Traditional Processing Strongly Affects Metabolite Composition by Hydrolysis in *Rehmannia glutinosa* Roots

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Received September 29, 2010; accepted February 1, 2011; published online February 18, 2011

The processing of biological raw materials is considered to have an important role in the therapeutic application in Traditional Chinese Medicine. The root of *Rehmannia glutinosa* has to be processed by nine cycles of rice wine immersing, steaming and drying before using in clinical applications. In order to understand the chemical changes resulting from the processing, a comprehensive analysis of Rehmanniae radix was made using ^{1}H-NMR and Fourier transform (FT)-mass spectrometry in combination with multivariate data analysis. After ^{1}H-NMR and principle component analysis, hydrolysis was found to be the major chemical process during the treatments. Catalpol, raffinose and stachyose levels gradually decreased during processing, whereas monosaccharides including galactose and glucose were found to be higher in processed roots. The metabolic profile changed gradually through the processing cycles although the differences became smaller after the fifth processing cycle. The positive and negative ion-mode mass spectra by high resolution FT-MS revealed several series of ion clusters with mass differences of 162.053 Da, indicating a difference of a hexose moiety. During the processing, the number and signal intensity of the smaller glycosides were increased. Therefore, these results indicate that the fresh Rehmanniae radix is rich in polysaccharides, which are hydrolyzed during the processing.

Key words *Rehmannia glutinosa*; traditional processing; Fourier-transform-mass spectrometry; principle component analysis

The processing of medicinal plant materials has a history as long as Traditional Chinese Medicine (TCM) itself. The importance of the processing of materials has already been mentioned in the Huang Di Nei Jing (The Yellow Emperor's Internal Classic, 475-221 B.C.) and Shen Nong Ben Cao Jing (Divine Husbandman's Classic of the Materia Medica, *c.* 220 A.D.) .[1)] Depending on the therapeutic application, the same plant material can be processed differently. The processing of materials is thought to have the following functions: reduction of toxicity and side effects, to potentiate biological effects, to change properties or functions, to preserve the active constituents, to facilitate the administration, to correct an unpleasant taste or to increase its purity by reduction of contaminations such as soil.[2)] In current TCM practice, all the materials are strictly required to be properly processed before using for therapeutic application. Understanding the metabolic changes during processing is of great important for quality control Chinese medicinal herbal materials.

Rehmannia glutinosa Libosch. (Scrophulariaceae) is one of the most popular medicinal plants whose roots used in TCM for treating of metabolic related disorders. According to the traditional uses, Rehmanniae radix has curing activity on disorders of liver and kidneys, hectic fever, night sweat and dizziness.[3)] It has been reported that Rehmanniae radix has a wide range of clinical activities including haemostatic, promoting blood coagulation, cardiotonic, diuretic and anti-inflammatory activities.[4)] Hypoglycemic effects,[5,6)] anti-tumor activity,[7,8)] immediate type allergic reaction inhibition,[9)] and tumor necrosis factor-α (TNF-α) secretion inhibiting activity[3)] were also found in the extracts of *R. glutinosa* roots. In addition, Rehmanniae radix stimulates the proliferation and activities of osteoblasts, suggesting a potential to treat osteoporosis by enhancing the metabolism.[10)]

There are two types of Rehmanniae radix used as medicinal herb, a non-processed (dried root) and a processed one. They are used in quite different therapeutic applications and the choice is strictly defined in TCM theory and practice. The dried, non-processed Rehmanniae radix has a "cold" property according to TCM theory. It means that Rehmanniae radix is able to cure "heat" symptoms. In the modern literature, these non-processed roots have reported to have the following major pharmacological effects such as the effects on plasma corticosterone concentration and preventing the adrenal cortex from atrophy in rabbits[11)]; a cardiac effect in the isolated frog heart model[12)]; anti-inflammatory and immunosuppressive effects in rat[12)] and an hypoglycemic activity in spontaneous diabetic mice.[13)] However, the processed Rehmanniae radix treated by steaming and drying for several cycles (generally nine times) have a slightly "warm" property that means Rehmanniae radix is good for treatment of "cold" symptoms. The major pharmacological effect has been reported to prevent an induction of impediment in the peripheral microcirculation against various chronic diseases through the improvement of hemorheology,[14,15)] Recently, three 5-hydroxymethylfurfural (5-HMF) derivates have been found in processed Rehmanniae radix only. 5-Hydroxymethyl-2-furfural, one of three 5-HMF derivates which obtained by thermal degradation of saccharine is a phase I clinical trials agent for sickle cell anemia.[16)] A pharmacological study of Rehmanniae radix, indicated that the processed

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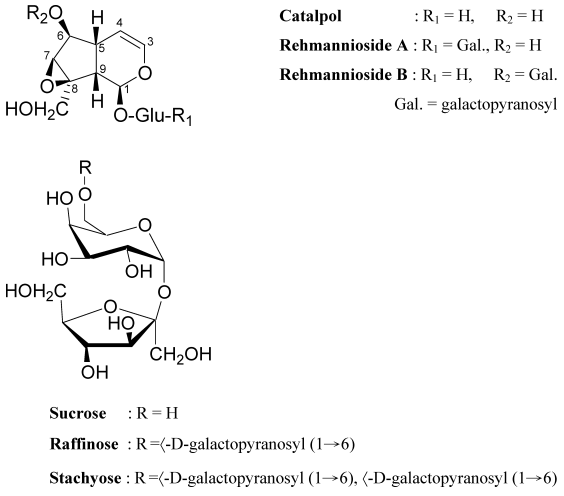

Fig. 1. Chemical Structures of Typical Iridoids and Carbohydrates in *Rehmannia glutinosa* Roots

roots have a stronger hypoglycemic effect in the type-II diabetes mice model induced by fructose/STZ than the non-processed one. The more processing cycles, the more potent the roots are.[17] Consequently, the obvious discrepancy between the features of two types of materials in therapeutic applications has raised great interest in the metabolic alterations during the processing. Understanding the metabolites changes during the processing is a base for further investigation the bioactivity of herbal materials.

The main constituents of Rehmanniae radix are iridoid glycosides, including catalpol (Fig. 1), leonuride and rehmannioside A, B, C and D, as well as rehmaglutins *etc.*[18—21] In addition to these iridoids, high levels of monosaccharides and amino acids were reported as its constituents.[22]

Although both quantitative and qualitative changes in the metabolic profile are assumed to occur during processing, previous research has been limited to sugars and catalpol contents.[17] It was reported that the amounts of several major components in Rehmanniae radix have changed in processed products, in particular, catalpol and stachyose. After every processing cycle, the amount of these two components is lower.[17] However, because of the complex mixture of compounds, it is difficult to know which compounds contribute to the bioactivity, and the changes in concentration of major and even minor compounds may have a significant impact.[23] Some studies regarding the comprehensive analysis of metabolites of Rehmanniae radix after different processing on biological activity steps have been reported, *e.g.*, infrared (IR) spectroscopy was applied in quality control of Rehmanniae radix preparations.[24,25] We have reported the GC-MS analysis combined with multivariate statistics to measure the metabolite fingerprint of Rehmanniae radix from growth conditions in different processing cycles.[26] This study provided a holistic strategy on the metabolic profile changes during processing of Rehmanniae radix. To have a complete view on metabolic changes, other methods with a different coverage of molecular types and ranges are needed as well.

To extend our previous GC-MS study, nuclear magnetic resonance (NMR) and mass spectrometry (MS) are applied in this study since it is currently accepted as general tools for metabolomic analysis. NMR spectroscopy has the advantage of a macroscopic and quantitative metabolic differentiation tool due to its high reproducibility and ease of quantization. Mass spectrometry has the advantage of high sensitivity and high resolution and certain metabolites might be detected as ng level. Consequently, the combination of high resolution MS with NMR is expected to provide more diverse and detailed metabolic information. Here, we report the use of NMR and high resolution Fourier-transform-MS (FT-MS) in combination with multivariate data analysis for studying the effect of processing on the metabolite composition of Rehmanniae radix. In combination with our previous GC-MS study and the present study (NMR, FT-MS), we are able to provide a comprehensive technology platform for studying the changes of metabolites of Rehmanniae radix during processing. This is an important basis for establishment of a tool for quality control of herbal medicines in TCM. By eventually coupling bioactivity data with the metabolomic data it will be possible to define a metabolomic quality profile.

Results and Discussion

^{1}H-NMR spectra of fresh Rehmanniae radix extract showed a wide array of metabolites including iridoids, monosaccharides, oligosaccharides, and amino acids. In order to observe the macroscopic metabolic changes in plants, principle component analysis (PCA) was applied to ^{1}H-NMR spectra of all samples from the two growth conditions (22, 25 °C) and from the different processing cycles. For the data set from the analysis of the extracts, a six-component model was able to explain 92.7% of the variance.

Examination of the score plots in PCA for PC1 *vs.* PC2 revealed that the various Rehmanniae radix samples were clearly separated from each other. Changes in the composition due to the processing are mainly described by PC1. An increasing number of processing cycles moved the Rehmanniae radix samples to the lower PC1 values in the score plot (Fig. 2A).

For evaluation of contributing metabolites, bi-plots containing correlations of all variables (^{1}H-NMR resonances) and scores of Rehmanniae radix samples were investigated (Fig. 2B). Using the bi-plot of correlations, resonances of anomeric protons of carbohydrates at δ 4.4—5.5 were proven to be the characteristic fingerprint region for each sample. The H-1 signal of the glucose moiety in raffinose, stachyose, and sucrose were detected at δ 5.40 (d, J=3.8 Hz). Anomeric signals of internal α-galactose and α-glucose were clearly visible at δ 5.0 (d, J=3.8 Hz). Clusters at δ 5.18 (d, J=3.8 Hz) and δ 4.58 (d, J=8.0 Hz) were identified as H-1 of the α- and β-form of monosaccharides such as glucose and galactose, respectively. The overlapping ^{1}H-NMR resonances in this region were identified by two dimensional J-resolved spectra (Fig. 3) and confirmed by ^{13}C-chemical shifts obtained from the heteronuclear single quantum coherence (HSQC) spectra. Raffinose and stachyose are more abundant in the dried roots than in the processed ones. This might be due to the possible hydrolysis of these oligosaccharides during processing of Rehmanniae radix.

The resonances of H-3 at δ 6.40 (dd, J=6.0, 1.8 Hz), H-4 at δ 5.13 (dd, J=5.9, 4.6 Hz), H-1′ at δ 5.04 (d, J=9.8 Hz), H-9 at δ 2.60 (dd, J=9.6, 7.7 Hz), and H-5 at δ 2.29 (m) were identified by comparison with the spectra of a reference sample of catalpol. Loading values of catalpol signals are

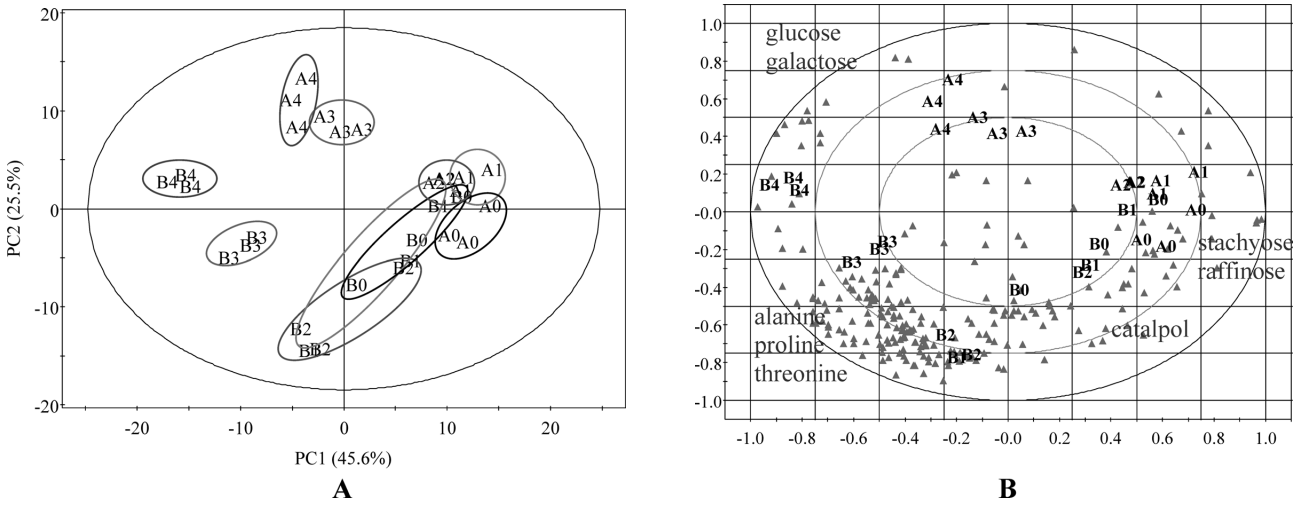

Fig. 2. Score Plot (A) and Correlation Loading Plot (B) of Principal Component Analysis Based on ^{1}H-NMR Spectra of *Rehmannia glutinosa* Roots

A group: plants root grown at 22 °C; B group: plants grown at 25 °C; 0: fresh roots; 1: dried roots, 2: roots processed by rinsing with Chinese rice wine 15 min and steamed for 60 min, then baked at 55 °C for dry (repeated by one cycle); 3: roots processed in the same way and repeated by five cycles); 4: roots processed in the same way and repeated by nine cycles). The ellipse represents the Hotelling T2 with 95% confidence in score plots.

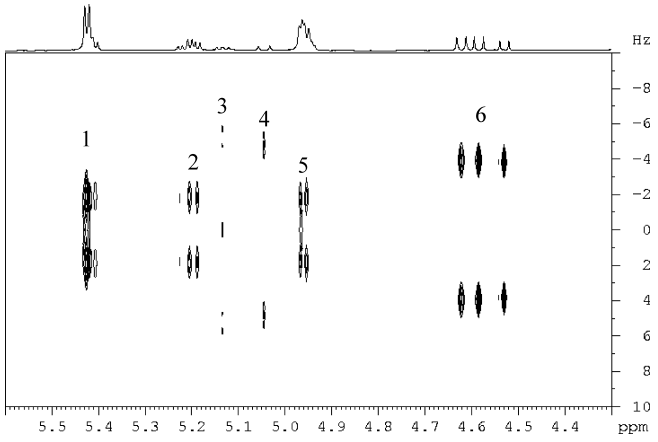

Fig. 3. Two Dimensional *J*-Resolved NMR Spectra of Processed *Rehmannia glutinosa* Roots in the Range of δ 4.3—5.6

1: H-1 of raffinose, stachynose and sucrose; 2: H-1 of α-glucose and α-galactose; 3: H-4 of catalpol; 4: H-1′ of catalpol; 5: H-1 of internal α-glucose and α-galactose moiety of raffinose and stachynose; 6: H-1 of β-glucose and β-galactose.

placed close to scores of non-processed roots in a bi-plot of correlations (Fig. 2B). It indicates that non-processed Rehmanniae radix contains more catalpol-type iridoids than processed ones. The oxidative degradation of the aglycon might occur during processing.

In order to confirm the oxidative degradation of the aglycon during processing, ^{1}H-NMR spectra of Rehmanniae radix samples were directly compared as shown in Fig. 4. It confirmed that catalpol, raffinose, and stachyose levels gradually decrease by processing but monosaccharides levels including galactose and glucose, increase in processed roots. Although the H-1 of sucrose could not be clearly distinguished from the H-1 of raffinose and stachyose in PCA plot because the resonances of those anomeric protons were in the same bucket, the differences between Rehmanniae radix samples from different growth conditions could clearly be observed by visual inspection of the NMR spectra.

The growth conditions of the root were reported to influence the metabolic composition. Although there is little difference between dried Rehmanniae radix (A1 *vs.* B1), PC2 obviously separated the two groups after the first processing cycle (A2 *vs.* B2) as shown in Fig. 2A. After the first processing cycle, the amino acid alanine (δ 1.48, s, J=7.2 Hz), threonine (δ 1.32, s, J=6.8 Hz) and proline (δ 2.34, m and δ

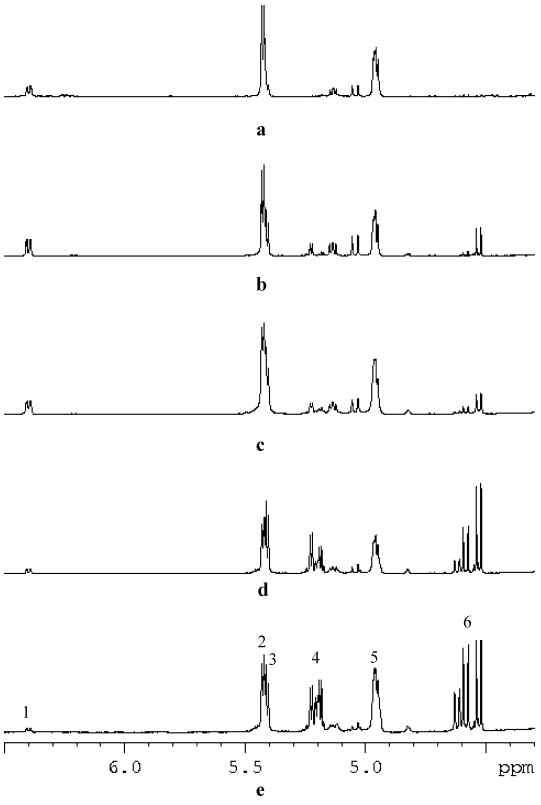

Fig. 4. ^{1}H-NMR Spectra of Processed *Rehmannia glutinosa* Roots in the Range of δ 4.3—6.5

(a) Fresh root; (b) dried root; (c) one cycle processing (rinsed with Chinese rice wine, steamed and baked); (d) five cycles processing; (e) nine cycles processing. 1: H-3 of catalpol; 2: H-1 of raffinose and stachynose; 3: H-1 of sucrose; 4: H-1 of α-glucose and α-galactose; 5: H-1 of internal α-glucose and α-galactose moiety of raffinose and stachynose; 6: H-1 of β-glucose and β-galactose.

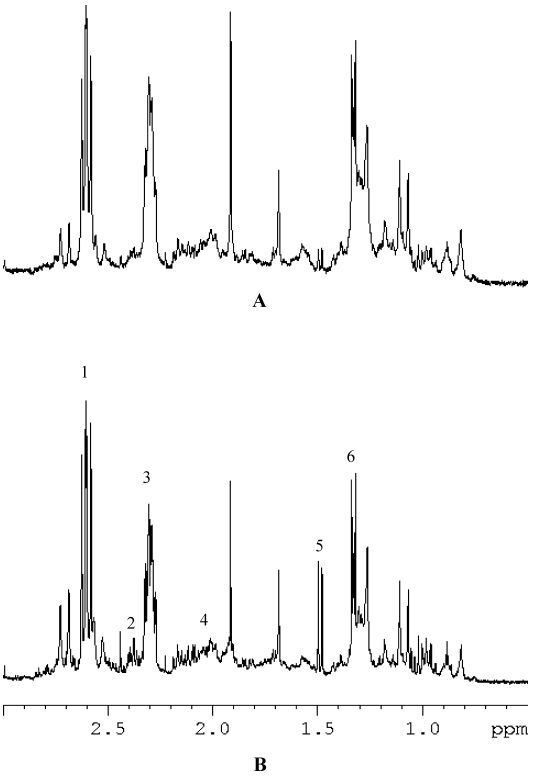

Fig. 5. [1]H-NMR Spectra of Processed *Rehmannia glutinosa* Roots in the Range of δ 0.5—3.0

(A) Processed root grown at 22 °C; (B) processed root grown at 25 °C. 1: H-9 of catalpol; 2: H-3 of proline; 3: H-5 of catalpol; 4: H-3′ of proline; 5: H-3 of alanine; 6: H-5 of threonine.

2.04, m) in Rehmanniae radix grown at 25 °C were found to be more abundant than in the plants grown at 22 °C. This was confirmed by direct comparison of [1]H-NMR spectra (Fig. 5). In reality, there was no significant difference of the level of alanine in the other samples except for the Rehmanniae radix processed by one cycle.

Based on PC1 (related to carbohydrates levels), the difference between five and nine cycles processing appeared to be small. The differences in metabolite profiles became smaller after five processing cycles suggesting that a relative stable composition can be obtained after five cycles of processing.

The presence of stachyose, and raffinose, abundant metabolites in the dried Rehmanniae radix as observed by NMR analysis was confirmed by FT-MS analysis. Processing of the roots led to hydrolysis of stachyose, leading to the formation of products with successive losses of hexose moieties. Besides hydrolysis of stachyose, for several other metabolites hydrolytic losses of hexose moieties were observed as well. Table 1 summarizes some (partly identified) metabolites and their respective hydrolytic products. In the fresh root (A0), catalpol was detected as K^+ adduct at *m/z* 401.0845. Rehmaionoside A and/or B ($C_{19}H_{34}O_8$) were detected as K^+ adduct at *m/z* 429.1886. Rehmaionoside C ($C_{19}H_{32}O_8$) was found with low intensity as K^+ adduct at *m/z* 427.1730. Rehmannioside A and/or B ($C_{21}H_{32}O_{15}$) were detected at low intensity at *m/z* 563.1382 as K^+ adduct. However, these metabolites were only found with lower intensity in the nine cycles processed root (A4). After processing, sucrose (including other hexoses) was detected at *m/z* 381.0794 (most intense) as K^+ adduct. At the higher mass end, several hydrolytic processes were also found *e.g.* *m/z* 1209.3548 (K^+ adduct)→1047.3012 (-hexose)→885.2489 (-hexose)→723.1959 (-hexose). Similar series were detected at *m/z* 1350.4602→1188.4227 (-hexose)→1026.3703 (-hexose)→864.3186 (-hexose)→702.2659 (-hexose) as well. In the nine cycles processed root (A4), no hydrolysable products could be identified.

The negative ion spectrum of the fresh root (A0) was dominated by an intense ion at *m/z* 725.2350. Medium intense ions were found at *m/z* 827.2678, *m/z* 1331.4364 and 1391.4587. During the subsequent processing cycles the metabolite at *m/z* 725.2360 remained the major compound but its relative abundance was reduced. The mass shifts indicated the hydrolytic loss of one, respectively two, hexose moieties. Even so other metabolites were found to be hydrolyzed (loss of hexose) intensively.

Table 1. Metabolites and Hydrolytic Products Observed by FT-MS Analysis of *R. glutinosa* Roots at Different Cycles of Processing

Positive ion mode (*m/z*)	Identity	Observed ions (*m/z*) during processing; sequential neutral losses of hexose moieties
381.0794	[sucrose+K]$^+$	
401.0845	[catalpol+K]$^+$	
427.1730	[rehmannionoside C+K]$^+$	
429.1886	[rehmannionoside A/B+K]$^+$	
543.1323	[raffinose+K]$^+$	
705.1850	[stachyose+K]$^+$	543.1323 (-1 hexose)
		381.0794 (-2 hexoses)
		219.0266 (-3 hexoses)
563.1382	[rehmannioside A/B+K]$^+$	
846.3071	Unknown	684.2548 (-1 hexose)
		522.2027 (-2 hexoses)
1209.3548	Unknown (K^+ adduct)	1047.3012 (-1 hexose)
		885.2489 (-2 hexoses)
		723.1959 (-3 hexoses)
1350.4602	Unknown	1188.4227 (-1 hexose)
		1026.3703 (-2 hexoses)
		864.3186 (-3 hexoses)
		702.2659 (-4 hexoses)

Negative ion mode (*m/z*)	Identity	Observed ions (*m/z*) during processing; sequential neutral losses of hexose moieties
725.2360	Unknown	563.1830 (-1 hexose)
		401.1301 (-2 hexoses)
827.2678	Unknown	665.2146 (-1 hexose)
		503.2146 (-2 hexoses)
		341.1089 (-3 hexoses)
1331.4364	Unknown	1169.3824 (-1 hexose)
		1007.3294 (-2 hexoses)
		845.2779 (-3 hexoses)
		683.2256 (-4 hexoses)
1391.4587	Unknown	1229.4037 (-1 hexose)
		1067.3500 (-2 hexoses)
		905.2984 (-3 hexoses)
		743.2465 (-4 hexoses)

Identification of metabolites was based on high resolution mass spectrometry only (however in parallel NMR analysis similar metabolites were identified). In the positive ion mode, most of the glycosides were observed as proton, sodium and potassium adducts; only the most intense ion is presented. The neutral loss of one hexose moiety is 162.0528 Da. *m/z* 1391.4587→1229.4037→1067.3500→905.2984→743.2465. *m/z* 1331.4364→1169.3824→1007.3294→845.2779→683.2256. *m/z* 827.2678→665.2146→503.2146→341.1089.

By comparison of the positive and negative ion spectra, the presence of potassium adducts was confirmed. Several metabolites were detected in both ion modes with a mass difference of 40 amu, *e.g.* 341/381 (sucrose), 503/543 (raffinose), 665/705 (stachyose), 683/723, 845/885, 1007/1047 and 1169/1209, respectively.

Chinese materia medica underlines the importance of processing for the effect and the quality of herbal materials. Although the mechanisms of processing have not yet been fully elucidated chemically and pharmacologically, the therapeutic effects of each material in the clinic are still strictly indicated following the different processing methods. For quality control of these materials, linking the biological activities with metabolites fingerprints of herbal materials is the ultimate basis.[27] As the relevant metabolites to predict biological activity are not known clearly yet, a holistic analysis of plant metabolites is required. The metabolomic approach in plants is based on the observable chemical profile or fingerprint of the metabolites using a wide spectrum of chemical analysis techniques in whole tissues combined with pattern recognition tools.[28] ^{1}H-NMR spectroscopy, in particular in combination with multivariate data analysis has proven to be a powerful approach for metabolic fingerprinting.[29—35] In metabolomics data analysis, after data preprocessing, an important approach is an unbiased clustering method, principle component analysis (PCA) which does not require knowledge of the data set and reduces the dimensionality of multivariate data, preserving most of the variance within it. In our study, PCA was applied to ^{1}H-NMR spectra of all Rehmanniae radix samples from two growth conditions (22, 25 °C) and from the different processing cycles. The PCA result is able to discriminate metabolic profiles significantly under different processing cycles. Based on the first two principle components, the difference between five and nine cycles processing of Rehmanniae radix samples appeared to be small. It means the metabolic composition change is stable between these two processing cycles. This result is in accordance with the report obtained by GC-MS coupled with ASCA.[26] Besides the processing, the growth conditions influence the metabolites in plants. It was reported that for commercial agriculture of *R. glutinosa*, a temperature of 21—22 °C is optimal.[36] The samples from two growth conditions, including different temperature (22, 25 °C) and light density (2700, 9000 lux) respectively were used in our study. Indeed, the growth condition highly affected the Rehmanniae radix metabolome which was particularly apparent after the first processing. The reason why only the first processing step makes a bigger difference between the two groups is unclear. It might be caused by the processing of heat steaming. Furthermore, for evaluation of contributing metabolites, the biplots containing corrections of all variables and scores of Rehmanniae radix samples were investigated in this study. The characteristic fingerprint region for each sample was recognized and more abundant oligosaccharides, such as raffinose family of oligosaccharides (RFOs), *e.g.* the trisaccharide raffinose and the tetrasaccharide stachyose, were found in the non-processed samples at higher level than in the processed ones. This might be caused by hydrolysis of these oligosaccharides. The occurrence of hydrolysis during processing is supported by higher levels of fructose, galactose, and glucose, which are assumed to be hydrolysis products of raffinose, stachyose, and sucrose in processed samples.

Although NMR spectroscopy provides detailed and quantitative metabolite profiles, its inherent lower sensitivity and overlapping signals are limiting the coverage of the metabolome. For instance, only catalpol was identified and some other minor iridoids were detected, while many other iridoid glycosides have been isolated from Rehmanniae radix.[18—21] Therefore, high resolution FT-MS was employed in this study for further coverage of the metabolome. The positive and negative ion-mode mass spectra revealed several series of ion clusters with mass differences of 162.053 Da, indicating a difference of a hexose moiety. The positive ion spectrum of the non-processed Rehmanniae radix (A0) root showed intense signals at *m*/*z* 684.2548 and 705.1850, which were decreasing in intensity during the subsequent processing cycles, accompanied by an increase in intensity of hydrolyzed products. Stachyose was found to be an abundant metabolite by NMR analysis. The ion *m*/*z* 705.1850 was identified as the K^+ adduct of stachyose as well. Processing of the Rehmanniae radix led to hydrolysis, resulting in the formation of *m*/*z* 543.1323 (K^+ adduct, neutral loss of one hexose), *m*/*z* 381.0794 (K^+ adduct, loss of two hexoses) and *m*/*z* 219.0266 (K^+ adduct, loss of three hexoses). The ion at *m*/*z* 543.1323 indicates also the K^+ adduct of raffinose, also detected by NMR analysis. Processing of the metabolite at *m*/*z* 684.2548 (H^+ adduct, no other adducts detected) may result in the formation of *m*/*z* 522.2027 (neutral loss of one hexose). This metabolite itself may be formed from *m*/*z* 846.3071, which contains an extra hexose.

The hydrolytic processes occurring during the processing were also clearly observed by the series of consequent neutral losses of hexose moieties in the negative ion mode. By comparison of the positive and negative ion spectra in MS data, the relative intensity of the smaller oligoglycosides strongly increased during the different cycles in the processing. This observation is similar to the previous report by Kitagawa *et al.*[37] It has indicated the content of monosaccharides and oligosaccharides were increased during processing using gas liquid chromatography (GLC). It can be concluded that the fresh roots are rich in the larger glycosides and polysaccharides, which were hydrolyzed during the processing. In combination with our previous GC-MS study on growth conditions and processing of the Rehmanniae radix,[26] the present study of this material with ^{1}H-NMR, FT-MS spectrometry adds to our knowledge in depth on the range of metabolites and changes during the processing.

In conclusion, we have done the study of effect of growth conditions and processing on *R. glutinosa* using GC-MS platform. The results clearly showed the effect of different factors on growth and indicated the directions for processing of *R. glutinosa* roots.[26] However, we couldn't get the details of direction which occurred during the processing. In order to ascertain the direction of processing, we have applied multidisciplinary analytical instruments, ^{1}H-NMR and FT-MS, to indicate the changes of metabolites composition during the processing of Rehmanniae radix. The results show that the combination of ^{1}H-NMR, FT-MS spectrometry and multivariate analysis (as PCA) allows a consistent method for quality control of processed Rehmanniae radix based on metabolite profiles. In addition, it was shown that the metabolite profiles of Rehmanniae radix was stabilized after

five cycles of processing. It indicates that the nine cycles of processing for Rehmanniae radix materials according to the traditional descriptions in TCM are not necessary. The quality of Rehmanniae radix after five cycles of processing has stabilized. Considering the clear difference between processed and non-processed extracts, a systems biology type of approach seems of interest to identify the compounds with activity in bioassays. Quality control of processed Rehmanniae radix products could then be based on the metabolomic analysis ensuring the pharmacological activities. The methods reported here are of interest for an unbiased examination of quality control for this medicinal material, an important step towards the modernization of TCM.

Experimental

Plant Material *Rehmannia glutinosa* Libosch. var. *purpurea* Makino plants were generously donated by Prof. Chong-Chuan Chen (School of Chinese Medicine Resources, China Medical University, Taichung, Taiwan) and grown in the growth chamber under 22/18 °C, 16/8 h in light/dark, 2700 lux light density in the daytime with 80% humidity, or 25 °C, 16/8 h in light/dark, 9000 lux with 50% humidity.

Processing of Rehmanniae Radix Three individual fresh Rehmanniae radix grown under the two conditions (22, 25 °C) were collected after 4 months and crowns and fibrous roots were removed. The samples A0 grown at 22 °C and B0 grown at 25 °C were obtained from these fresh roots and frozen in liquid nitrogen immediately. The remaining roots were dried in an oven at 55 °C for 3 d (dried, non-processed root: A1 grown at 22 °C; B1 grown at 25 °C). The dried, roots were immersed in Chinese rice wine (Michiu, Taiwan Tobacco & Liquor Corp., Taipei, Taiwan) for 15 min, then steamed with water in a bamboo steam boiler for 60 min until it became soft. After this, samples were dried overnight at 55 °C. This procedure was repeated for one cycle (A2 grown at 22 °C, B2 grown at 25 °C), five cycles (A3 grown at 22 °C, B3 grown at 25 °C), and nine cycles (A4 grown at 22 °C, B4 grown at 25 °C). After subsequent processing, especially after nine cycles, the inside of the roots became softer, sticky and oily-black colored.

NMR Measurements Fifty milligrams of Rehmanniae radix was transferred to a 2 ml-microtube. Seven hundred fifty microliters of KH_2PO_4 in D_2O buffer containing 0.1% w/w TSP and 750 μl of methanol-d_4 were added to the tube followed by vortexing for 1 min and sonication for 20 min. The mixture was centrifuged at 13000 rpm at 4 °C for 2 min. Eight hundred microliters of the supernatant was taken for NMR analysis.

^{1}H-NMR spectra were recorded at 25 °C on a 400 MHz Bruker AV-400 spectrometer operating at a proton NMR frequency of 400.13 MHz. TSP was used as an internal standard. The NMR parameters of ^{1}H, *J*-resolved, ^{1}H–^{1}H-correlated spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC) spectra were the same as in our previous studies.[31—34]

Data Analysis The ^{1}H-NMR spectra were automatically reduced to ASCII files using AMIX (v. 3.7, Bruker Biospin). Spectral intensities were scaled to total intensity and reduced to integrated regions of equal width (δ 0.04) corresponding to the region of δ 0.40—10.00. The region of δ 4.80—4.90 was excluded from the analysis because of the residual signal of water. Principle component analysis (PCA) was performed with the SIMCA-P software (v. 11.0, Umetrics, Umeå, Sweden).

Mass Spectrometry A LTQ-FTMS (Thermo Electron, Bremen, Germany) was used for analysis. Sodium trifluoroacetate was used for calibration in the positive ion mode. The same calibration file was used for measuring in the negative ion mode. The positive ion spectra were recalibrated using potassium adducts of hexose, dihexose, tetrahexose and a higher carbohydrate ($C_{36}H_{64}O_{32}$) at *m*/*z* 219.0266, 381.0794, 705.1850 and 1047.3012 respectively. Data processing was performed using Xcalibur software, version 1.4, extended with tools for recalibration (Thermo Electron).

Using a Surveyor autosampler (Thermo Electron), 20 μl of the extract was injected, using 50% MeOH containing 1% AcOH as eluent at a flow rate of 20 μl/min without using a separation column. Between 0.6 and 3 min after injection, FT-MS spectra were recorded and averaged (77 scans in positive ion mode, 136 scans in negative ion mode). Instrument settings were as follows: sheath gas flow 25, auxiliary gas flow 5, spray voltage 4 kV, capillary temperature 275 °C, capillary voltage 11 V, and tube lens 120 V.

Acknowledgements We would like to thank Prof. C. C. Chen (School of Chinese Medicine Resources, China Medical University, Taichung, Taiwan) for generous donation of *R. glutinosa* plants. The "Van Leersumfonds" is gratefully acknowledged for financial support.

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