_{16}H_{18}O_9$	353.0878	353.0878	0.00	191.0547 (100), 85.0294 (6.1)	chlorogenic acid (3-CQA)
20	6.874	$C_9H_8O_4$	179.0350	179.0350	0.00	135.0437 (100), 89.0395 (5.7)	caffeic acid
21	7.632	$C_{16}H_{18}O_9$	353.0878	353.0878	0.00	191.0535 (60.7), 179.0324 (70.3), 173.0426 (100), 135.0431 (80.9), 93.0335 (27.6)	cryptochlorogenic acid (4-CQA)
22	7.885	$C_{16}H_{24}O_{10}$	375.1297	375.1303	1.60	213.0764 (39.1), 195.0660 (38.4), 169.0877 (47.9), 151.0770 (95.2), 125.0605 (80.7), 107.0510 (100)	mussaenosidic acid
23	8.307	$C_{23}H_{34}O_{15}$	585.1592[b]	585.1584	-1.37	549.1815 (12.3), 225.0769 (69.3), 207.0657 (13.4), 123.0449 (100), 101.0243 (64.9)	genipin gentiobioside
24	8.56	$C_8H_8O_2$	135.0452	135.0453	0.74	120.0244 (5.5), 108.0206 (10.8), 92.0279 (100)	4'-hydroxyacetophenone
25	9.487	$C_{17}H_{24}O_{10}$	423.1063[b]	423.1070	1.65	387.1282 (5.5), 225.0762 (100), 207.0653 (13.5), 123.0447 (85.3), 101.0243 (74.5)	geniposide
26	10.161	$C_{16}H_{26}O_8$	381.1322[b]	381.1309	-3.41	345.1464 (11.9), 183.1020 (86.1), 165.0908 (100), 109.0290 (46.7), 89.0231 (58.9)	jasminoside B
27	11.173	$C_{27}H_{30}O_{15}$	593.1512	593.1520	1.35	549.1610 (52.4), 343.1053 (11.6), 265.0722 (19.1), 207.0295 (100), 163.0397 (21.6)	rheinoside D/rheinoside C

28	11.763	$C_{27}H_{30}O_{15}$	593.1512	593.1522	1.69	549.1606 (45.2), 343.1052 (13.9), 265.0719 (14.8), 207.0299 (100), 163.0401 (12.7)	rheinoside C/rheinoside D
29	12.606	$C_{22}H_{22}O_{12}$	477.1038	477.1020	-3.77	313.0554 (40.3), 169.0133 (100), 163.0388 (29.33), 125.0237 (13.77)	*p*-coumaroyl-O-galloyl-glucose
30	13.454	$C_{25}H_{28}O_{12}$	519.1508	519.1511	0.58	355.1029 (8.9), 307.0824 (2.3), 211.0606 (18.2), 193.0504 (22.4), 167.0716 (27.8), 163.0397 (84.3), 149.0601(33.2), 145.0299 (100), 123.0445 (52.2)	6'-O-*trans*-coumaroyl geniposidic acid
31	15.562	$C_{21}H_{20}O_{12}$	463.0882	463.0876	-1.30	301.0330 (64.82), 300.0265 (100), 283.0252 (1.3), 271.0240 (37.85), 255.0293 (16.3), 151.0022 (8.6)	hyperoside/isoquercitrin
32	15.726	$C_{27}H_{30}O_{16}$	609.1461	609.1457	-0.66	301.0357 (95.9), 300.0274 (100), 151.0028 (5.3)	rutin
33	15.894	$C_{21}H_{18}O_{11}$	445.0776	445.0773	-0.67	283.0238 (80.8), 239.0347 (100), 211.0413 (18.7)	rhein monoglucoside
34	16.068	$C_{21}H_{20}O_{12}$	463.0882	463.0874	-1.73	301.0331 (49.3), 300.0271 (100), 271.0244 (36.2), 255.0297 (17.2), 151.0034 (8.5)	isoquercitrin/hyperoside
35	17.417	$C_{25}H_{24}O_{12}$	515.1195	515.1207	2.33	353.0878 (100), 317.0665 (18.7), 299.0552 (21.1), 255. 0658 (15.6), 203.0349 (57.5), 191.0558 (12.9), 179.0354 (49.5), 173.0456 (57.6), 135.0467 (7.4)	1,4-dicaffeoylquinic acid
36	17.923	$C_{25}H_{24}O_{12}$	515.1195	515.1207	2.33	353.0872 (100), 335.0766 (25.3), 191.0561 (47.6), 179.0354 (58.3), 173.0460 (71.9), 135.0452 (10.3)	4,5-dicaffeoylquinic acid
37	18.26	$C_{25}H_{24}O_{12}$	515.1195	515.1207	2.33	353.0874 (100), 191.0551 (78.8), 179.0349 (29.3), 135.0445 (8.5)	3,5-dicaffeoylquinic acid
38	18.424	$C_{27}H_{34}O_{13}$	601.1693[b]	601.1695	0.33	565.1893 (14.6), 385.1127 (24.8), 325.0924 (100), 295.0824 (54.1), 265.0727 (79.7), 223.0604 (55.7), 205.0507 (25.3)	11-(6-O-*trans*-sinapoyl glucopyranosyl) gardendiol
39	18.424	$C_{22}H_{36}O_{12}$	527.1901[b]	527.1897	-0.76	491.2130 (13.7), 167.1064 (100), 89.0238 (23.6)	jasminoside H/jasminoside I
40	18.929	$C_{22}H_{22}O_{12}$	477.1038	477.1037	-0.21	357.0598 (14.1), 314.0421 (100), 285.0438 (42.9), 271.0237 (36.9), 243.0325 (50.3)	isorhamnetin-3-glucoside

41	19.908	$C_{22}H_{36}O_{12}$	527.1901[b]	527.1899	-0.38	491.2130 (25.5), 167.1066 (100), 89.0234 (31.9)	jasminoside I/jasminoside H
42	20.031	$C_{25}H_{24}O_{12}$	515.1195	515.1207	2.33	353.0880 (100), 191.0569 (11.43), 179.0354 (34.4), 173.0458 (50.8), 135.0455 (5.1)	3,4-dicaffeoylquinic acid
43	20.278	$C_{27}H_{34}O_{13}$	601.1693[b]	601.1697	0.67	565.1907 (28.9), 385.1119 (25.1), 325.0927 (100), 295.0815 (62.2), 265.0711 (91.3), 223.0609 (64.4), 205.0504 (25.2)	10-(6-O-*trans*-sinapoyl glucopyranosyl) gardendiol
44	20.784	$C_{32}H_{40}O_{17}$	731.1960[b]	731.1947	-1.78	695.2186 (100), 469.1347 (10.1), 367.1018 (3.4), 225.0769 (12.7), 163.0397 (9.6), 145.0294 (9.4), 123.0451 (20.6), 101.0246 (13.4)	6"-O-*trans*-coumaroyl genipin gentiobioside
45	21.121	$C_{22}H_{22}O_{11}$	461.1089	461.1082	-1.52	313.0552 (100), 169.0129 (41.4), 147.0437 (52.4), 125.0234 (8.1), 103.0544 (12.4)	2-cinnamoyl-1-galloylglucose
46	21.374	$C_{34}H_{44}O_{19}$	791.2171	791.2168	-0.38	755.2393 (100), 529.1560 (9.6), 427.1242 (1.6), 225.0763 (16.6), 223.0607 (11.6), 207.0657 (3.1), 205.0498 (6.3), 123.0449 (21.4), 101.0252 (13.4)	6"-O-*trans*-sinapoyl genipin gentiobioside
47	22.217	$C_{31}H_{32}O_{16}$	659.1618	659.1618	0.00	497.1291 (100), 353.0863 (40.2), 335.0829 (69.3), 273.0965 (10.5), 233.0653 (14.9), 191.0548 (82.9), 161.0440 (62.8)	3,5-di-O-caffeoyl-4-O-(3-hydroxy-3-methyl)-glutaroylquinic acid
48	22.639	$C_{27}H_{28}O_{13}$	559.1457	559.1453	-0.72	397.1131 (100), 223.0602 (11.2), 173.0446 (71.6)	5-O-caffeoyl-4-O-sinapoylquinic acid
49	22.892	$C_{44}H_{64}O_{24}$	1011.3482[b]	1011.3479	-0.30	651.2650 (100), 327.1596 (39.9), 283.1696 (8.6), 179.0556(3.5)	crocin-I
50	23.061	$C_{28}H_{34}O_{14}$	629.1643[b]	629.1640	-0.48	593.1878 (100), 367.1036 (9.7), 225.0766 (23.8), 223.0609 (27.5), 207.0659 (10.3), 205.0507 (64.3), 123.0451 (46.8), 101.0249 (31.7)	6'-O-*trans*-sinapoyl geniposide
51	23.566	$C_{15}H_{10}O_7$	301.0354	301.0347	-2.33	178.9974 (40.2), 151.0028 (100), 121.0293 (31.9), 107.0135 (35.7), 65.0039 (46.7)	quercetin

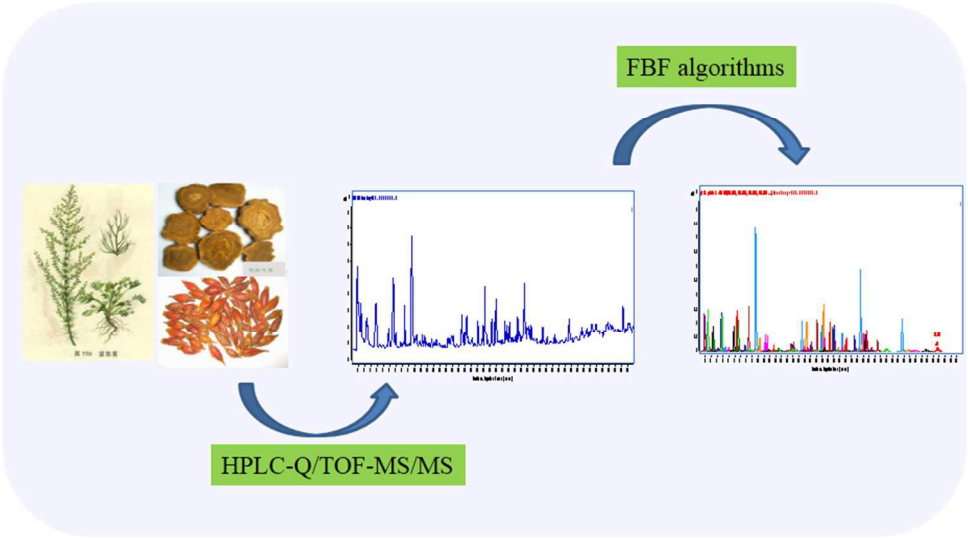
52	23.735	$C_{22}H_{22}O_{11}$	461.1089	461.1091	0.43	313.0561 (100), 169.0138 (40.8), 147.0446 (53.2), 125.0243 (8.1), 103.0555 (13.6)	6-cinnamoyl-1-galloylglucose
53	24.325	$C_{38}H_{54}O_{19}$	849.2959[b]	849.2951	-0.94	651.2651 (100), 489.2115 (25.5), 327.1587 (74.5), 283.1711 (23.7)	crocin-II
54	24.41	$C_{26}H_{30}O_{12}$	533.1664	533.1659	-0.94	325.0926 (56.2), 307.0819 (37.7), 225.0767 (81.9), 207.0669 (27.3), 163.0406 (100), 145.0287 (33.6), 123.0455 (64.1), 101.0241 (58.7)	6'-O-*trans*-coumaroyl geniposide
55	24.41	$C_{29}H_{26}O_{15}$	613.1199	613.1209	1.63	465.0664 (100), 313.0564 (33.8), 271.0458 (28.2), 169.0142 (41.6)	cinnamoyl-di-O-galloyl-glucose
56	24.747	$C_{16}H_{10}O_7$	313.0354	313.0354	0.00	269 (100), 241 (10.1), 225 (11.2)	laccaic acid D
57	25.084	$C_{21}H_{20}O_{10}$	431.0989	431.0987	-0.46	269 (100), 240 (54.1), 223 (17.4)	aloe-emodin monoglucoside
58	25.506	$C_{32}H_{40}O_{16}$	715.2010[b]	715.2002	-1.12	679 (100), 531 (20.9), 453 (11.2), 225 (82.2), 207 (22.6), 147 (28.7), 123 (35.1), 101 (16.5)	6''-O-*trans*-cinnamoyl genipin gentiobioside
59	26.433	$C_{24}H_{22}O_{13}$	517.0988	517.0978	-1.93	473 (20.2), 269 (100)	emodin-8-O-(6'-O-malonyl)-glucoside
60	26.601	$C_{48}H_{64}O_{23}$	1007.3766	1007.3761	-0.50	683 (100), 327 (6.9), 283 (6.4)	crocin-I-acid
61	27.023	$C_{15}H_{10}O_4$	253.0506	253.0509	1.19	225 (100), 210 (17.1), 195 (16.8), 182 (28.8), 154 (8.1)	chrysophanol
62	27.192	$C_{22}H_{22}O_{10}$	481.0907[b]	481.0911	0.83	283 (100), 240 (30.3)	physcion monoglucoside
63	27.445	$C_{21}H_{20}O_{10}$	431.0984	431.0989	1.16	269 (100), 225 (12.5)	emodin monoglucoside
64	27.866	$C_{32}H_{44}O_{14}$	687.2425[b]	687.2415	-1.46	651 (13.9), 327 (100), 283 (70.6), 239 (20.3)	crocin-III
65	28.035	$C_{44}H_{64}O_{24}$	1011.3482[b]	1011.3479	-0.30	651 (100), 327 (39.4), 283 (8.3), 179 (3.1)	13-*cis*-crocin-I
66	28.288	$C_{16}H_{10}O_6$	297.0405	297.0408	1.01	253 (100), 225 (58.16), 182 (15.6)	3-(1,8-Dihydroxy-6-methyl)anthraquinonecarboxylic acid
67	28.541	$C_{19}H_{24}O_4$	315.1607	315.1599	-2.54	297 (43.2), 271 (33.7), 241 (100), 186 (15.6)	Capillartemisin A
68	28.878	$C_{16}H_{12}O_5$	283.0617	283.0610	-2.47	240 (100), 212 (12.5), 184 (7.3)	physcion

69	29.215	$C_{15}H_{10}O_5$	269.0455	269.0450	-1.86	240 (100), 223 (20.2), 211 (78.1), 195 (14.5), 183 (61.7), 167 (26.4)	aloe-emodin
70	29.384	$C_{38}H_{54}O_{19}$	849.2953[b]	849.2954	0.12	651 (85.5), 489 (29.7), 327. (100), 283 (19.6)	13-*cis*-crocin-II
71	30.395	$C_{15}H_8O_6$	283.0248	283.0247	-0.35	239 (100), 211 (44.1), 183 (91.5)	rhein
72	31.829	$C_{32}H_{44}O_{14}$	687.2425[b]	687.2422	-0.44	651 (12.3), 489 (2.9), 327 (100), 283 (46.8), 239 (13.4)	13-*cis*-crocin-III
73	34.511	$C_{15}H_{10}O_5$	269.0455	269.0450	-1.86	241 (34.6), 225 (100), 210 (20.7), 195 (36.5), 105 (21.9)	emodin
74	35.628	$C_{30}H_{22}O_8$	509.1242	509.1247	0.98	254 (100)	palmidin A
75	36.808	$C_{30}H_{20}O_9$	523.1035	523.1037	0.38	485 (4.4), 254 (100), 224 (9.84)	rheidin
76	38.916	$C_{30}H_{22}O_7$	493.1293	493.1290	-0.61	476 (20.1), 462 (52.5), 254 (100), 238 (88.9), 152 (24.6)	palmidin B
77	40.602	$C_{30}H_{22}O_7$	493.1293	493.1294	0.20	254 (100), 152 (5.68)	palmidin C

[a] Differences between the measured and theoretical values, ppm.

[d] gave $[M+Cl]^-$ as precursor ions

* identified with the reference compounds



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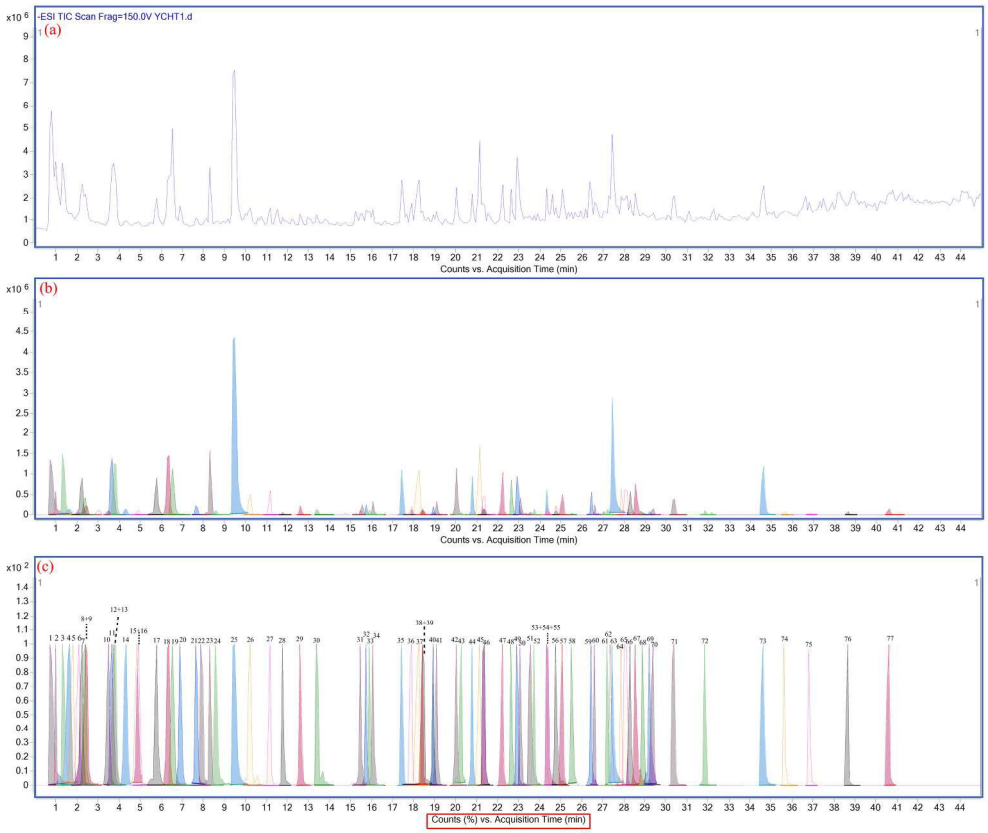
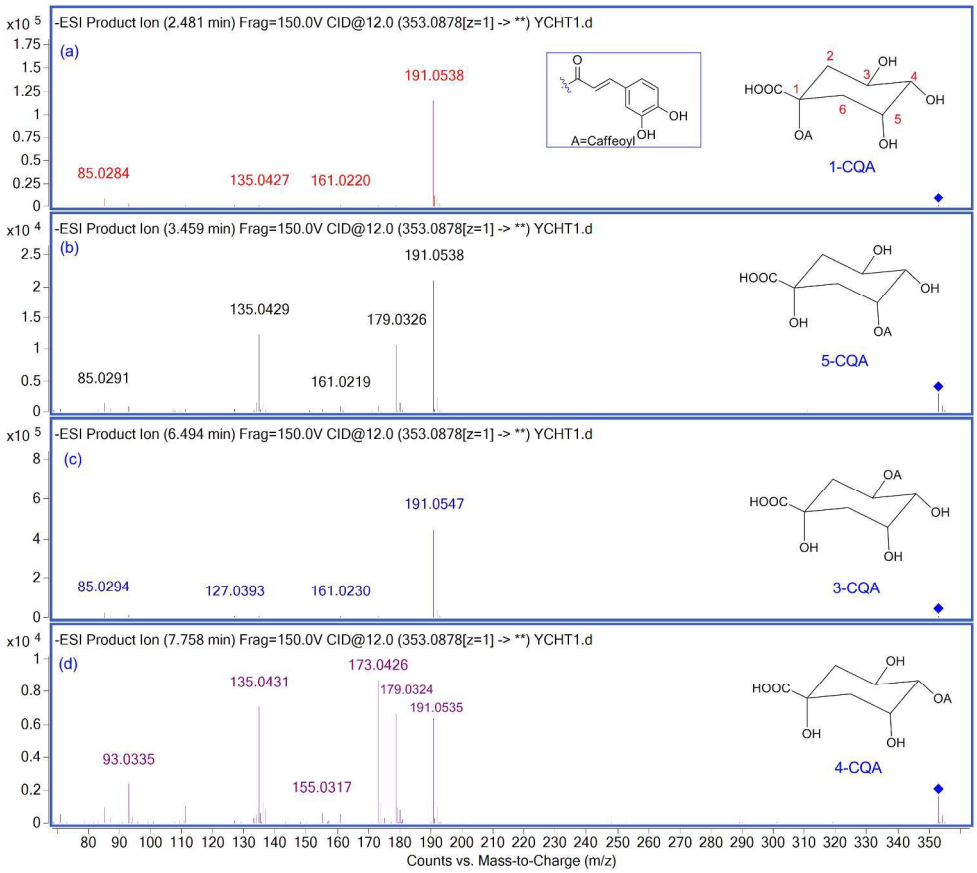


The extract ion chromatogram (EIC) of YCHD in negative ion mode (a); the processed extract ion chromatogram (p-EIC) of YCHD in negative ion mode (b); the scaled to largest for each chromatogram of p-EIC (c).
225x187mm (300 x 300 DPI)



The MS/MS spectra of the [M-H]- ions at m/z 353.0878 for 1-CQA (a); 5-CQA (b); 3-CQA (c) and 4-CQA (d).
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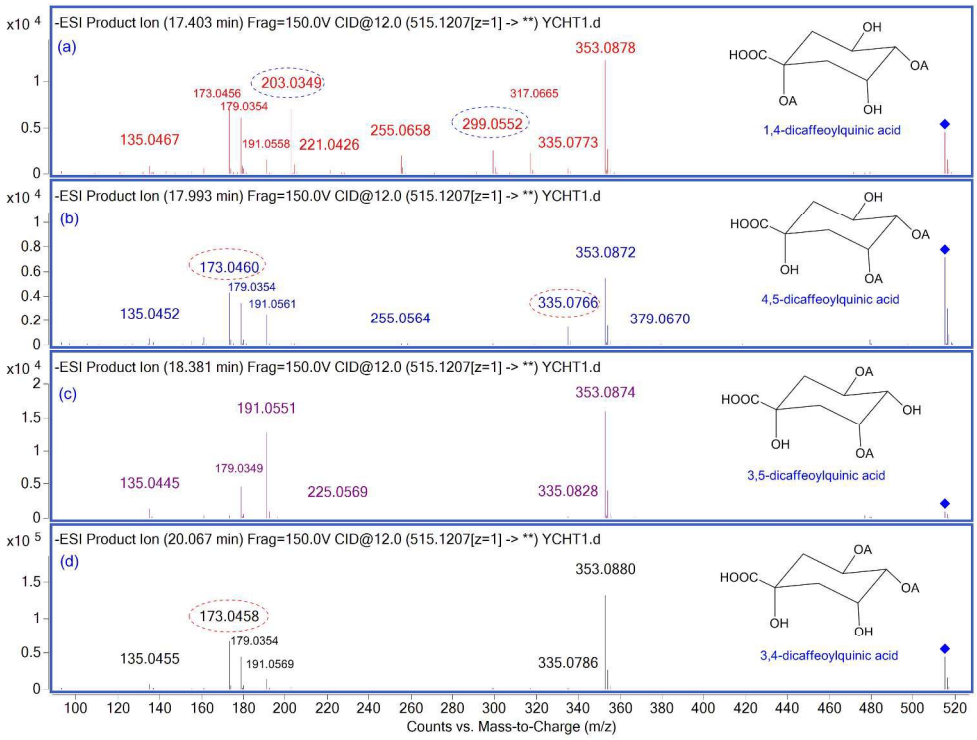

The MS/MS spectra of the [M-H]- ions at m/z 515.1207 for 1,4-dicaffeoylquinic acid (a); 4,5-dicaffeoylquinic acid (b); 3,5-dicaffeoylquinic acid (c) and 3,4-dicaffeoylquinic acid (d).
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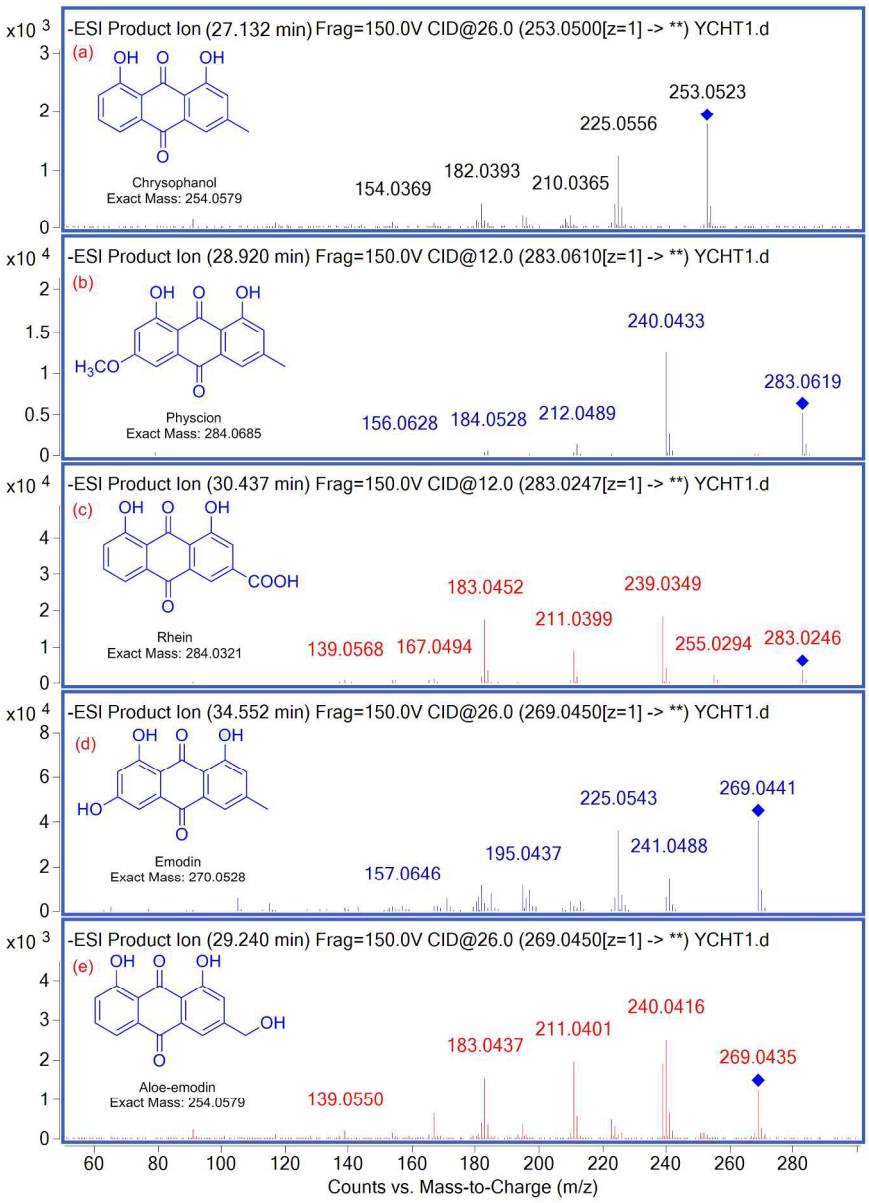
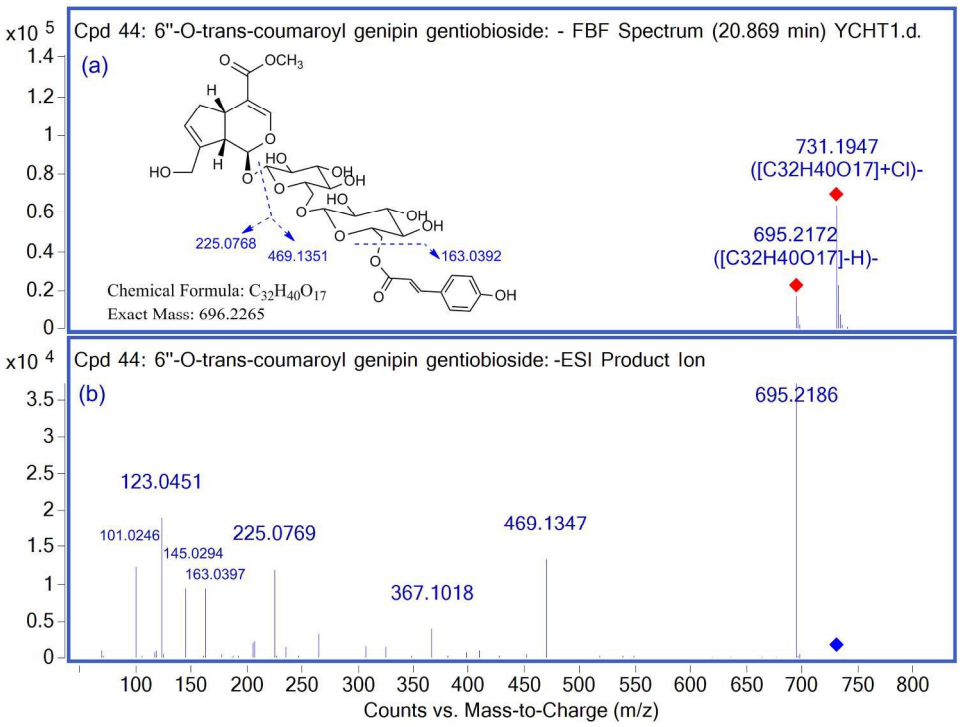
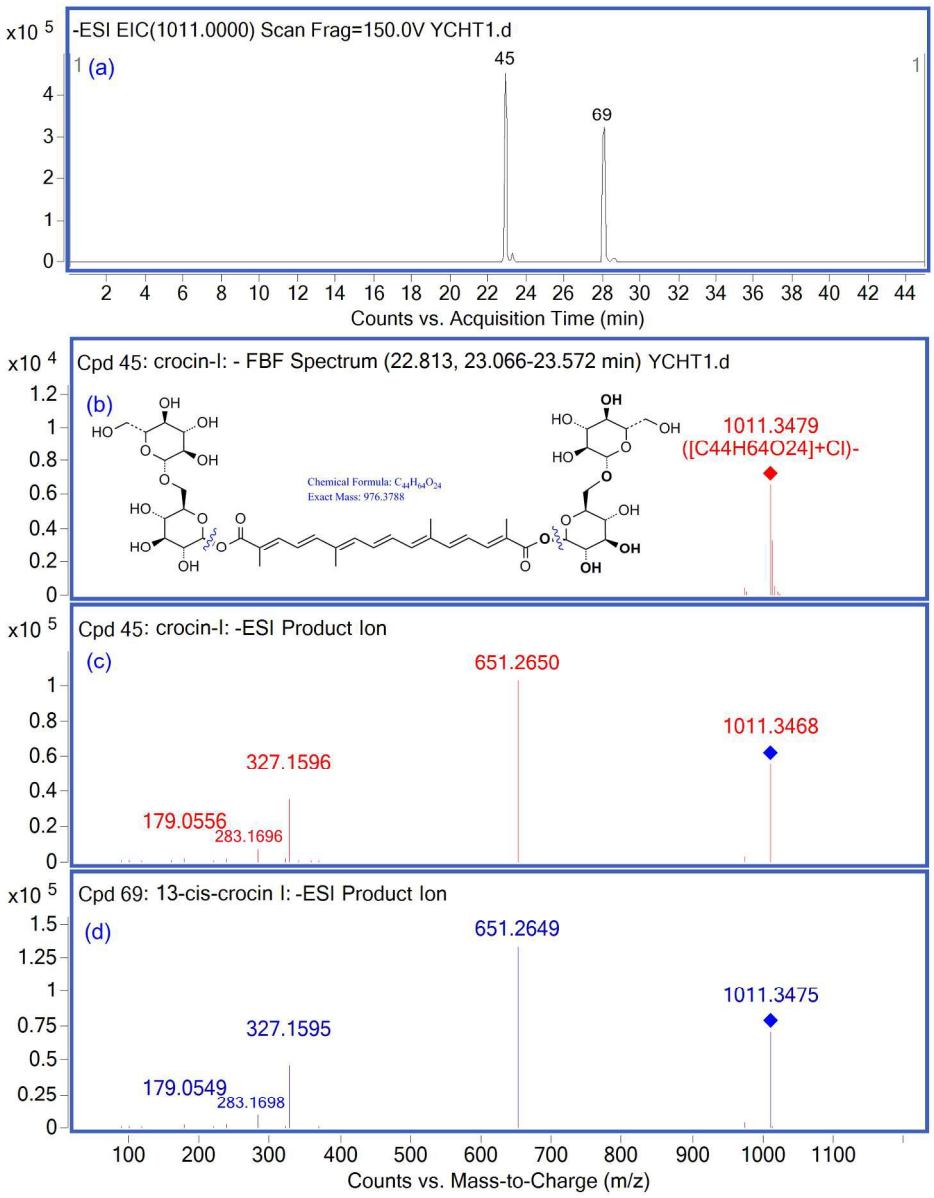


The MS/MS spectra of the [M-H]- ions at m/z 253.0500 for chrysophanol (a); m/z 283.0610 for physcion (b); m/z 283.0247 for rhein (c); m/z 269.0450 for emodin (d) and m/z 269.0450 for aloe-emodin (e).
184x245mm (300 x 300 DPI)



The FBF spectrum for 6''-O-trans-coumaroyl genipin gentiobioside (a) and its MS/MS spectrum of the [M+Cl]- ion at m/z 731.1947 (b).
191x139mm (300 x 300 DPI)



The EIC of m/z 1011 in negative ion mode (a), the FBF spectrum for crocin-I (b); the MS/MS spectra of the [M+Cl]- ion at m/z 1011.3468 for crocin-I (c) and m/z 1011.3475 for 13-cis-crocin-I (d).
188x233mm (300 x 300 DPI)