Journal of Pharmaceutical and Biomedical Analysis 51 (2010) 812–823

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

A novel strategy to rapidly explore potential chemical markers for the discrimination between raw and processed Radix Rehmanniae by UHPLC–TOFMS with multivariate statistical analysis

Song-Lin Li[a], Jing-Zheng Song[a], Chun-Feng Qiao[a], Yan Zhou[a], Keduo Qian[b], Kuo-Hsiung Lee[b], Hong-Xi Xu[a,*]

[a] Chinese Medicine Laboratory, Hong Kong Jockey Club Institute of Chinese Medicine, Shatin, N. T., Hong Kong, PR China
[b] Natural Products Research Laboratories, School of Pharmacy, University of North Carolina, Chapel Hill, NC 27599, USA

ARTICLE INFO

Article history:
Received 7 August 2009
Received in revised form
29 September 2009
Accepted 2 October 2009
Available online 9 October 2009

Keywords:
Herb processing
Chemical marker
UHPLC–TOFMS
Radix Rehmanniae
Multivariate statistical analysis

ABSTRACT

In traditional Chinese medicine, raw and processed herbs are used to treat different diseases. Suitable chemical markers are crucial for the discrimination between raw and processed herbs. In this study, a novel strategy using UHPLC–TOFMS coupled with multivariate statistical analysis to rapidly explore potential chemical markers was proposed and validated. Using Radix Rehmanniae as a model herb, batches of raw and processed samples were determined by UHPLC–TOFMS. The datasets of t_R–m/z pair, ion intensity and sample code were subjected to principal component analysis (PCA) and orthogonal partial least squared discriminant analysis (OPLS-DA) to holistically compare the difference between raw and processed samples. Once a clear cluster was found, extended statistics was performed to generate S-plot, in which the variables (t_R–m/z pair) contributing most to the difference were clearly indicated as points at the two ends of "S", and the components that correlate to these ions should be the processing-induced transformed components. These transformed components could be regarded as the potential chemical markers that can be used to distinguish between raw and processed herbs. The identity of the potential markers can be identified by comparing the mass/UV spectra and retention time with that of reference compounds and/or tentatively assigned by matching empirical molecular formula with that of the known compounds published. Using this proposed strategy, leonuride or its isomer and 5-(α-D-glucopyranosyl-(1-6)-α-D-glucopyranosyloxymethyl)-2-furancarboxaldehyde were rapidly explored as the most characteristic markers of raw and processed Radix Rehmanniae, respectively. This newly proposed strategy can not only be used to explore chemical markers but also to investigate the chemical transforming mechanisms underlying traditional herb processing.

1. Introduction

The processing of traditional Chinese herbs is a common practice and usually occurs before most herbs are prescribed. There are a variety of traditional ways for processing herbs, such as frying with sand or oil, sauteing with rice wine or wheat bran, steaming with water or rice wine, and braising with rice wine or licorice liquids, etc. [1]. According to the theory of traditional Chinese medicine (TCM), the main purposes of herb processing are to increase potency, reduce toxicity and alter effects. The main mechanisms underlying herb processing were found to be mainly related to the changes in the composition and/or activity of the components in the herbs [2]. One typical example is frying Semen Strychni (Maqianzi), the seeds of *Strychnos nux-vomica* L., with sand or oil, to reduce its toxicity and increase the analgesic potency [3], which has been proved by modern phytochemical, toxicological and pharmacological investigations [4,5].

As raw and processed herbs are used for different clinical purposes, the quality control, in particular the discrimination between raw and processed herbs, is very important for the safe and effective use of medicinal herbs. Chemical markers are crucial for this purpose as they can be used to distinguish between raw and processed herbs and to evaluate the extent of processing. Ideally, the processing-induced transforming components would be potential chemical markers for the quality control of processed herbs.

During the last decades, large numbers of studies have been reported on the quality control of raw herbs, and statutory standards on the raw herbs (crude drugs), such as that in the Chinese Pharmacopoeia, have been successfully issued. By contrast, little progress has been made on the quality control of processed herbs. The main reason for this is that there are few extensive and sys-

* Corresponding author. Tel.: +852 3406 2873; fax: +852 3551 7333.
E-mail addresses: xuhongxi@hkjcicm.org, songlinli64@126.com (H.-X. Xu).

0731-7085/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2009.10.002

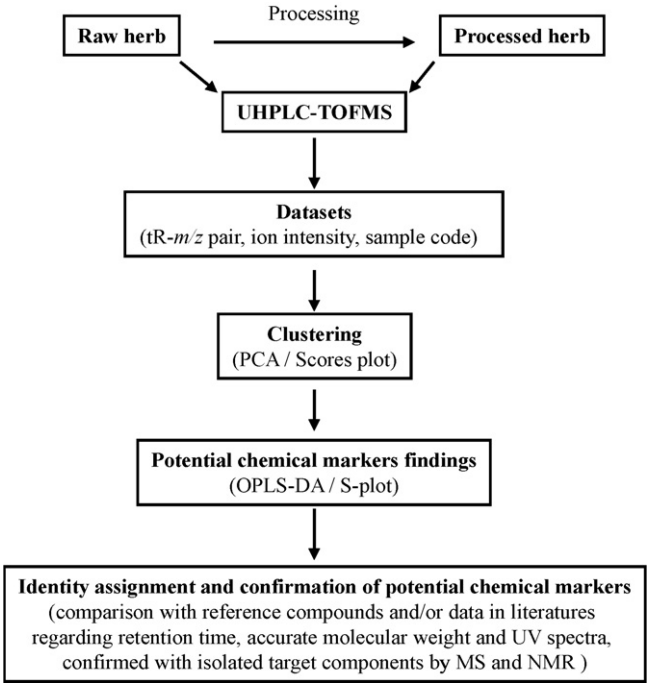
tematic phytochemical studies on processed herbs that can reveal process-induced chemical changes. Thus, limited chemical information is available to select suitable characteristic components as markers for the quality control of processed herbs. Conventional phytochemical strategies for selecting chemical markers, such as extraction, isolation and structure elucidation, are tedious and time-consuming. An effective and efficient approach for determining the processing-induced transforming components is therefore needed.

Ultra high performance liquid chromatography coupled with time-of-flight mass spectrometry (UHPLC–TOFMS) is a newly developed hyphenated technique. The enhanced retention time reproducibility, high chromatographic resolution, improved sensitivity and increased operation speed of UHPLC [6] as well as the accurate and precise mass values of TOFMS have made UHPLC–TOFMS a powerful tool for the metabolomics studies [7–10]. In addition, the accurate mass values of TOFMS can produce candidate empirical formulae which, at the mass error less than 5 ppm, significantly reduce the number of possible structures of putative compounds [11]. In recent years, (U)HPLC–DAD-TOFMS has been increasingly used for rapid global chemical profiling of medicinal herbs [12–14].

Radix Rehmanniae (Dihuang), which is derived from the root of *Rehmannia glutinosa* Libosch, is one of the most important Chinese medicinal herbs. Two types of Radix Rehmanniae are commonly used in TCM practice: raw Radix Rehmanniae, obtained from fresh or dried root of *R. glutinosa*, and processed Radix Rehmanniae, obtained by braising Radix Rehmanniae with rice wine. Traditionally, raw Radix Rehmanniae has been known to "reduce *heat* in blood, nourish *yin* and promote the production of body fluids", and used for treating maculation, nosebleeds, rash, and skin eruptions, while processed Radix Rehmanniae can "nourish *yin* and replenish blood, reinforcing essence and marrow", and has been used for treating anemia, diabetes, dizziness, tinnitus, nocturnal emission and palpitation [15].

The two kinds of Radix Rehmanniae have different effects, but have similar physical characteristics in that they both have irregular shapes, a sticky texture, and are black in color, which make them difficult to distinguish. Although many types of components were successively identified from these two kinds of Radix Rehmanniae, such as iridoids glycosides [16–18], ionone glycosides [19], phenethylalcohol glycosides [20,21], and furfural derivatives [22], etc. (Fig. 1), only 5-hydroxymethyl-2-furadehyde (5-HMF) was used as chemical marker for the quality evaluation of processed Radix Rehmanniae [15]. However, 5-HMF was found to be a common component of other processed herbs [23–25] and so is not characteristic to processed Radix Rehmanniae. More recently, a FT-IR spectroscopy coupled with nonnegative independent component analysis method was developed for characterizing the processing procedure of Radix Rehmanniae [26]; however, this method could not be used to find characteristic chemical markers. Therefore, it is necessary to find more characteristic markers for the discrimination and quality control of these two kinds of Radix Rehmanniae.

In this study, using Radix Rehmanniae as an example, an approach using UHPLC–TOFMS along with multivariate statistical analysis was developed to rapidly find potential chemical markers for the quality control of processed herbs. The protocol was shown in Scheme 1. Batches of raw and processed samples were determined by UHPLC–TOFMS. The datasets of t_R–m/z pair, ion intensity and sample code were subjected to multivariate statistical analysis to holistically compare the difference between raw and processed samples. Once a clear cluster was found, extended statistics was performed to find the variables (ions) that contribute most to the difference. The components that correlate to these ions should be the processing-induced transformed components, and could be regarded as potential chemical markers for the discrimination between raw and processed herbs. The identity of the potential markers can be determined by comparing the mass/UV spectra and retention time with those of the reference compounds and/or tentatively assigned by matching the empirical molecular formula with that of the published compounds.



Scheme 1. The strategy proposed for rapidly exploring potential chemical markers for discrimination of raw and processed herbs by UHPLC–TOFMS coupled with multivariate statistical analysis.

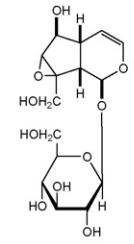
2. Experimental

2.1. Chemicals, solvents and herbal materials

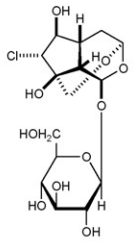
Catalpol (**1**) was purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). 5-(α-D-glucopyranosyl-(1-6)-α-D-glucopyranosyloxymethyl)-2-furancarboxaldehyde (5-GGMF, **4**), 5-(α-D-glucopyranosyloxymethyl)-furancarboxaldehyde (5-GMF, **6**) and 5-HMF (**8**) were previously isolated and identified from Radix Rehmanniae. Their identities were elucidated by UV, MS, NMR analysis [22], and the purity was determined to be more than 98% by HPLC–DAD(MS).

Radix Rehmanniae was collected from Wuzhi County, Henan Province, one of its indigenous cultivating regions in China. Raw Radix Rehmanniae (CMED-0002-01 and CMED-0002-03) was obtained by sun-drying the fresh root of *R. glutinosa*. The processed Radix Rehmanniae (CMED-0002-02) was produced by braising raw Radix Rehmanniae (CMED-0002-01) with rice wine for 20 h, according to the processing method described in Chinese Pharmacopoeia [27]. In addition, commercial Radix Rehmanniae samples (CMED-0002-04 to CMED-0002-22) were purchased from different herbal shops in Hong Kong and mainland China. The voucher specimens were deposited in Chinese Medicine Laboratory, Hong Kong Jockey Club Institute of Chinese Medicine (Table 1).

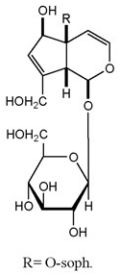
HPLC–MS grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany), and MS grade formic acid from Sigma–Aldrich. Other chemicals and solvents were of analytical



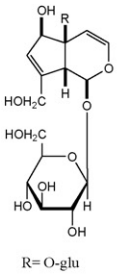
Catalpol (1)



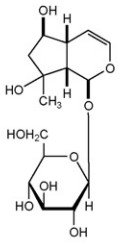
Glutinoside (7)



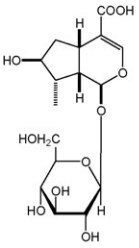
Rehmannioside D (9)



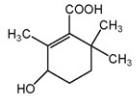
Melittoside (10)



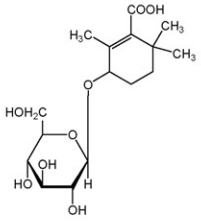
Leonuride (12)



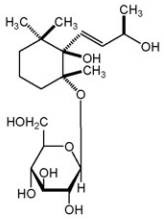
8-Epiloganic acid (16)



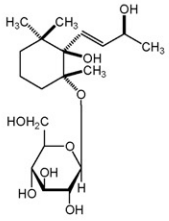
3-hydroxy-2,6,6-trimethyl-1-cyclohexene -1-carboxylic acid (19)



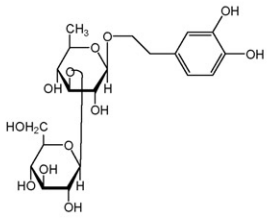
Rehmapicroside (17)



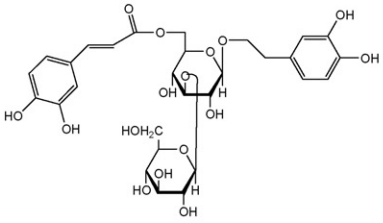
Rehmaionoside A (20 22) 22)



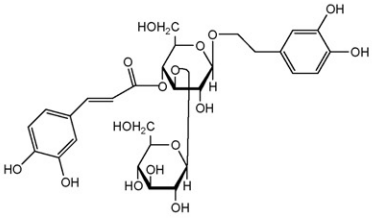
Rehmaionoside B B (20 ,22)



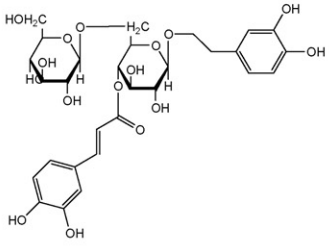
Decaffeoyl-verbascoside (15)



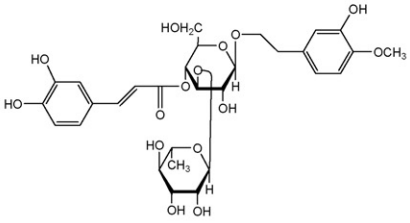
Isoacteoside (13, 23, 25)



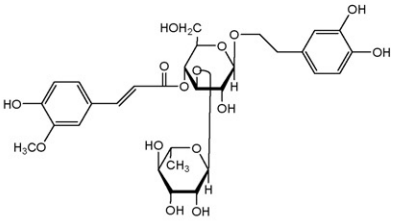
Acteoside (13, 23, 25)



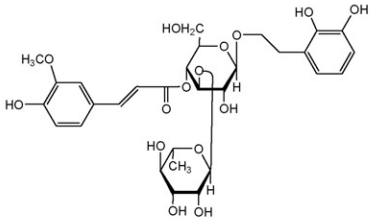
Forsythoside A A (13 ,23 25)



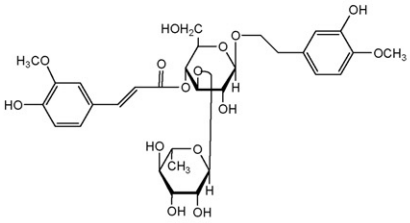
Jionoside D (26)



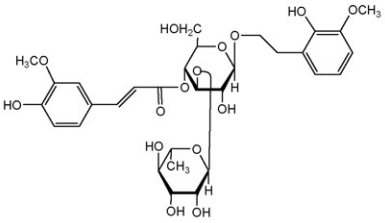
Leucosce ptoside A (26)



Leucosceptoside (26)

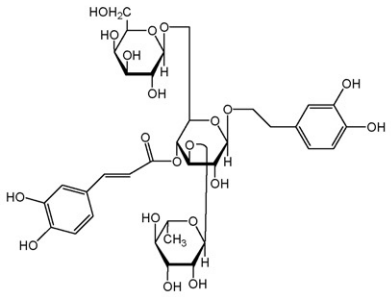


Martynoside (27, 30)

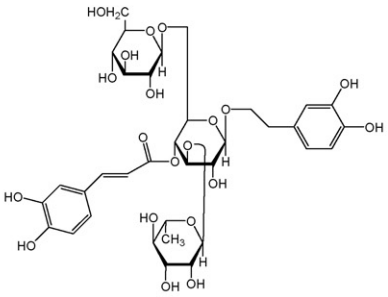


Martynoside isomer (27, 30)

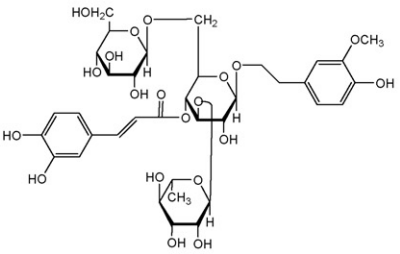
Fig. 1. Chemical structures of major components identified from *Rehmannia glutinosa*. The compound numbers represent the same meanings as in Table 2.



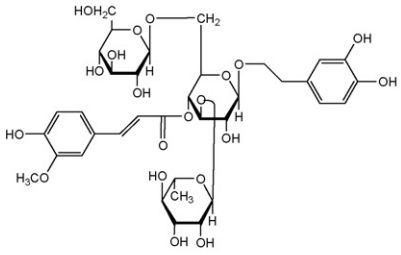
Purpureaside C (18)



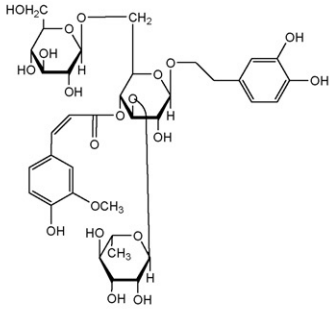
Echinacoside (18)



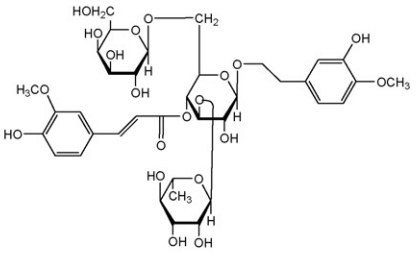
Cistanoside A (21)



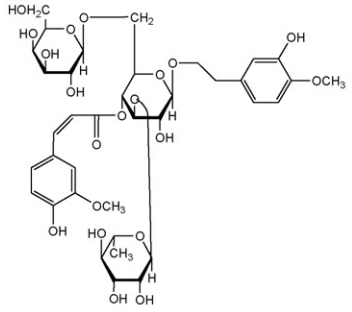
Jionoside A1 (21)



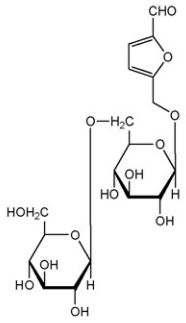
Jionoside A2 (21)



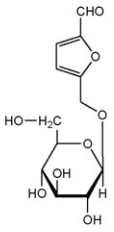
Jionoside B1(24)



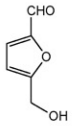
Jionoside B2 (24)



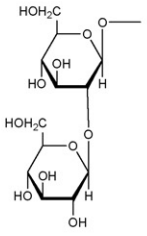
5-(α-D-glucopyranosyl-(1-6)-α-D-glucopyranosyloxymethyl)-2-furancarboxaldehyde (4)



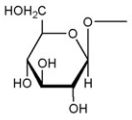
5-(α-D-glucopyranosyloxymethyl)-furancarboxaldehyde (6)



5-hydroxymethyl-2-furfural (7)



sophorosyl



glycosyl

Fig. 1. (*Continued*).

grade. Purified water was prepared in-house with Millipore (Bedford, MA, USA).

2.2. Liquid chromatography

UHPLC was performed using a Waters Acquity UPLC system (Waters, Milford, MA, USA), equipped with a binary solvent delivery system, auto-sampler, and a photodiode-array detection (DAD) system. The chromatography was performed on a Waters Acquity HSS T3 column (100 mm × 2.1 mm I.D., 1.8 μm, Waters, Milford, MA, USA). The mobile phase consisted of (A) water containing 0.1% formic acid and (B) acetonitrile containing 0.1% formic acid. The UHPLC eluting conditions were optimized as follows: isocratic 2% B (0–1 min), linear gradient from 2% to 5% B (1–2 min), 5% to 12%

Table 1
Samples of Radix Rehmanniae analyzed in present study.

Code no.	Type	Location of collection or purchase	Time
CMED-0002-01	Raw (Dried)	Wuzhi County, Henan Province, China	2004-11-02
CMED-0002-02	Processed	Processed from raw Radix Rehmanniae (CMED-0002-01)	2004-11-02
CMED-0002-03	Raw (Dried)	Wuzhi County, Henan Province, China	2005-11-28
CMED-0002-04	Raw (Dried)	One herbal shop, Hong Kong	2005-11-28
CMED-0002-05	Raw (Dried)	Gwong Jyun Taai herbal shop, Hong Kong	2009-06-30
CMED-0002-06	Processed	Gwong Jyun Taai herbal shop, Hong Kong	2009-06-30
CMED-0002-07	Raw (Dried)	Can Fan Gei herbal shop, Hong Kong	2009-06-30
CMED-0002-08	Processed	Can Fan Gei herbal shop, Hong Kong	2009-06-30
CMED-0002-09	Raw (Dried)	Jyu Hing Coeng herbal shop, Hong Kong	2009-06-30
CMED-0002-10	Processed	Jyu Hing Coeng herbal shop, Hong Kong	2009-06-30
CMED-0002-11	Raw (Dried)	Dak Hap Sing herbal shop, Hong Kong	2009-06-30
CMED-0002-12	Processed	Dak Hap Sing herbal shop, Hong Kong	2009-06-30
CMED-0002-13	Raw (Dried)	Ging Hing herbal shop, Hong Kong	2009-06-30
CMED-0002-14	Processed	Ging Hing herbal shop, Hong Kong	2009-06-30
CMED-0002-15	Raw (Dried)	Ming Hing Hou herbal shop, Hong Kong	2009-06-30
CMED-0002-16	Processed	Ming Hing Hou herbal shop, Hong Kong	2009-06-30
CMED-0002-17	Raw (Dried)	Hong Wang herbal shop, Hong Kong	2009-06-30
CMED-0002-18	Processed	Hong Wang herbal shop, Hong Kong	2009-06-30
CMED-0002-19	Raw (Dried)	Dung Sing herbal shop, Hong Kong	2009-06-30
CMED-0002-20	Processed	Dung Sing herbal shop, Hong Kong	2009-06-30
CMED-0002-21	Raw (Dried)	Hang Hing Pharmaceutical company, Hong Kong	2009-06-30
CMED-0002-22	Processed	Hang Hing Pharmaceutical company, Hong Kong	2009-06-30

B (2–5 min), 12% to 20% B (5–10 min), 20% to 30% B (10–12 min), 30% to 50% B (12–13 min), 50% to 100% B (13–15 min), isocratic 100% B for 1 min, and then back to 2% B in 1 min. The flow rate was 0.5 ml/min. The column and auto-sampler were maintained at 35 and 10 °C, respectively. Each wash cycle consisted of 200 μl of strong solvent (80% ACN) and 600 μl of weak solvent (30% ACN). The injection volume is 2 μl. The monitoring UV wavelength was set at 283 nm, and the scan range for DAD was 190–400 nm.

2.3. Mass spectrometry

Mass spectrometry was performed using a Waters QTOF Premier (Micromass MS Technologies, Manchester, UK) equipped with an electrospray ionization (ESI) source operating in positive and negative ion mode, respectively. The nebulization gas was set to 600 l/h at temperature of 300 °C, the cone gas set to 50 l/h, and the source temperature set to 100 °C. The capillary voltage and cone voltage were set to 2700 V and 35 V, respectively. The TOF acquisition rate was set to 0.2 s, with a 0.01 s inter-scan delay. Argon was employed as the collision gas at a pressure of 7.066×10^{-3} Pa.

2.4. Accurate mass measurement

All MS data were acquired using the LockSpray™ to ensure mass accuracy and reproducibility. The $[M-H]^-$ and $[M+H]^+$ ions of Leucine-enkephalin at m/z 554.2615 and m/z 556.2771 were used as the lock mass in negative and positive electrospray ionization mode, respectively. The concentration of Leucine-enkephalin was 50 pg/μl and the infusion flow rate was 10 μl/min. Centroided data were acquired for each sample from 100 to 1200 Da, and dynamic range enhancement (DRE™) was applied throughout the MS experiment to ensure accurate mass measurement over a wide dynamic range.

2.5. Sample preparation

The standard solutions of catalpol, 5-HMF, 5-GGMF and 5-GMF were prepared in 50% (v/v) methanol. The pulverized samples of Radix Rehmanniae from different collections were accurately weighed (approximately 0.5 g), and ultrasonic-extracted with 5.0 ml methanol for 25 min. Then, 5.0 ml water was added, and the mixture was ultrasonic-extracted for another 25 min. The resulting solutions were centrifuged at 3000 r/min, the supernatants were filtered through a 0.2 μm PTFE syringe filter, and an aliquot (2 μl) of each filtrate was subjected to UHPLC–DAD-TOFMS analysis.

Blank 50% (v/v) methanol (2 μl) was injected between selected analysis to validate inter-sample cross-talking effect.

2.6. Establishment of in-house library and peak assignment

By searching from databases, such as PubMed of the U.S. National Library Medicine and the National Institutes of Health, SciFinder Scholar of American Chemical Society and Chinese National Knowledge Infrastructure (CNKI) of Tsinghua University, all components reported in the literatures on Radix Rehmanniae and other *Rehmannia* species were summarized in a Microsoft Office Excel table to establish an in-house library, which includes the name, molecular formula, UV maximum wavelength, chemical structure and literatures of each published known compound. The "*Find*" function of Microsoft Office Excel was used to match the empirical molecular formula with that of published known compounds in the library. The empirical molecular formula was deduced from and short listed by comparing the accurately measured mass value to the exact mass value of putative deprotonated molecular ions $[M-H]^-$ at the mass accuracy of less than 5 ppm.

2.7. Multivariate statistical analysis

The UHPLC–TOFMS data of all determined samples were analyzed by MarkerLynx software (Waters, Manchester, UK) to identify the potential chemical markers for discrimination and quality control of raw and processed Radix Rehmanniae. For data collection, the method parameters were set as follows: retention time range 1.5–8 min, mass range 100–1200 Da, retention time tolerance 0.01 min, mass tolerance 0.01 Da. For peak integration, peak width at 5% height was 1 s, peak-to-peak baseline noise 0.1, peak intensity threshold 10. No specific mass or adduct was excluded. For data analysis, a list of the intensities of the detected peaks was generated using retention time (t_R) and mass data (m/z) pairs as the identifier of each peak. An arbitrary ID was assigned to each of these t_R–m/s pairs in the order of their UHPLC elution for data alignment. This was repeated for each run until the final sample. The data for the entire batch of samples was sorted such that, for each sample, the correct peak intensity data for each t_R–m/s pair was aligned in a table. The ion intensities for each detected peak

were normalized against the sum of the peak intensities within that sample using MarkerLynx. Ions of different samples were considered to be the same ion when they demonstrated the same t_R (tolerance of 0.01 min) and m/s value (tolerance of 0.01 Da). If a peak was not detected in a sample, the ion intensity was documented as zero in the table. The resulting three-dimensional data comprising of peak number (t_R–m/s pair), sample name and ion intensity were analyzed by principle component analysis (PCA) and orthogonal partial least squared discriminant analysis (OPLS-DA) within the MarkerLynx software.

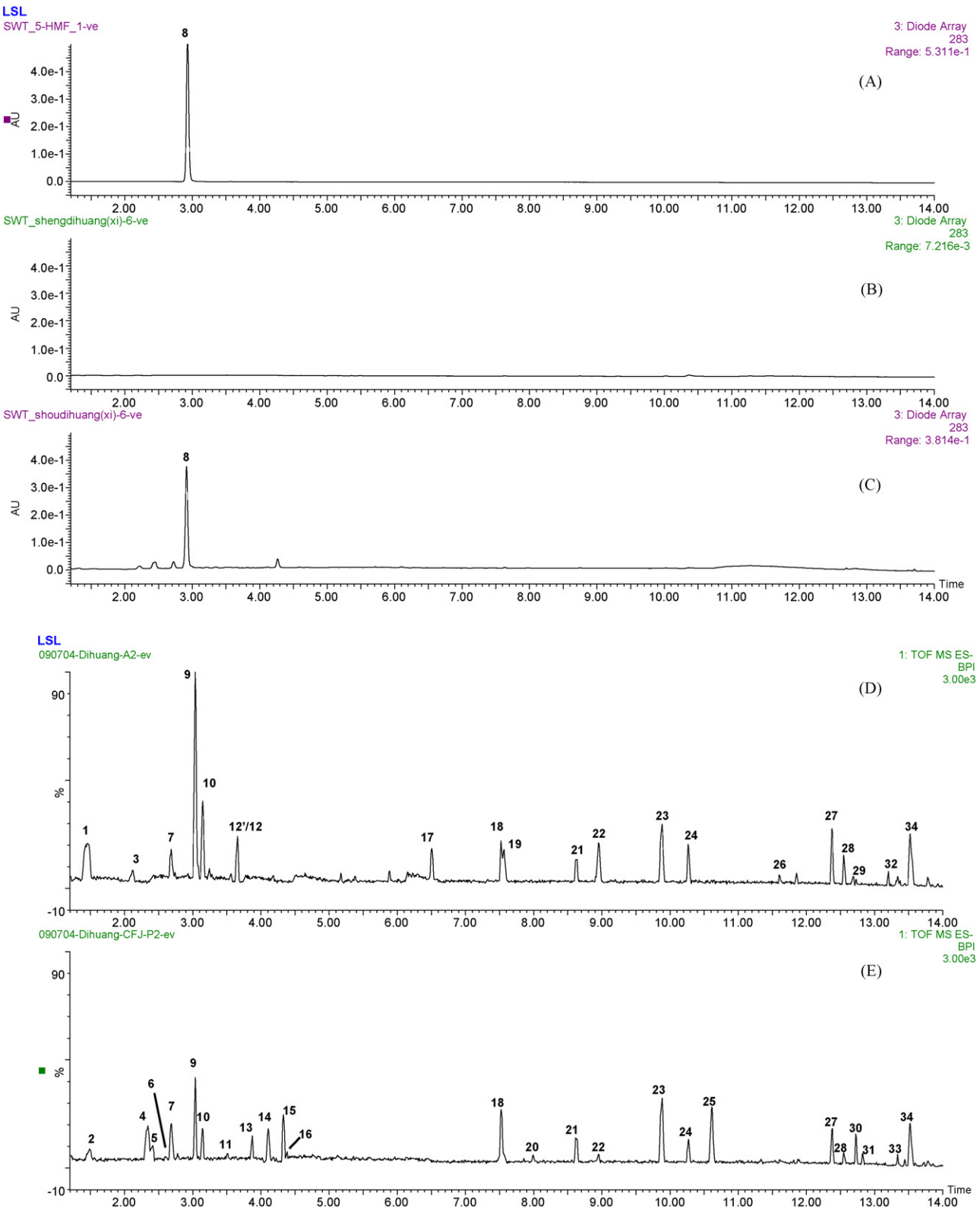


Fig. 2. Representative chromatograms of Radix Rehmanniae analyzed on Waters ACQUITY UPLC HSS T3 (100 mm × 2.1 mm, 1.8 μm) column. A: 5-HMF; B, D: raw Radix Rehmanniae (CMED-0002-01); C, E: processed Radix Rehmanniae (CMED-0002-012); A, B, C: monitored at UV 283 nm; D, E: ES-BPI chromatograms. The peak numbers represent the same meanings as in Table 2.

3. Results and discussion

3.1. Sample preparation

To explore potential chemical markers for the discrimination between raw and processed Radix Rehmanniae, both hydrophilic and hydrophobic components should be extracted and determined. To do this, different concentrations of methanol solvents were tested, and it was found that samples extracted with 50% aqueous methanol showed the greatest number of detectable components in Radix Rehmanniae. In our previous study, glycoside components in medicinal herbs were degraded during extraction with 50% methanol, but when the herbal materials were moistened with 100% methanol before extraction, this degradation was prevented. This phenomenon is probably due to glucosidase evolved hydrolysis of glycoside components [28]. To avoid possible glucosidase evolved hydrolysis of iridoid glycosides, ionone glycosides and phenethylalcohol glycosides in Radix Rehmanniae during extraction, all samples were extracted with 100% methanol first to denature the glucosidase and then equal volumes of water were added to form 50% methanol solvent for the extensive extraction.

3.2. Chromatographic conditions and TOFMS method development

In our preliminary test, two columns, ACQUITY HSS T3 (100 mm × 2.1 mm, 1.8 μm) and ACQUITY BEH C18 (100 mm × 2.1 mm, 1.7 μm), were tested. It was found that the ACQUITY HSS T3 (100 mm × 2.1 mm, 1.8 μm) column has more peak capacity and stronger retention ability as well as better resolution of major components, especially for those hydrophilic components of Radix Rehmanniae under the optimized conditions; thus ACQUITY UPLC HSS T3 (100 mm × 2.1 mm, 1.8 μm) column was chosen for this study. By optimized gradient elution, the main components were separately eluted within 14 min on this column. The representative UV and BPI chromatograms of raw and processed Radix Rehmanniae were illustrated in Fig. 2B–E. From Fig. 2B and C, it was found that few peaks could be monitored by DAD due to the poor UV absorption or low content of most components in Radix Rehmanniae. Only 5-HMF could be detected in processed Radix Rehmanniae, which was confirmed by comparing its retention time and UV spectrum with that of the reference compound (Fig. 2A and C). However, the components of Radix Rehmanniae could be detected by MS in both positive and negative modes (Fig. 2D and E), indicating that the sensitivity of MS under the optimized conditions was adequate for this study.

The sensitivities of the components in Radix Rehmanniae were found to be higher in the negative ion mode. Since the quality of mass spectra is critical in chemical profiling and multivariate statistical analysis, data monitored in negative ion mode was used for the multivariate statistical analysis and component characterization. The positive mode was used to generate accompanying spectra to aid structural elucidation of peaks of interest. Unfortunately, under the present MS conditions, 5-HMF was poorly ionized both in positive and negative ion modes.

3.3. Multivariate statistical analysis and characteristic markers exploring

To compare the difference between raw and processed Radix Rehmanniae, unsupervised principal component analysis (PCA) and supervised orthogonal partial squared discriminant analysis (OPLS-DA) were performed. After Pareto scaling with mean-centering, the data were displayed as scores plot (Fig. 3). The

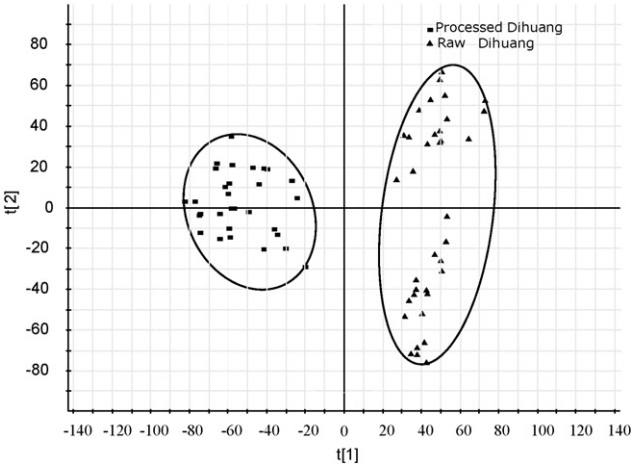


Fig. 3. PCA/Scores plot of raw and processed Radix Rehmanniae samples obtained using Pareto scaling with mean centering.

scores plot shows that the determined samples clearly clustered into two groups, i.e. the raw and the processed Radix Rehmanniae, indicating that the processing procedures caused changes in the composition and/or content of components in Radix Rehmanniae.

To find the potential chemical markers for the discrimination between raw and processed Radix Rehmanniae, the extended statistical analysis was performed to generate S-plot (Fig. 4). In the S-plot, each point represents an ion t_R–m/z pair; the X axis represents variable contribution, where the farther the distance the ion t_R–m/z pair points from zero, the more the ion contributes to the difference between two groups; the Y axis represents variable confidence, where the farther the distance the ion t_R–m/z pair points from zero, the higher the confidence level of the ion to the difference between two groups. So, the t_R–m/z pair points at the two ends of "S" represent characteristic markers with the most confidence to each group.

The first three ions (**a**–**c**) at the bottom left corner of "S" are the ions of processed Radix Rehmanniae contributing most to difference between raw and processed Radix Rehmanniae. The ion intensity trends of these ions in analyzed samples were shown in Fig. 5. It was found that ion **a** (t_R 2.34 min, m/z 449.1283) was detected with higher intensity in all 30 processed samples, but was undetectable in all 36 raw samples. Ion **b** (t_R 4.11 min, m/z 236.0553) and **c** (t_R 2.41 min, m/z 465.1236) were detectable with higher intensity in all 30 processed samples, but were undetectable

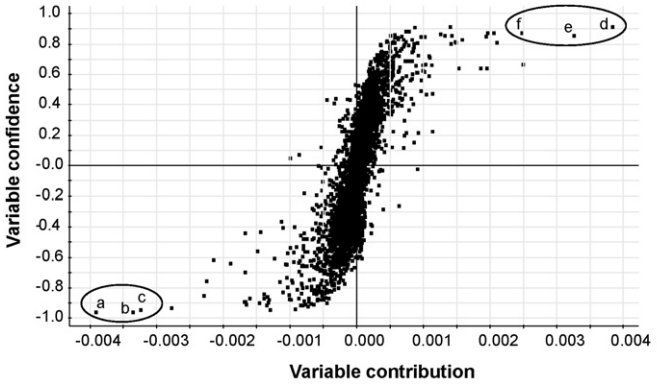


Fig. 4. OPLS-DA/S-Plot of raw and processed Radix Rehmanniae samples. a (t_R 2.34 min, m/z 449.1283); b (t_R 4.11 min, m/z 236.0553); c (t_R 2.41 min, m/z 465.1236); d (t_R 3.66 min, m/z 393.1360); e (t_R 3.64 min, m/z 393.1361); f (t_R 6.51 min, m/z 345.1549).

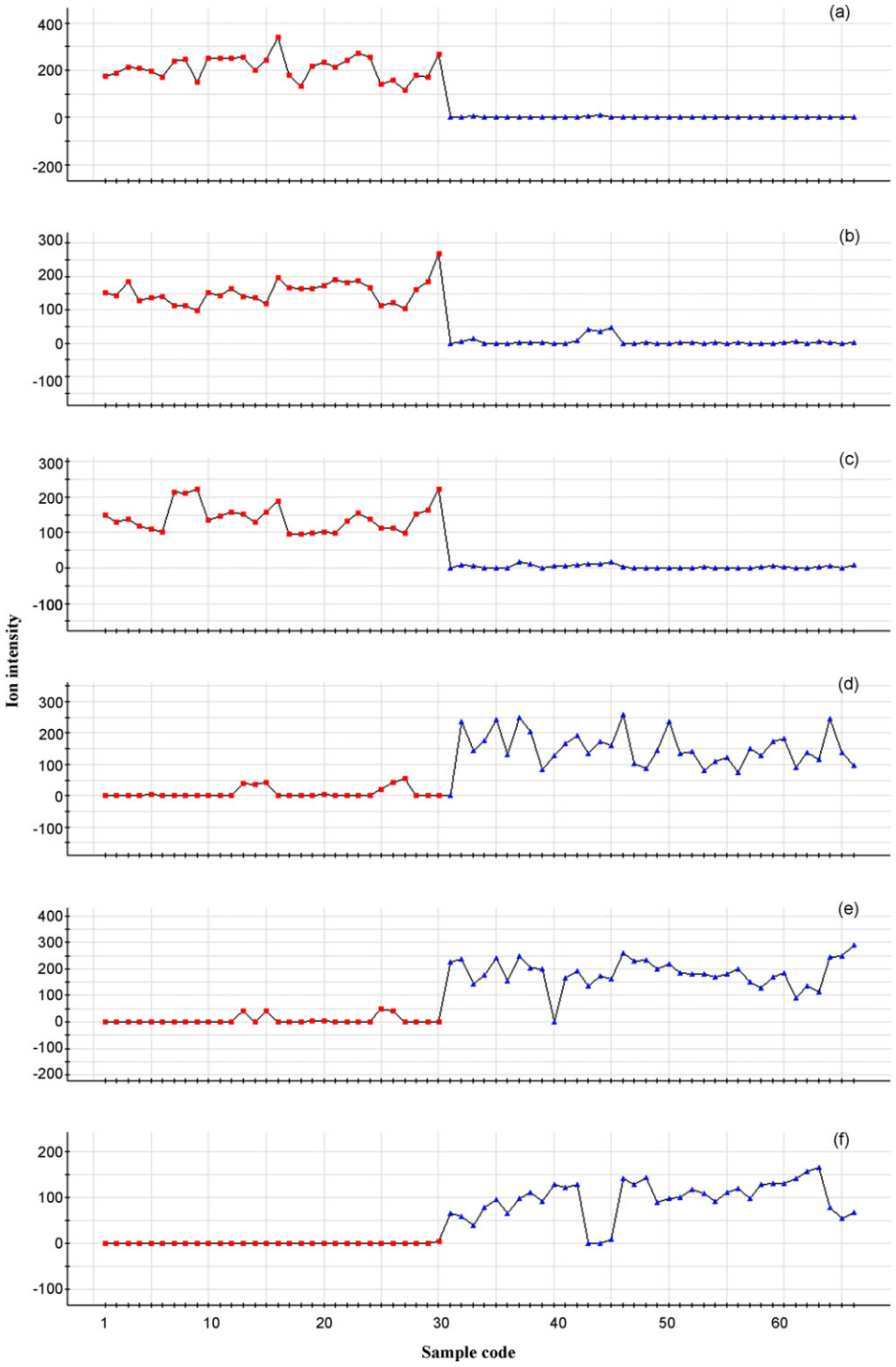


Fig. 5. Selected ion intensity trend plots. a (t_R 2.34 min, m/z 449.1283); b (t_R 4.11 min, m/z 236.0553); c (t_R 2.41 min, m/z 465.1236); d (t_R 3.66 min, m/z 393.1360); e (t_R 3.64 min, m/z 393.1361); f (t_R 6.51 min, m/z 345.1549). ■: processed Radix Rehmanniae samples; ▲: raw Radix Rehmanniae samples

in 33 of the 36 raw samples and in low intensity in the other three raw samples. The ion intensity trends suggested that components that correlate to ion **a**–**c** could be used as potential characteristic markers to distinguish processed Radix Rehmanniae from raw Radix Rehmanniae. In particular, the component that correlates to ion **a** would be the most suitable chemical marker to identify processed Radix Rehmanniae.

Similarly, first three ions **d** (t_R 3.66 min, m/z 393.1360), **e** (t_R 3.64 min, m/z 393.1361) and **f** (t_R 6.51 min, m/z 345.1549) at the top right corner of "S" are the ions of raw Radix Rehmanniae which contribute most to the difference between raw and processed Radix Rehmanniae. The intensity trends showed that these three ions could be detected with higher intensity in 33–36 of the 36 raw samples, but was undetectable in 24–30 of the 30 processed samples. So, the components that correlate to ions **d**–**f** could be regarded as potential chemical markers for the discrimination of raw Radix Rehmanniae from processed Radix Rehmanniae. In particular, the component that correlates to ion **d** would be the most suitable chemical marker to identify raw Radix Rehmanniae.

Table 2
Components identified from *Rehmannia glutinosa*.

Peak no.	t_R (min)	Assigned identity	Molecular formula	UV λ_{max} (nm)	$[M-H]^-$ m/z			$[M+HCOO]^-$ m/z	$[2M-H]^-$ m/z	Reference
					Mean measured mass (Da)	Theoretical exact mass (Da)	Mass accuracy (ppm)			
1#	1.45	Catalpol	$C_{15}H_{22}O_{10}$		361.1131	361.1135	−1.1	407.1182		[17]
2	1.49	Unknown	$C_{22}H_{35}O_{19}$		603.1773	603.1772	−0.2	677.1995		
3	2.12	Unknown	$C_{39}H_{41}O_{16}$		765.2361	765.2395	−4.4			
4#	2.34	5-(α-D-glucopyranosyl-(1-6)-α-D-glucopyranosyloxymethyl)-2-furancarboxaldehyde	$C_{18}H_{26}O_{13}$	283	449.1283	449.1295	−2.7	495.1375		[22]
5	2.41	Unknown	$C_{18}H_{26}O_{14}$	258	465.1236	465.1244	−1.7		931.2740	
6#	2.59	5-(α-D-glucopyranosyloxymethyl)-2-furancarboxaldehyde	$C_{12}H_{16}O_8$	283	287.0764	287.0767	−1.0	333.0847		[22]
7	2.68	Glutinoside	$C_{15}H_{23}ClO_{10}$		397.0900	397.0902	−0.5	443.0965		[29,30]
8#	2.92	5-hydroxymethyl-2-furfural	$C_6H_6O_3$	283						[22]
9	3.04	Rehmannioside D	$C_{27}H_{42}O_{20}$		685.2207	685.2191	2.3	731.2234		[16]
10	3.15	Melittoside	$C_{21}H_{32}O_{15}$		523.1672	523.1663	1.7	569.1704	1047.3507	[31]
11	3.51	Unknown	$C_{11}H_{11}NO_5$		236.0551	236.0559	−3.4			
12	3.64	Leonuride or isomer	$C_{15}H_{24}O_9$		347.1334	347.1342	−2.3	393.1361		[30]
12′	3.66	Leonuride or isomer	$C_{15}H_{24}O_9$		347.1334	347.1342	−2.3	393.1360		[30]
13	3.87	Isoacteoside/Acteoside/Forsythoside A	$C_{26}H_{40}O_{17}$		623.2183	623.2187	−0.6			[32]
14	4.11	Unknown	$C_{11}H_{11}NO_5$		236.0553	236.0559	−3.8			
15	4.33	Decaffeoyl-verbascoside	$C_{20}H_{30}O_{12}$		461.1656	461.1659	−0.7			[21]
16	4.39	8-Epiloganic acid	$C_{16}H_{24}O_{10}$		375.1286	375.1291	−1.3			[17,18]
17	6.51	Rehmapicroside	$C_{16}H_{26}O_8$		345.1549	345.1549	0		691.3695	[33]
18	7.52	Purpureaside C/Echinacoside	$C_{35}H_{46}O_{20}$		785.2510	785.2510	0.8			[32]
19	7.57	3-Hydroxy-2,6,6-trimethyl-1-cyclohexene-1-carboxylic acid	$C_{10}H_{16}O_3$		183.1023	183.1021	2.7			[34]
20	7.99	Rehmaionoside A/Rehmaionoside B	$C_{19}H_{34}O_8$		389.2185	389.2175	2.6			[33]
21	8.64	Cistanoside A/Jionoside A1/Jionoside A2	$C_{36}H_{48}O_{20}$		799.2665	799.2661	0.5			[20]
22	8.95	Rehmaionoside A/Rehmaionoside B	$C_{19}H_{34}O_8$		389.2188	389.2175	3.3	435.2179		[33]
23	9.88	Isoacteoside/Acteoside/Forsythoside A	$C_{29}H_{36}O_{15}$		623.1970	623.1976	−1.0			[20]
24	10.27	Jionoside B1/Jionoside B2	$C_{37}H_{50}O_{20}$		813.2822	813.2817	0.6			[20]
25	10.61	Isoacteoside/Acteoside/forsythoside A	$C_{29}H_{36}O_{15}$		623.1971	623.1976	−0.8			[20]
26	11.60	Jionoside D/Leucosceptoside A/Leucosceptoside	$C_{30}H_{38}O_{15}$		637.2143	637.2132	1.7			[20]
27	12.38	Martynoside	$C_{31}H_{40}O_{15}$		651.2286	651.2289	−0.5			[20]
28	12.55	Unknown	$C_{28}H_{30}O_3$		413.2125	413.2117	1.7		827.4477	
29	12.69	Unknown	$C_{36}H_{55}O_{16}$		743.3499	743.3490	1.2	789.3608		
30	12.72	Martynoside isomer	$C_{31}H_{40}O_{15}$		651.2290	651.2289	0.2			[20]
31	12.82	Unknown	$C_{28}H_{40}O_{10}$		535.2528	535.2543	−2.8			
32	13.20	Unknown	$C_{28}H_{40}O_{10}$		535.2552	535.2543	1.7			
33	13.34	Unknown	$C_{20}H_{34}O_9$		417.2119	417.2125	−1.4			
34	13.52	Unknown	$C_{18}H_{34}O_5$		329.2328	329.2328	0			

Identified with reference compounds.

3.4. Identity assignment and confirmation of chemical markers **a** and **d**

The identities of potential markers **a** and **d** together with major peaks detected in two types of Radix Rehmanniae were identified or tentatively assigned by comparison with the reference compounds or matching the empirical molecular formula with that of the published compounds of Radix Rehmanniae or *Rehmannia* species [29–34]. Catapol (peak 1), 5-GGMF (peak 4), 5-GMF (peak 6) and 5-HMF (peak 8) were confirmed by comparing the mass/UV spectra and retention time with that of reference compounds. The details of the identified components were summarized in Table 2.

As shown in Table 2, the mass accuracy for all assigned components was less than 5 ppm. When several empirical molecular formulae matched the same formula, those isomeric components previously reported from Radix Rehmanniae or other *Rehmannia* species would be preferentially selected as the putative components with chemotaxonomy considerations. In negative ion mode, owing to the addition of formic acid to the mobile phase as a modifier, the adduct ions $[M+HCOO]^-$ could be observed in the mass spectra of many components. In addition, dimmer ions $[2M-H]^-$ were found in the mass spectra of some components. In positive ion mode, adduct ions $[M+Na]^+$, $[M+K]^+$ and $[M+NH_4]^+$, and occasionally fragment ions could be found in the mass spectra of many components (Fig. 6). All these quasi-molecular ions or fragment ions could help the confirmation of $[M-H]^-$ or $[M+H]^+$ ions for the generation of empirical molecular formula.

Fig. 6 shows the representative mass spectra in both positive and negative modes of potential marker **a** (peak 4). The mass spectra and accurate deprotonated molecular weight in negative mode suggested that its empirical molecular formula is most likely (with mass accuracy −2.7 ppm) $C_{18}H_{26}O_{13}$, which matches that of 5-

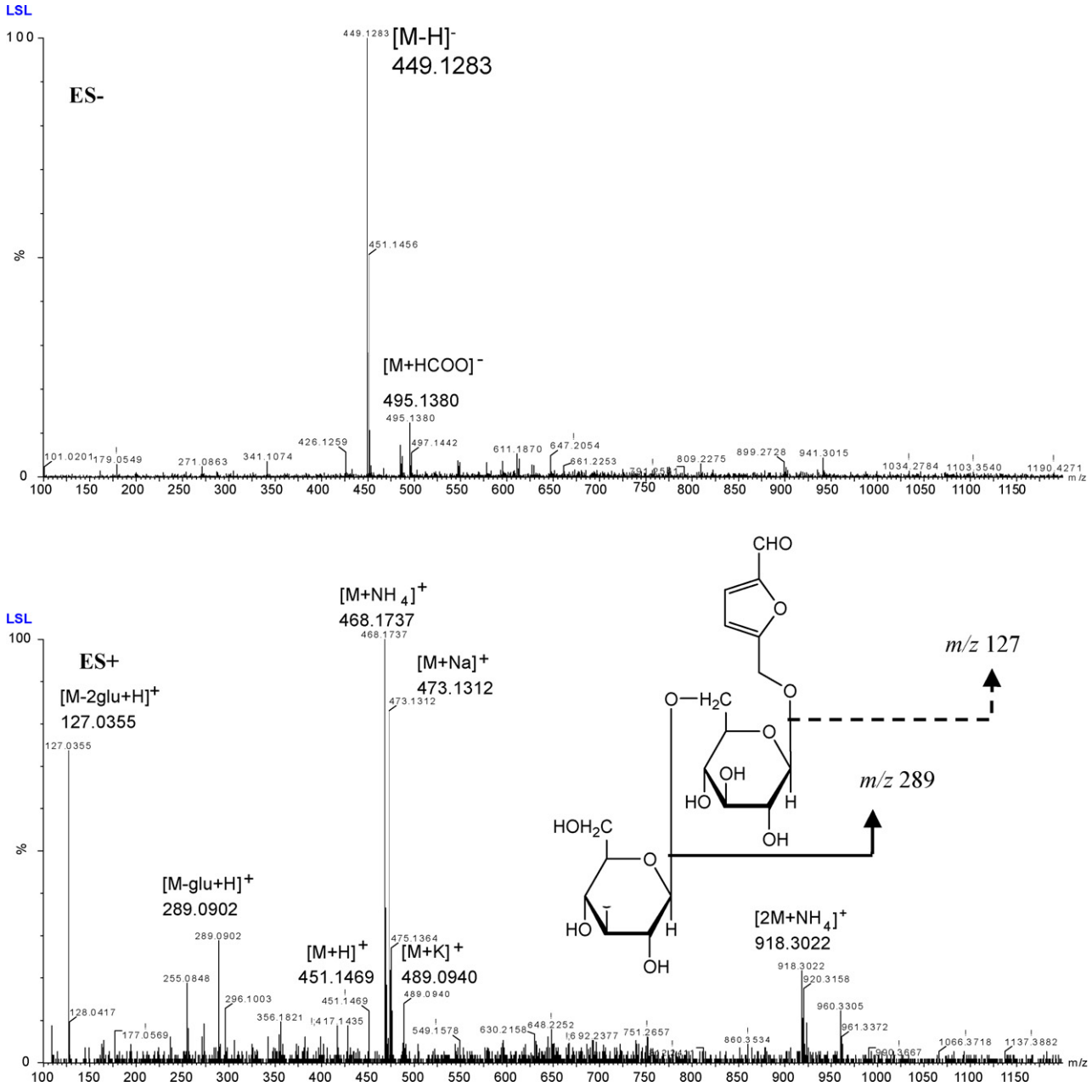


Fig. 6. Representative mass spectra of marker **a** detected in both positive and negative mode.

GGMF, a furfural derivative previously identified from processed Radix Rehmanniae in our lab [22]. In the mass spectrum of positive ion mode, fragment ions of m/z 289.0902 and m/z 127.0355 indicated the successive losses of two glucosyl units (162 Da), further supporting that this component is 5-GGMF. The assignment was extensively confirmed by comparing the retention time, UV and mass spectra with that of previously isolated reference compounds which were elucidated with UV, MS and NMR analysis by our team [22].

Similarly, in the mass spectrum of potential marker **d** (peak 12′), two ions, m/z 347.1334 and m/z 393.1360, were found. These two ions were deduced to be $[M-H]^-$ and $[M+HCOO]^-$, respectively, and the empirical molecular formula was presumed to be $C_{15}H_{24}O_8$, which matches that of leonuride [30]. Since ion **e** (peak 12) has almost identical retention time (3.64 min) and mass value (m/z 393.1361) to ion **d** (3.66 min, m/z 393.1360), the components correlated to ions **d** and **e** were tentatively assigned as leonuride or its isomer. Therefore, the most characteristic components of raw and processed Radix Rehmanniae were identified to be leonuride/leonuride isomer and 5-GGMF, respectively. These two components can be used as potential chemical markers to discriminate between raw and processed Radix Rehmanniae, and evaluate the extent of processing.

4. Conclusion

A novel strategy to rapidly determine potential chemical markers for the discrimination and quality control of raw and processed Chinese medicinal herbs by UHPLC–TOFMS coupled with multivariate statistical analysis was proposed, and validated using Radix Rehmanniae as a model herb. Unlike conventional phytochemical approaches, which require the tedious and time-consuming characterization of large numbers of components, most of which are contained in both raw and processed herbs, this new approach can avoid replication in isolation, purification and identification of the identical components in both raw and processed herbs, and is therefore a cost-effective way to determine potential chemical markers of processed herbs. Furthermore, with multivariate statistic analysis of large numbers of samples, the determined markers should be more representative. The successful identification of markers for the discrimination of processed Radix Rehmanniae from raw ones suggests that this strategy might also be used for the investigation of chemical transforming mechanisms underlying herb processing.

Acknowledgement

This research is funded by the Hong Kong Jockey Club Charities Trust.

References

[1] S.Y. Jin, Q. Wang, Studies on Processing of Chinese Medicinal Yinpian and Its Clinical Application, Press of Chemistry Industry, Beijing, 2004.
[2] B.C. Cai, Q.F. Gong, Processing of Chinese Medicinal Herbs, People Health Press, Beijing, 2009.
[3] Chinese Pharmacopeia Commission, Pharmacopeia of the People's Republic of China, vol. 1, Chemical and Industrial Publisher, Beijing, 2005, p. 34.
[4] B.C. Cai, H. Wu, S. Kadota, M. Hattori, T. Namba, Processing of Nux vomica. III. Effects of seven different processing of Nux vomica on chemical constituents and acute toxicity, J. Trad. Med. 11 (1994) 134–141.
[5] B.C. Cai, T. Nagasawa, S. Kadota, M. Hattori, T. Namba, Y. Kuraishi, Processing of Nux vomica. VII. Antinociceptive effects of crude alkaloids from the processed and unprocessed seeds of Strychnos nux-vomica in mice, Biol. Pharm. Bull. 19 (1996) 127–131.
[6] I.C. Mona, C.T. Nathan, R.M. Larry, R.D. Daniel, Improving LC–MS sensitivity through increases in chromatographic performance: comparisons of UPLC–ES/MS/MS to HPLC–ES/MS/MS, J. Chromatogr. B 825 (2005) 134–143.
[7] M. Brown, W.B. Dunn, P. Dobson, Y. Patel, C.L. Winder, S. Francis-McIntyre, P. Begley, K. Carroll, D. Broadhurst, A. Tseng, N. Swainston, I. Spasic, R. Goodacre, D.B. Kell, Mass spectrometry tools and metabolite-specific databases for molecular identification in metabolomics, Analyst 134 (2009) 1322–1332.
[8] E. Zelena, W.B. Dunn, D. Broadhurst, S. Francis-McIntyre, K.M. Carroll, P. Begley, S. O'Hagan, J.D. Knowles, A. Halsall, I.D. Wilson, D.B. Kell, Development of a robust and repeatable UPLC–MS method for the long-term metabolomic study of human serum, Anal. Chem. 81 (2009) 1357–1364.
[9] P.A. Guy, I. Tavazzi, S.J. Bruce, Z. Ramadan, S. Kochhar, Global metabolic profiling analysis on human urine by UPLC–TOFMS: issues and method validation in nutritional metabolomics, J. Chromatogr. B 871 (2008) 253–260.
[10] A. Nordström, G. O'Maille, C. Qin, G. Siuzdak, Nonlinear data alignment for UPLC–MS and HPLC–MS based metabolomics: quantitative analysis of endogenous and exogenous metabolites in human serum, Anal. Chem. 78 (2006) 3289–3295.
[11] E.C.Y. Chan, S.L. Yap, A.J. Lau, P.C. Leow, D.F. Toh, H.L. Koh, Ultra-performance liquid chromatography/time-of-flight mass spectrometry based metabolomics of raw and steamed *Panax notoginseng*, Rapid Commun. Mass Spectrom. 21 (2007) 519–528.
[12] F.M. Li, X.M. Lu, H.P. Liu, M. Liu, Z.L. Xiong, A pharmaco-metabonomic study on the therapeutic basis and metabolic effects of *Epimedium brevicornum* Maxim. on hydrocortisone-induced rat using UPLC–MS, Biomed. Chromatogr. 21 (2007) 397–405.
[13] E. Grata, J. Boccard, G. Glauser, P.A. Carrupt, E.E. Farmer, J.L. Wolfender, S. Rudaz, Development of a two-step screening ESI-TOF-MS method for rapid determination of significant stress-induced metabolome modifications in plant leaf extracts: the wound response in Arabidopsis thaliana as a case study, J. Sep. Sci. 30 (2007) 2268–2278.
[14] S.L. Li, J.Z. Song, F.F.K. Choi, C.F. Qiao, Y. Zhou, Q.B. Han, H.X. Xu, Chemical profiling of Radix Paeoniae evaluated by ultra-performance liquid chromatography/photo-diode-array/quadrupole time-of-flight mass spectrometry, J. Pharm. Biomed. Anal. 49 (2009) 253–266.
[15] Chinese Pharmacopeia Commission, Pharmacopeia of the People's Republic of China, vol. 1, Chemical and Industrial Publisher, Beijing, 2005, pp. 82–83.
[16] H. Oshio, H. Inouye, Iridoid glycosides of *Rehmannia glutinosa*, Phytochemistry 21 (1981) 133–138.
[17] T. Morota, H. Sasaki, H. Nishimura, K. Sugama, M. Chin, H. Mitsuhashi, Chemical and biological studies on Rehmanniae radix. Part 4. Two iridoid glycosides from Rehmannia glutinosa, Phytochemistry 28 (1989) 2149–2153.
[18] H. Nishimura, K. Sugama, T. Morota, M. Chin, H. Mitsuhashi, Chemical and biological studies on Rehmanniae radix. Part 3. Six iridoid glycosides from Rehmannia glutinosa, Phytochemistry 28 (1989) 2705–2709.
[19] M. Yoshikawa, Y. Fukuda, T. Taniyama, B.C. Cha, I. Kitagawa, Absolute configurations of rehmaionosides A, B, and C and rehmapicroside three new ionone glucosides and a new monoterpene glucoside from Rehmanniae radix, Chem, Pharm. Bull. 34 (1986) 2294–2297.
[20] H. Sasaki, H. Nishimura, M. Chin, T. Morota, H. Mitsuhashi, Hydroxycinnamic acid esters of phenethylalcohol glycosides from *Rehmannia glutinosa* var. *Purpurea*, Phytochemistry 28 (1989) 875–879.
[21] H. Nishimura, H. Sasaki, T. Morota, M. Chin, H. Mitsuhashi, Six glycosides from *Rehmannia glutinosa* var. *Purpurea*, Phytochemistry 29 (1990) 3303–3306.
[22] A.S. Lin, K.D. Qian, Y. Usami, L. Lin, H. Itokawa, C.H. Susan, L. Morris-Natschke, K.H. Lee, 5-Hydroxymethyl-2-furfural, a clinical trials agent for sickle cell anemia, and its mono/di-glucosides from classically processed steamed Rehmanniae Radix, J. Nat. Med. 62 (2008) 164–167.
[23] W.F. Du, H. Cai, M.Y. Wang, Ding, H. Yang, B.C. Cai, Simultaneous determination of six active components in crude and processed Fructus Corni by high performance liquid chromatography, J. Pharm. Biom. Anal. 48 (2008) 194–197.
[24] P.Y. Liao, D. Wang, Y.J. Zhang, C.R. Yang, Dammarane-type glycosides from steamed notoginseng, J. Agric. Food Chem. 56 (2008) 1751–1756.
[25] Q. Xu, Y.H. Li, X.Y. Lu, Investigation on influencing factors of 5-HMF content in *Schisandra*, J. Zhejiang Univ. Sci. B 8 (2007) 439–445.
[26] G.Q. Wang, C.H. Dong, Y.K. Shang, Y.A. Sun, D.X. Fu, J.B. Zhao, Characterization of radix rehmanniae processing procedure using FT-IR spectroscopy through nonnegative independent component analysis, Anal. Bioanal. Chem. 394 (2009) 827–833.
[27] Chinese Pharmacopeia Commission, Pharmacopeia of the People's Republic of China, vol. 1, Chemical and Industrial Publisher, Beijing, 2005, Appendix II D, Appendix 20.
[28] S.L. Li, Q.B. Han, C.F. Qiao, J.Z. Song, C.L. Cheng, H.X. Xu, Chemical markers for the quality control of herbal medicines: an overview, Chin. Med. 3 (2008) 1–16.
[29] M. Yoshikawa, Y. Fukuda, T. Taniyama, I. Kitagawa, Absolute stereostructures of rehmaglutin C and glutinoside a new iridoid lactone and a new chlorinated iridoid glucoside from Chinese rehmanniae radix, Chem. Pharm. Bull. 34 (1986) 1403–1406.
[30] I. Kitagawa, Y. Fukuda, T. Taniyama, M. Yoshikawa, Chemical studies on crude drug processing. VIII. On the constituents of Rehmanniae Radix. (2): absolute stereostructures of rehmaglutin C and glutinoside isolated from Chinese Rehmanniae Radix, the dried root of Rehmannia glutinosa Libosch, Chem. Pharm. Bull. 43 (1995) 1096–1100.
[31] T. Morota, H. Sasaki, K. Sugama, H. Nishimura, M. Chin, Chemical and biological studies on Rehmanniae Radix. Part 6. Two nonglycosidic iridoids from *Rehmannia glutinosa*, Phytochemistry 29 (1990) 523–526.
[32] H. Sasaki, H. Nishimura, T. Morota, M. Chin, H. Mitsuhashi, Y. Komatsu, H. Maruyama, G.R. Tu, W. He, Y.L. Xiong, Chemical and biological studies on rehmanniae radix. Part 1. Immunosuppressive principles of Rehmannia glutinosa var. hueichingensis, Planta Med. 55 (1989) 458–462.

[33] M. Yoshikawa, Y. Fukuda, T. Taniyama, I. Kitagawa, Chemical studies on crude drug processing. IX. On the constituents of Rehmanniae Radix. (3). Absolute stereo-structures of rehmaionosides A, B, and C, and rehmapicroside, biologically active ionone glucosides and a monoterpene glucoside isolated from Chinese Rehmanniae Radix, Chem. Pharm. Bull. 44 (1996) 41–47.

[34] H. Sasaki, T. Morota, H. Nishimura, T. Ogino, T. Katsuhara, K. Sugama, M. Chin, H. Mitsuhashi, Chemical and biological studies on Rehmanniae Radix. Part 8. Norcarotenoids of *Rehmannia glutinosa* var. *hueichingensis*, Phytochemistry 30 (1991) 1997–2001.