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# Comparison of two officinal Chinese pharmacopoeia species of *Ganoderma* based on chemical research with multiple technologies and chemometrics analysis

Juan Da, Wan-Ying Wu*, Jin-Jun Hou, Hua-Li Long, Shuai Yao, Zhou Yang, Lu-Ying Cai, Min Yang, Bao-Hong Jiang, Xuan Liu, Chun-Ru Cheng, Yi-Feng Li, De-An Guo**

*Shanghai Research Center for Modernization of Traditional Chinese Medicine, National Engineering Laboratory for TCM Standardization Technology, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China*

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ABSTRACT

*Aim of the study:* To investigate the chemical differences between *Ganoderma lucidum* (*G. lucidum*, Chizhi) and *Ganoderma sinense* (*G. sinense*, Zizhi).

*Materials and methods:* Thirty two batches of commercial *Ganoderma* samples were collected, including 20 batches of *G. lucidum* and 12 batches of *G. sinense* cultivated in different geographical regions. Chemical substances in aqueous extract and alcoholic extract, mainly polysaccharides and triterpenes respectively, were investigated. Determination of polysaccharides was carried out with a high performance liquid chromatography with an variable wavelength detector. Meanwhile, analysis of triterpenes were performed on an ultraviolet spectrophotometer, an ultra performance liquid chromatography and a rapid resolution liquid chromatograph combined with an electrospray ionization mass spectrometer. Chromatograms and spectra for all batches and reference standards of main components were obtained and used for direct comparison. Further discussion was made on the basis of the result of principal component analysis (PCA).

*Results:* Significant difference of triterpenes was shown between *G. lucidum* and *G. sinense*. In 20 batches of *G. lucidum*, 12 main components, including eight ganoderic acids and four ganoderenic acids were identified and ten of them were quantitatively determined, with the total content from 0.249% to 0.690%. However, none of those triterpenes was found in either batch of *G. sinense*. As for constituents of polysaccharides, seven monosaccharides were identified and four main components among them were quantitatively determined. Difference of polysaccharides was not directly observed, but latent information was revealed by PCA and the discrimination became feasible.

*Conclusions: G. lucidum* and *G. sinense* were chemically different, which might result in pharmacological distinction. Preparations of traditional Chinese medicine (TCM) from *Ganoderma* should make accurate specification on the origin of species.



*Abbreviations: G. lucidum* (Chizhi), *Ganoderma lucidum* (Leyss.ex Fr.) Karst.; *G. sinense* (Zizhi), *Ganoderma sinense* Zhao, Xu et Zhang; TCM, traditional Chinese medicine; GC2, ganoderic acid C2; GG, ganoderic acid G; GB, ganoderic acid B; GK, ganoderic acid K; GA, ganoderic acid A; GH, ganoderic acid H; GD, ganoderic acid D; GF, ganoderic acid F; GEC, ganoderenic acid C; GEB, ganoderenic acid B; GEA, ganoderenic acid A; GED, ganoderenic acid D; Glu, D-glucose anhydrous; GluA, D-glucuronic acid; Man, D-mannose; Xyl, D-xylose; Ara, arabinose; Gal, D-galactose; Lyx, D-lyxose; Rib, D-ribose; Fuc, L-(−)-fucose; PCA, principle component analysis.

* Corresponding author at: Shanghai Research Center for Modernization of Traditional Chinese Medicine, National Engineering Laboratory for TCM Standardization Technology, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Haike Road 501, Shanghai 201203, China. Tel.: +86 21 20231000x2221; fax: +86 21 50272789.

** Corresponding author at: Shanghai Research Center for Modernization of Traditional Chinese Medicine, National Engineering Laboratory for TCM Standardization Technology, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Haike Road 501, Shanghai 201203, China. Tel: +86 21 50271516; fax: +86 21 50272789.

*E-mail addresses:* wanyingwu@mail.shcnc.ac.cn (W.-Y. Wu), daguo@mail.shcnc.ac.cn (D.-A. Guo).

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## 1. Introduction

*Ganoderma* (Lingzhi) is officially recorded as the dried sporophore of *Ganoderma lucidum* (Leyss.ex Fr.) Karst. and *Ganoderma sinense* Zhao, Xu et Zhang in current Chinese Pharmacopoeia (2010 edition) [1], with the common name Chizhi and Zizhi, respectively. In Chinese history, it was described as a miraculous drug in legend and literatures, and recently has been confirmed by modern research on its antitumor, cardio-protective, immunomodulatory, hypoglycemic, anti-inflammatory and hepatoprotective effects [2]. Compared with tremendous amount of research data regarding *G. lucidum*, reports on *G. sinense* are relatively fewer, but pharmacological and chemical differences between the two species have been observed. Ethanol extracts of both species have antitumoral proliferation effect, but targeting on the different phases of cell cycle arresting [3]. Difference between the both species was also shown through hierarchical clustering analysis based on nine components (including ganoderic acid A and DM, ganoderic acid Y + ganoderol B, ganoderol A, methyl ganoderate D, G, ganoderal A and ergosterol) from 11 samples [4]. On the other hand, price of *G. sinense* is apparently higher than that of *G. lucidum* in the current herbal medicine market in China, particularly *G. sinense* is even regarded as precious "wild Lingzhi" in folk conception. However, from the data base of Chinese State Food and Drug Administration (www.sfda.gov.cn), for over 30 registered medicines entitled with "Lingzhi" and produced by more than 100 factories, no species designation was clarified.

For *G. lucidum*, triterpenes and polysaccharides are the two major types of active substances [5]. Among the hundreds of triterpenes isolated (many of which were oxygenated tetracyclic triterpenoids), some have been reported to possess antitumor activity, such as ganoderic acid F, K, B, D and AM1 [6,7], ganoderic acid DM [8], ganoderic acid A, F and H [9]. Other potential activities included their anti-inflammatory [10,11], anti-HIV-1 and anti-HIV-1 protease [12,13], anti-hepatitis B [14], and inhibitory activity on human aldose reductase [15,16], osteoclastic differentiation and structural criteria [17]. Polysaccharides were also extensively studied in Lingzhi, with antitumor [18], immunomodulatory [19], antiherpetic [20], antioxidant [21] and antidiabetics activities [22]. Thus, experiments focusing on those components might be useful in discrimination and classification of the two species.

Current HPLC methods available for analysis of triterpenes in *G. lucidum* are always time-consuming, even without a base-line separation [4,23,24]. Development of chromatographic method for *Ganoderma* is in urgent demand. PCA has been already applied for the discrimination of *G. lucidum* from different origins, but only for methanol extract, using peak areas instead of content, and these peaks were not identified [25]. PCA of polysaccharides was not previously reported, partly because UV detection for total polysaccharides recorded in Chinese Pharmacopoeia (2010 edition) could not provide sufficient information. However, as *Ganoderma* was commonly used as decoction in TCM, analysis of aqueous extract should not be neglected.

In the present study, methods were explored for better identification and quantitative investigation of physiologically active components in *Ganoderma*, which laid basis for chemical comparison, including UPLC-PDA and RRLC-ESI-$MS^n$ for triterpenes, and HPLC-VWD method for polysaccharides [26]. Further information was revealed by PCA, based on two different series of data sets: content of main components and peak area of all visual peaks in chromatograms. Besides, in order to evaluate the information of triterpenes and polysaccharides simultaneously, combination of the data separately obtained with different methods was attempted for PCA.

## 2. Experimental

### 2.1. Materials and reagents

Twenty batches of *G. lucidum* and twelve batches of *G. sinense* from different provinces in China were collected, including *G. lucidum* from Anhui (No. C1-11), Shandong (No. C12, 13), Jiangsu (No. C14, 15), Jilin (No. C16, 17), Fujian (No. C18), Henan (No. C19) and Guangxi (No. C20); and *G. sinense* from Anhui (No. Z1-6), Jilin (No. Z7), Fujian (No. Z8, 9), Guangxi (No. Z10), Jiangxi (No. Z11) and Hainan (No. Z12). All samples were identified by Professor De-An Guo (Shanghai Institute of Materia Medica, Chinese Academy of Sciences), following the methods described in American Herbal Pharmacopoeia and Therapeutic Compendium [2], Chinese Pharmacopoeia 2010 [1], and illustrated handbook of *Ganoderma* in China [27]. Macroscopic and microscopic characteristics are shown in Figs. S1 and S2.

Twelve triterpenes, namely ganoderic acid C2 (GC2), ganoderic acid G (GG), ganoderic acid B (GB), ganoderic acid K (GK), ganoderic acid A (GA), ganoderic acid H (GH), ganoderic acid D (GD), ganoderic acid F (GF) and ganoderenic acid C (GEC), ganoderenic acid B (GEB), ganoderenic acid A (GEA), ganoderenic acid D (GED) were prepared in our laboratory and identified with purity not less than 98%.

D-Glucose anhydrous (Glu, Lot 110833-200904), D-glucuronic acid (GluA, Lot 140648-200602), D-gannose (Man, Lot 140651-200602), D-xylose (Xyl, Lot 111508-200404), arabinose (Ara, Lot 1506-200001) were purchased from National Institute for Control of Biological and Pharmaceutical Products of China, D-galactose (Gal, Lot F20100517), D-lyxose (Lyx, Lot WALD20091016), D-ribose (Rib, Lot WF20061128) were obtained from Sinopharm Chemical Reagent Co., Ltd. (SCRC, Shanghai, China), L-(−)-Fucose, minimum (Fuc, Lot 014K10431) was purchased from Sigma–Aldrich. Co. (St. Louis, USA) with purity not less than 98%. 1-Phenyl-3-methyl-5-pyrazolone (CP) was purchased from SCRC; acetonitrile (HPLC, Lot K4WA1H,) and methanol (HPLC, Lot KBSG2H) were obtained from Honeywell (NJ, USA).

### 2.2. Determination of log ε for 11 triterpenes

Standard stock solutions with known concentrations were obtained by dissolving accurately weighted reference standards (GC2, GG, GB, GA, GH, GD, GF, GEA, GEC, GEB and GED, except for GK because of its trace amount) in methanol, respectively. Then reference standard solutions were prepared with those standard stock solutions and scanned in the entire UV range (200–400 nm) to determine the $\lambda_{max}$ and corresponding log ε values were measured.

### 2.3. Qualitative and quantitative analysis of triterpenes with UPLC-PDA

#### 2.3.1. Reference standard solutions and sample solutions

Use the reference standard solutions described in "Section 2.2". GK was dissolved in methanol to obtain a concentration of approximate 1 mg $mL^{-1}$.

Sample solutions: 2 g of sample powder (capable of passing a 4-mm sieve) were accurately weighted, extracted with 75 mL of alcohol for 45 min, evaporated under reduced pressure to dryness, and dissolved in 25 mL of alcohol. The 2 mL of solution were diluted with 18 mL of water, transferred to a solid-phase extraction column (Alltech, 200 mg, 5 mL) at the rate of 1 drop $s^{-1}$. (The column was initially conditioned with 5 mL of methanol and then 3 mL of water). The column was washed with 3 mL of water (the eluate was discarded), and then with 2 mL of methanol. The eluate was collected to a 2-mL of volumetric flask, diluted to volume with methanol and passed through a nylon filter having a 0.22-μm porosity.

*2.3.2. UPLC-PDA analysis of triterpenes*

Qualitative analysis was performed on a Waters ACQUITY ™ Ultra Performance LC (Waters Corp., Milford, MA, USA) comprised a binary solvent manager (C10UPB 139A), a sampler manager, a PDA detector, and Empower chemistation (W0BAXG026M). Separation was performed on an ACQUITY UPLC® HSS T3 Column (1.8 μm, 2.1 mm × 150 mm) with flow rate 0.4 mL min$^{-1}$ of mobile phase, which consisted of acetonitrile (A) and 0.075% aqueous solution of phosphoric acid (B) and followed the gradient program as 0/3/34/52 min, 20%/26.5%/26.5%/38.5% (A). The injection volume was 5 μL. Detection was performed at 257 nm, and UV spectra (210–400 nm) were recorded simultaneously.

Retention time and UV spectra were used for identification with the help of reference standards. Peak areas were applied in content determination with external standard method. Method validation was carried out following the guidelines in U.S. Pharmacopoeia (2010 USP(33)-NF(28), volume 1, general information ⟨1225⟩).

*2.4. Qualitative analysis of triterpenes with RRLC-ESI-MS$^n$*

*2.4.1. Reference standard solutions and sample solutions*

Use the reference standard solutions and sample solutions in "Section 2.3.1".

*2.4.2. RRLC-ESI-MS$^n$ analysis of triterpenes*

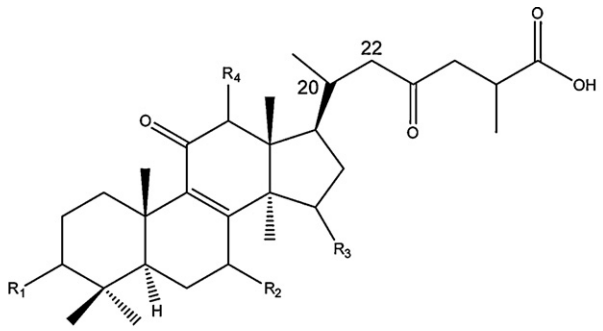
RRLC-ESI-MS$^n$ determination was performed on a BRUCKER HCT mass spectrometer (Bruker, Germany) connected to an Agilent 1200 RRLC system (Agilent, USA). Separation was performed on an ACQUITY UPLC® HSS T3 Column (1.8 μm, 2.1 mm × 150 mm) with flow rate 0.2 mL min$^{-1}$ of mobile phase, which consisted of acetonitrile (A) and 0.5% aqueous solution of formic acid (B) and followed the gradient program as 0/3/39/57/70 min, 23.5%/29.5%/29.5%/42.5%/42.5% (A). The injection volume was 5 μL. Then the effluent was introduced to an electrospray ionization source with split ratio of 1:2. Parameters of MS detector were: nebulizer gas, 40.0 psi; dry gas, 9.0 L min$^{-1}$; dry temperature, 350 °C; ionization voltage, 4000 V; full-scan ions were selected in the range of 50–1200 *m/z*. $[M-H]^-$ ions were selected and parameters for MS(n) were as follows: Auto MS: 4; scan mode: Ultra Scan; MS/MS Frag Ampl 1.00 V. MS spectra of reference standards and samples were obtained and compared.

*2.5. Qualitative and quantitative analysis of monosaccharides in polysaccharides with HPLC-VWD*

*2.5.1. Reference standard solutions and sample solutions*

Standard stock solutions with known concentrations were obtained by dissolving accurately weighted reference standards Man, Rib, GluA, Glu, Gal, Fuc, Lyx in water, respectively. Then reference standard solutions for each monosaccharide and mixed solution of Man, GluA, Glu, Gal, Lyx were prepared with those standard stock solutions. The solutions were transferred to a pressure vial respectively, added with 0.25 mL 4 M trifluoroacetic acid, and hydrolyzed at 110 °C for 4 h. Then, 0.5 mL of methanol was added for four times and evaporated to remove the trifluoroacetic acid. The residue was dissolved in 0.25 mL of water and 0.30 mL of 0.15 mol L$^{-1}$ sodium hydroxide, and 0.50 mL of 0.1 mol L$^{-1}$ methanolic solution of 1-phenyl-3-methyl-5-pyrazolone was added, and kept at 70 °C for 30 min. Then 0.30 mL of 0.15 mol L$^{-1}$ hydrochloric acid was added. Finally, the solution was transferred to a 2-mL of volumetric flask, diluted to volume and passed through a nylon filter having a 0.45-μm porosity.

Sample solutions: 2 g of sample powder (capable of passing a 4-mm sieve) were accurately weighted, extracted with 60 mL of water under reflux for two times, 4 h and 3 h respectively, evaporated on a water bath to dryness, and dissolved in 5 mL of water, then 75 mL of alcohol was added and mixed well. The solution was centrifuged (4000 rpm × 30 min) after standing for 12 h at 4 °C, and the precipitate was evaporated to dryness and dissolved in 10 mL of hot water. The solution was centrifuged (4000 rpm × 10 min), and the supernatant was prepared according to the method described in above reference standard solutions from "transferred to a pressure vial. . ."




| | R1 | R2 | R3 | R4 | Double Bound |
|---|---|---|---|---|---|
| GEC | β-OH | β-OH | α-OH | H | $\Delta_{20,22}$ |
| GC2 | β-OH | β-OH | α-OH | H | - |
| GG | β-OH | β-OH | =O | β-OH | - |
| GEB | β-OH | β-OH | =O | H | $\Delta_{20,22}$ |
| GB | β-OH | β-OH | =O | H | - |
| GEA | =O | β-OH | α-OH | H | $\Delta_{20,22}$ |
| GK | β-OH | β-OH | =O | β-OAc | - |
| GA | =O | β-OH | α-OH | H | - |
| GH | β-OH | =O | =O | β-OAc | - |
| GED | =O | β-OH | =O | H | $\Delta_{20,22}$ |
| GD | =O | β-OH | =O | H | - |
| GF | =O | =O | =O | β-OAc | - |

**Fig. 1.** Chemical structures of main triterpenes found in *G. lucidum*.

*2.5.2. HPLC-VWD analysis of monosaccharides in polysaccharides*

Qualitative analysis was performed on an Agilent HPLC (Agilent Corp., USA) comprised of a solvent manager, a sampler manager, a VWD detector, and an Agilent A 10.02 chemistation. Separation was performed on a Zorbax XDB C18 column (5 μm, 4.6 mm × 250 mm) with flow rate 1 mL min$^{-1}$ of mobile phase, which consisted of acetonitrile (A) and aqueous buffer solution of 0.05 M phosphate (pH 6.0) (B) and followed the gradient program as 0/30/55/60 min, 15%/16.5%/18%/18% (A). The injection volume was 10 μL. Detection was performed at 250 nm. Method validation was carried out following the guidelines in U.S. Pharmacopoeia (2010 USP(33)-NF(28), volume 1, general information ⟨1225⟩).

Retention time was used for identification with the help of reference standards. Peak areas were applied in content determination with external standard method.

### 2.6. PCA based on the data obtained from quantitative determination

PCA was carried out based on two different series of data sets: contents of main components and area of visible peaks in chromatograms, using SIMCA-P+12.0 software.

## 3. Results

### 3.1. Value of Log ε for 11 triterpenes

Chemical structures of main triterpenes found in *G. lucidum* are listed in Fig. 1.

The result of log ε values is shown in Table 1.

In general, for ganoderic acids, log ε was smaller than those of ganoderenic acids. For ganoderic acids, log ε values of GC2, GG, GB, GA and GD (MW516.7–532.7) were similar and larger than GH (MW572.7) and GF (MW570.7), in relation to molecular weight to some extent. For ganoderenic acids, log ε of GEA, GEB and GED (MW512.6–514.7) were similar, larger than GEC (MW516.7).

### 3.2. Qualitative analysis of triterpenes with UPLC-PDA

Chromatograms of triterpenes in *G. lucidum*, *G. sinense* and reference standards are shown in Fig. 2. Retention times and UV spectra were used for identification (Table 2).

High similarity was shown for 20 batches of *G. lucidum*. Through comparison of retention times with reference standards, main peaks in *G. lucidum* chromatograms (peaks 1–11) were identified as GEC, GC2, GG, GEB, GB, GK + GEA, GA, GH, GED, GD, and GF (difference < 0.2 min), respectively. None of such peaks was found in 12 batches of *G. sinense*. Double bond ($\Delta_{20,22}$) resulted in slightly bigger polarity of ganoderenic acids than corresponding ganoderic acids as retention times were relatively smaller.

Similar profiles of online-spectrograms for those triterpenes were shown based on assemble chromophores. UV $\lambda_{max}$ of ganoderenic acids were about 250 nm, compared to 257 nm of ganoderic acids, indicating blue shift caused by double bond ($\Delta_{20,22}$). Identification of peaks 1–4, 7, 9–11 was confirmed according to identical UV $\lambda_{max}$. However, further study was needed because 1.3 nm of discrepancy existed between peak 5 and GB, peak 8 and GH, and 2.5 nm between peak 6 and GEA.

### 3.3. Qualitative analysis of triterpenes with RRLC-ESI-$MS^n$

Negative TIC of *G. lucidum* and *G. sinense* are shown in Fig. 3. In general, base peak for each acid appeared as $[M-H]^-$ (molecular ion) or $[M-H-H_2O]^-$ (Table 3) [28]. For GEC, base peak appeared as $[M-H+HCOOH]^-$, and confirmed by positive mass spectrum with base peak of $[M+H]^+$ (Fig. 3E and F). MS1 and MS2 of peaks of *G. lucidum* were identical to the reference standards at their corresponding retention times, except peak 6, which was identified as a mixture of GEA and GK. Peaks 5 and 8 were finally determined as GB and GH, respectively. None of these ions appeared in TIC of *G. sinense* (Fig. 3B), in accordance with the result of UPLC.

### 3.4. Qualitative analysis of monosaccharide components in polysaccharides with HPLC-VWD

Chromatograms of monosaccharide components in polysaccharides in *G. lucidum*, *G. sinense* and reference standards are shown in Fig. 4.

Visible peaks 1, 3–8 in chromatograms of *G. lucidum* and *G. sinense* were identified as Man, Rib, GluA, Glu, Gal, Ara+Xyl, Fuc, respectively (peak 2 was Lyx used as internal standard). No difference in qualitative analysis of monosaccharides was shown between *G. lucidum* and *G. sinense*.

### 3.5. Quantitative analysis of triterpenes in G. lucidum with UPLC-PDA

The method for the assay of triterpenes was comparable to the requirements of method validation in U.S. Pharmacopoeia 33, and could be used in quality control.

#### 3.5.1. Method validation of triterpenes: precision, linearity, accuracy, specificity, stability and ruggedness

Ganoderic acid A was chosen as the single standard, and content of other triterpenes were calculated according to the conversion factor. The method was validated through investigations on the precision, linearity, accuracy, specificity, stability and ruggedness.

Precision (Repeatability and intermediate precision): Repeatability was assessed with 9 sample solutions of low, medium and high levels (1.0 g, 2.0 g and 3.0 g, respectively), each with three triplicates. Content was calculated with SSDMC method (Table S1). The RSDs of inner-day variation of three levels were in the range from 0.13 to 2.67% for individual component, and 0.04% for total content.

Intermediate precision was performed through investigation of three different days, three different analysts and two independent equipments, each with three triplicates (Table S2). The RSDs of intra-day variation were in the range from 0.89 to 2.54% for individual component, and 1.38% for total content. Significant difference was found between the content of GH obtained with different analysts (RSD = 3.88%) and GD obtained with different equipments (RSD = 20.04%), but the RSD for total content were 1.16% and 1.63%, respectively.

Good linearity was shown with correlation in the range from 0.9998 to 1.0000 (Table S3).

Accuracy was tested with percentage of recovery. Three different concentrations (low, medium and high) of reference standard were spiked to 1.0 g of GL, all in triplicates. Recovery was ranged from 94.02 to 102.56% (Table S4).

Specificity: retention times of 10 components in sample solution were corresponding to relative reference standard (Fig. 2). No peak appeared in the chromatogram of blank solvent.

Stability of sample solution was investigated after storage at room temperature for 0, 3, 6, 9, 12, 24 h and all components were found to be stable within 24 h (RSDs range from 0.23% to 1.02%, Table S5).

Result of ruggedness indicated that small variations to certain chromatographic parameters were permitted: firstly, concentration of $H_3PO_4$ in mobile phase could be reduced to 0.030%; secondly, the third part of elution program could be changed at least ±1% ACN and ±1 min; thirdly, volume of injection could be reduced to 2 μL; and wavelength error less than ±3 nm caused by different instruments was acceptable. Changes to other parameters were not permitted.

#### 3.5.2. Contents of 10 triterpenes in twenty batches of G. lucidum

Contents of 10 triterpenes in twenty batches of *G. lucidum* were determined (Fig. 5), except for peak 6 due to its impurity. For most batches of *G. lucidum*, content of ganoderic acid A was higher than that of other triterpene acids (except for C5, 9,16,18,20 with more ganoderic acid H). The total content of ganoderic acids varied from 0.225% to 0.595%, while ganoderenic acids from 0.009% to 0.095%. The content ratio was 3.47–22.78% of ganoderenic acids to ganoderic acids.

Average content of ganoderenic acids, ganoderic acids and total acids in *G. lucidum* from different resources were calculated, and six different ratios were calculated (Fig. S3), including ganoderenic acids, ganoderic acids and total acids of different regions to

**Table 1**
Value of Log ε for 11 triterpenes.

| | Concentration (mol/L) | Absorbance | Correl | Average log ε | RSD (%) |
|---|---|---|---|---|---|
| GEC | 2.64E-05–7.92E-05 | 0.351–0.989 | 0.9998 | 4.11 | 0.32 |
| GC2 | 1.89E-05–1.32E-04 | 0.158–0.958 | 0.9998 | 3.88 | 0.76 |
| GG | 3.55E-05–1.77E-04 | 0.308–1.231 | 0.9993 | 3.87 | 1.07 |
| GEB | 7.60E-06–6.08E-05 | 0.140–1.087 | 0.9999 | 4.26 | 0.15 |
| GB | 3.74E-05–1.31E-04 | 0.290–0.998 | 1.0000 | 3.88 | 0.09 |
| GEA | 7.28E-06–5.82E-05 | 0.133–0.972 | 0.9996 | 4.24 | 0.41 |
| GA | 1.93E-05–1.35E-04 | 0.155–1.017 | 0.9996 | 3.88 | 0.32 |
| GH | 3.43E-05–1.71E-04 | 0.223–1.046 | 1.0000 | 3.79 | 0.30 |
| GED | 7.83E-06–6.26E-05 | 0.143–1.076 | 1.0000 | 4.25 | 0.26 |
| GD | 2.04E-05–1.43E-04 | 0.165–1.100 | 0.9999 | 3.89 | 0.27 |
| GF | 1.91E-05–1.34E-04 | 0.153–0.875 | 0.9999 | 3.84 | 1.01 |

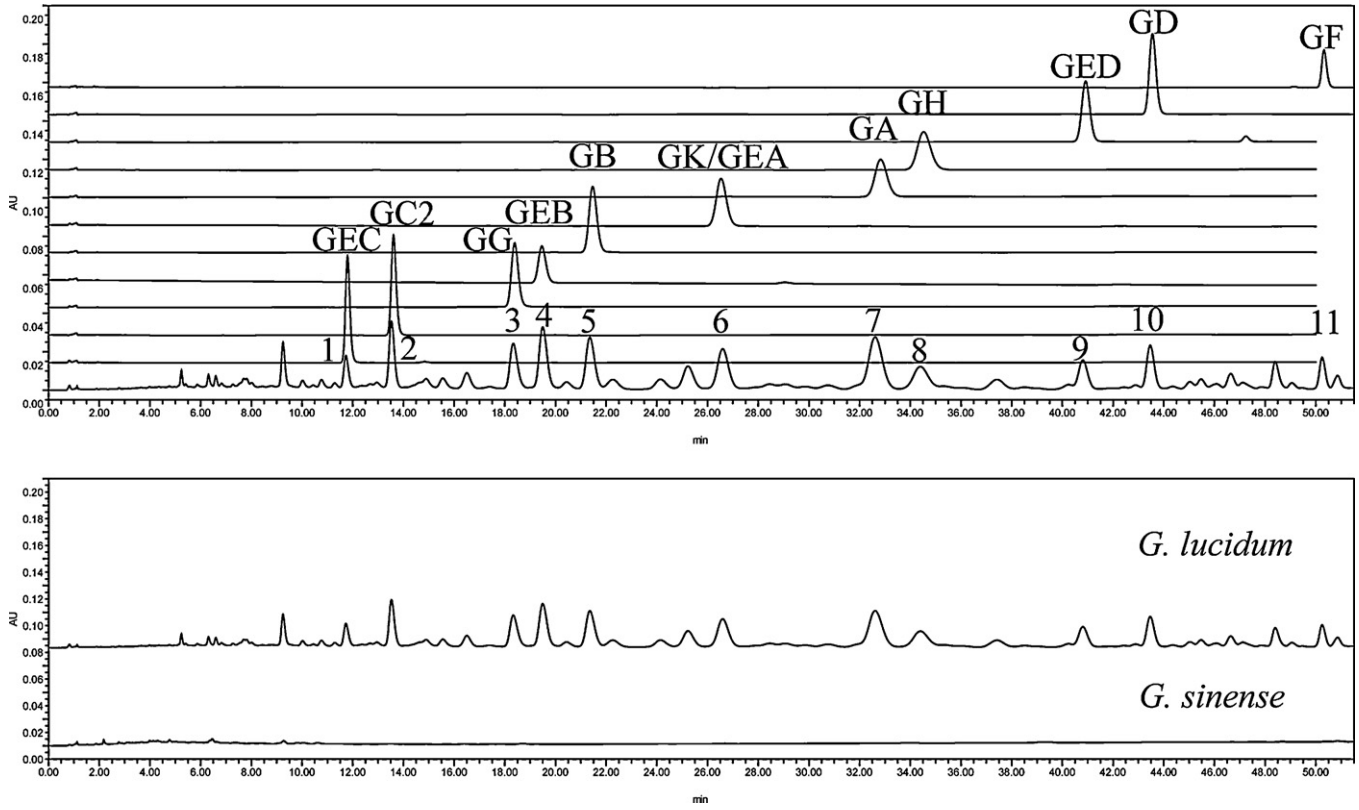



**Fig. 2.** Identification of triterpenes in *G. lucidum* and *G. sinense* with UPLC.

**Table 2**
Retention time and UV $\lambda_{max}$ of reference standards and samples.

| No. | Compounds | Retention time (min) | | UV $\lambda_{max}$ (nm) | | |
|---|---|---|---|---|---|---|
| | | Standard | *G. lucidum* | *G. sinense* | Standard | *G. lucidum* |
| 1 | GEC | 11.800 | 11.825 | – | 251.7 | 251.7 |
| 2 | GC2 | 13.616 | 13.661 | – | 257.9 | 257.9 |
| 3 | GG | 18.400 | 18.498 | – | 255.4 | 255.4 |
| 4 | GEB | 19.508 | 19.636 | – | 248.1 | 248.1 |
| 5 | GB | 21.475 | 21.544 | – | 257.9 | 256.6 |
| 6 | GEA + GK | 26.540 | 26.375 | – | 250.5 (GEA) | 253.0 |
| 7 | GA | 32.841 | 32.792 | – | 256.6 | 256.6 |
| 8 | GH | 34.533 | 34.493 | – | 262.8 | 261.5 |
| 9 | GED | 40.926 | 40.852 | – | 246.8 | 246.8 |
| 10 | GD | 43.564 | 43.525 | – | 256.6 | 256.6 |
| 11 | GF | 50.312 | 50.239 | – | 255.4 | 255.4 |

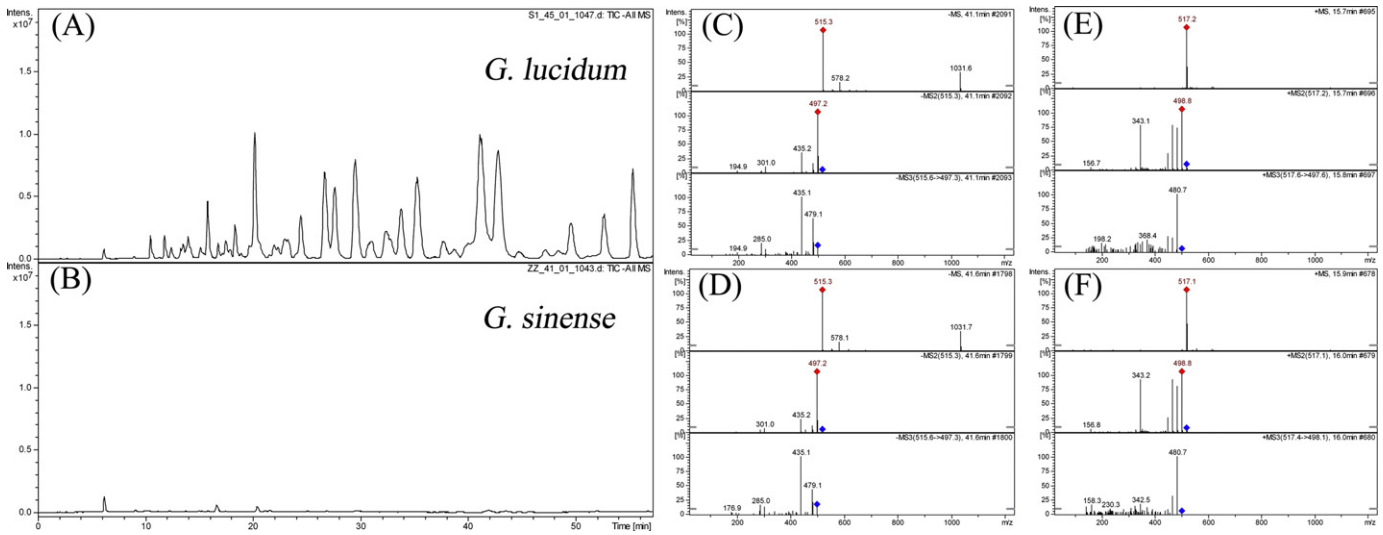



**Fig. 3.** Negative mode total ion chromatogram (TIC) of *Ganoderma* and $MS^n$ spectra of GA and GEC. The inlet: (A) negative TIC of *G. lucidum*; (B) negative TIC of *G. sinense*; (C) $MS^n$ spectra of *m*/*z* 515 for GA in sample solution of *G. lucidum* (negative mode); (D) $MS^n$ spectra of *m*/*z* 515 for GA in reference standard solution (negative mode); (E) $MS^n$ spectra of *m*/*z* 517 for GEC in sample solution of *G. lucidum* (positive mode); (F) $MS^n$ spectra of *m*/*z* 517 for GEC in reference standard solution (positive mode).

**Table 3**
$MS^1$ and $MS^2$ spectral information of reference standards and samples (m/z, negative mode).

| | | MW | $MS^1$ | | | $MS^2$ | | | Base peak (MS1) |
|---|---|---|---|---|---|---|---|---|---|
| | | | Standard | *G. lucidum* | *G. sinense* | Standard | *G. lucidum* | *G. sinense* | |
| 1 | GEC | 516.7 | 561.4 | 561.3 | – | 515.2 | 515.2 | – | $[M-H+HCOOH]^-$ |
| 2 | GC2 | 518.7 | 517.4 | 517.4 | – | 499.2 | 499.2 | – | $[M-H]^-$ |
| 3 | GG | 532.7 | 513.4 | 513.4 | – | 469.2 | 469.1 | – | $[M-H-H_2O]^-$ |
| 4 | GEB | 514.7 | 513.3 | 513.4 | – | 495.2 | 495.2 | – | $[M-H]^-$ |
| 5 | GB | 516.7 | 497.4 | 497.5 | – | 453.2 | 453.2 | – | $[M-H-H_2O]^-$ |
| 6 | GEA | 514.7 | 513.5 | 513.7 | – | 495.1 | – | – | $[M-H]^-$ |
| | GK | 574.7 | 555.1 | 555.4 | – | 468.7 | 469.2 | – | $[M-H-H_2O]^-$ |
| 7 | GA | 516.7 | 515.3 | 515.3 | – | 497.2 | 497.2 | – | $[M-H]^-$ |
| 8 | GH | 572.7 | 553.3 | 553.4 | – | 511.2 | 511.2 | – | $[M-H_2O]^-$ |
| 9 | GED | 512.6 | 511.3 | 511.3 | – | 493.2 | 493.1 | – | $[M-H]^-$ |
| 10 | GD | 514.7 | 495.4 | 495.4 | – | 451.2 | 451.2 | – | $[M-H-H_2O]^-$ |
| 11 | GF | 570.7 | 569.3 | 569.3 | – | 551.1 | 551.1 | – | $[M-H]^-$ |

average of 20 batches (ratios A–C), and ganoderic acids/total acids (ratio D), ganoderic acids/total acids (ratio E) and ganoderenic acids/ganoderic acids (ratio F) for each region.

Differences of ratios A–F were shown for different provinces. From ratio A, total acids of samples from Guangxi and Jiangsu are higher than the average of 20 batches of samples (about 20%), while those from Henan, Fujian and Shandong were opposite. Ratio A of samples from Jilin and Anhui were about 100%, but ratio B from Jilin was much higher. Samples from Anhui were thought to be good representatives for commercial *G. lucidum*, but partially because 11 batches of *G. lucidum* were obtained from this main production region. Ratio D for 7 regions was similar. For samples from Guangxi and Shandong, ratio C was higher than ratio B, opposite to that from other regions. Difference of ratio D and E for samples from Guangxi

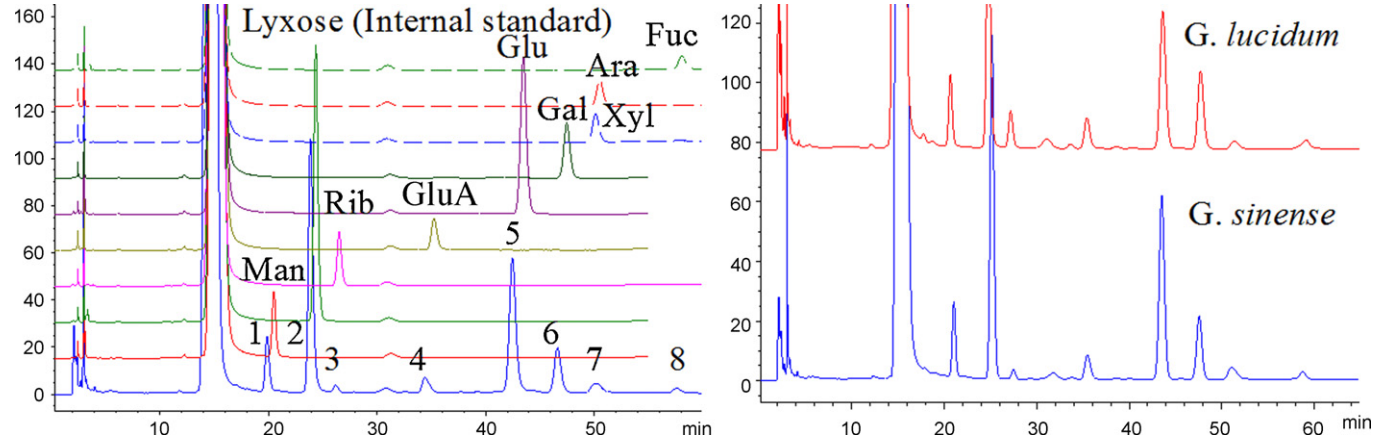



**Fig. 4.** Identification of monosaccharide components in polysaccharides in *G. lucidum* and *G. sinense* with HPLC.

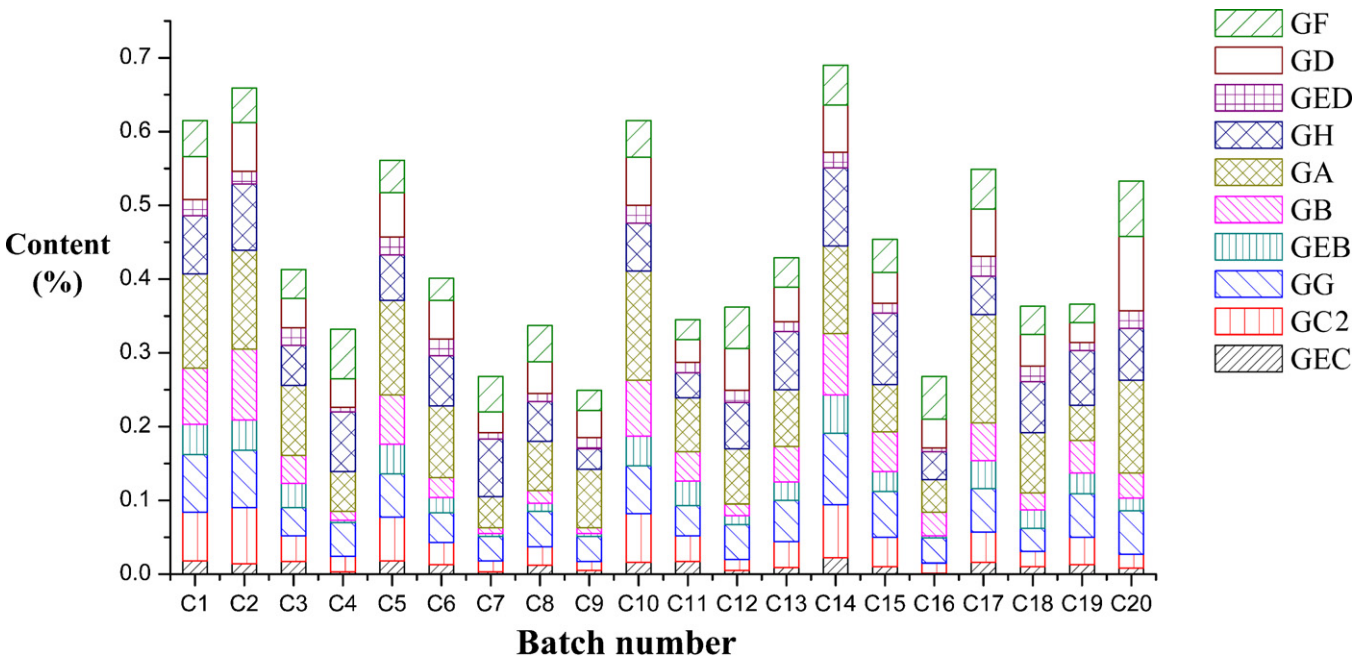



**Fig. 5.** Content of main triterpenes in *G. lucidum*.

were even more apparent. Thus *G. lucidum* from Guangxi might be far apart from others. However, because batch number from those regions is relatively small, more samples were needed for further analysis.

### 3.6. Quantitative analysis of monosaccharides in polysaccharides with HPLC-VWD

#### 3.6.1. Method validation of monosaccharides in polysaccharides: precision, linearity, accuracy, specificity, stability and ruggedness

Precision (repeatability and intermediate precision): Repeatability was assessed with 6 sample solutions. The RSDs of inner-day variation were in the range from 0.72 to 1.68% for individual component, and 0.77% for total content (Table S6).

Intermediate precision was performed through investigation of three different days, three different analysts and three independent equipments, each with three triplicates (Table S7). The RSDs of intra-day variation were in the range from 0.61 to 1.34% for individual component, and 0.67% for total content.

Good linearity of all triterpenes was shown with correlation in the range from 0.9995 to 0.9999 (Table S8).

Because monosaccharides would be lost in the procedure of alcohol precipitation during sample preparation, these reference standards were spiked at three different levels after alcohol precipitation but before hydrolysis. Average recovery of these monosaccharides was ranged from 99.91 to 101.58% (Table S9).

Specificity: retention times of 4 components in sample solution were corresponding to relative standard solution (Fig. 4). No interfering peaks appeared in blank sample.

Stability of sample solution was investigated after storage at room temperature for 0, 1, 2, 3, 4, 6, 8, 10, 12, 24 h (Table S10). Man, Glu and Gal were found to be stable within 24 h (RSDs range from 0.25% to 0.76%) while GluA was stable within 8 h (RSD = 1.47%).

Result of ruggedness indicated that pH value and ratio of components in mobile phase should be strictly controlled. Injection volume should not be over 20 μL. But the wavelength, flow rate, injection volume, column length, column temperature and concentration of phosphate buffer can be slightly adjusted in method application.

#### 3.6.2. Contents of polysaccharides in thirty-two batches of Ganoderma

Four main monosaccharides constituting polysaccharides in *Ganoderma*, including Man, GluA, Glu and Gal were quantitatively determined (Fig. 6A). The average content for Man, GluUA, Glu, and Gal of polysaccharides were 0.072%, 0.039%, 0.741%, and 0.078% for *G. lucidum* and 0.079%, 0.057%, 0.674%, and 0.131% for *G. sinense*. Total content of polysaccharides were 0.930% and 0.942%, respectively. Species have no statistically significant differences on total content according to the result of *F*-test and *T*-test ($F = 5.21 > F_{0.10(19,11)}$, $t' = 0.243$, $\nu \approx 28$, $P > 0.50$). Content and ratio of main monosaccharides constituting polysaccharides in *G. lucidum* and *G. sinense* were analyzed (Fig. 6B and C). Content of Glu was much higher than the others. For both *G. lucidum* and *G. sinense*, the contents of other monosaccharides were as follows (from high to low): Gal, Man and GluA.

Samples from Anhui province were quantitatively closer to average value. Content of *G. lucidum* from Shandong, Jilin and Henan provinces were lower than average level, but the contents of triterpenes were not higher than the average level, suggesting that the content of those components were not in negative correlation.

### 3.7. Discrimination of G. lucidum and G. sinense with PCA

#### 3.7.1. PCA of triterpenes and polysaccharides in 32 samples

PCA was efficient in summarizing multivariate variation into a few principle components remaining maximum possible variability [25]. Content of 10 triterpenes and 4 monosaccharide components in polysaccharides were simultaneously calculated and scatter plots of first two principle components is shown in Fig. 7A. Because small peaks were ignored, another process of PCA of all visible peaks in chromatograms was performed as a supplement (Fig. 7B), but pre-treatment was necessary [29]. As most peaks were baseline separated through optimization of chromatographic conditions, chromatograms were regarded as herbal fingerprints and the evaluation techniques for fingerprints were used [30,31]. A software recommended by Chinese Pharmacopoeia Commission for quality control of TCM, namely “similarity evaluation system for chromatographic fingerprint of TCM, 2004 edition” was applied for

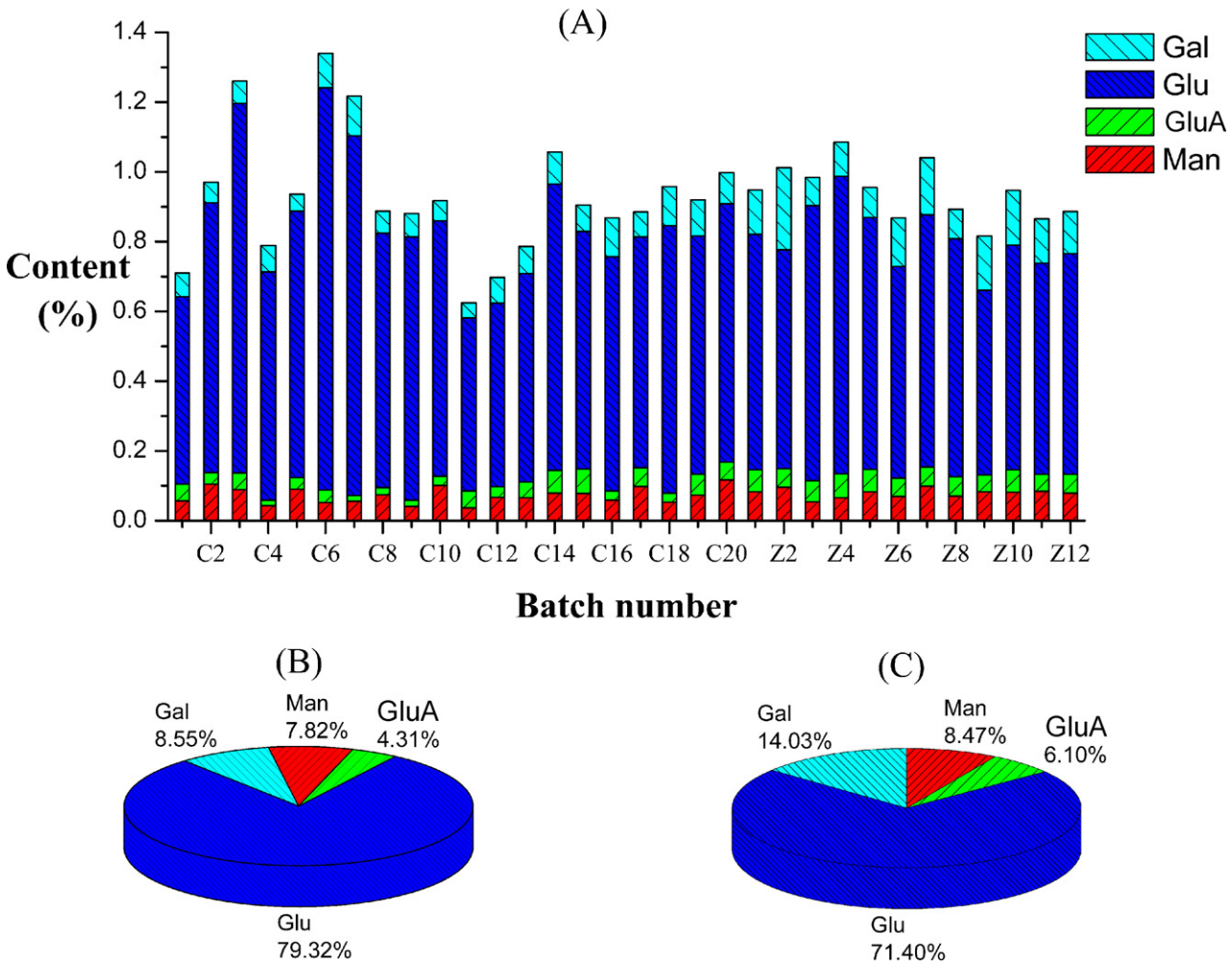



**Fig. 6.** Content and ratio of main monosaccharide components in polysaccharides in *Ganoderma*. (A) content of 32 batches; (B) average ratio of each monosaccharide in total content of 4 monosaccharides in *G. lucidum*; (C) average ratio of each monosaccharide in total content of 4 monosaccharides in *G. sinense*.

peak alignment, similarity analysis and data generation. In PCA of content, four principle components (PCs) were considered to be significant ($Y = 0.9047$) from accumulative reliabilities of initial 14 variables. On the contrary, in PCA of peak areas, ten principle components were accumulated when $Y = 0.9063$.

Thirty-two sample dots were successfully classified into group I and group II corresponding to *G. lucidum* and *G. sinense* (Fig. 7). Dots in group I were relatively nearer to each other, indicating a closer relationship among twelve batches of *G. sinense*, consistent to the undetection of main triterpenes in them. Dots in group II were relatively scattered, suggesting diversification of the 20 batches of samples. Because 2PCs only accounted for 77.08% and 56.79% of total variance, respectively, rules for dispersal was not clear and further analysis was needed.

### 3.7.2. PCA of polysaccharides in 32 samples

Three 3D plots were obtained based on the contents of polysaccharides (Fig. 8A), ratio of each monosaccharide in total content (Fig. 8B), and peak areas (use the data set of polysaccharides generated according to "Section 3.7.1", but not combined with data of triterpenes, Fig. 8C). Dots of *G. lucidum* and *G. sinense* occupied different PCs space. Overlapping did exist (Z3, Z5, Z8, C11, C19 in Fig. 8A, Z3, Z4, Z5, Z8 in Fig. 8B, and confounding of Z8 in Fig. 8C) but the trend of separation was still apparent. Thus discrimination of *G. lucidum* from *G. sinense* based on polysaccharides alone was feasible, despite not as perfect as PCA together with triterpenes.

### 3.8. Discrimination of G. lucidum from different geographical origins

Firstly, two 3D plots were obtained based on the content of triterpenic acids (Fig. S4A) and peak areas (Fig. S4B), with the hope to explain the dispersal described in "Section 3.7.1". Overlapping of dots of *G. lucidum* samples from different provinces indicated the geographical origins not to be the exclusive element influencing triterpenes. However, information was insufficient to make a better classification (such as strains of *G. lucidum*, harvest time, method of cultivation and the nutrient medium used, drying and storage), so classification with data of triterpenes was unsuccessful.

Discrimination based on polysaccharides was better. Some clues were shown in Fig. 8C: the dots on right of the green curve were all from Anhui province. From 3D plots obtained from the content of monosaccharide components in polysaccharides (Fig. 9A), discrimination of four different geographical origins were obvious, better than PCA of peak areas (Fig. 9B).

## 4. Discussion

Multiple chromatographic methods were explored and optimized in the present study in order to achieve a better analysis of main components in *G. lucidum* and *G. sinense*. To our best knowledge, it is the first UPLC method developed for the determination of so many ganoderic and ganoderenic acids, resulting in much

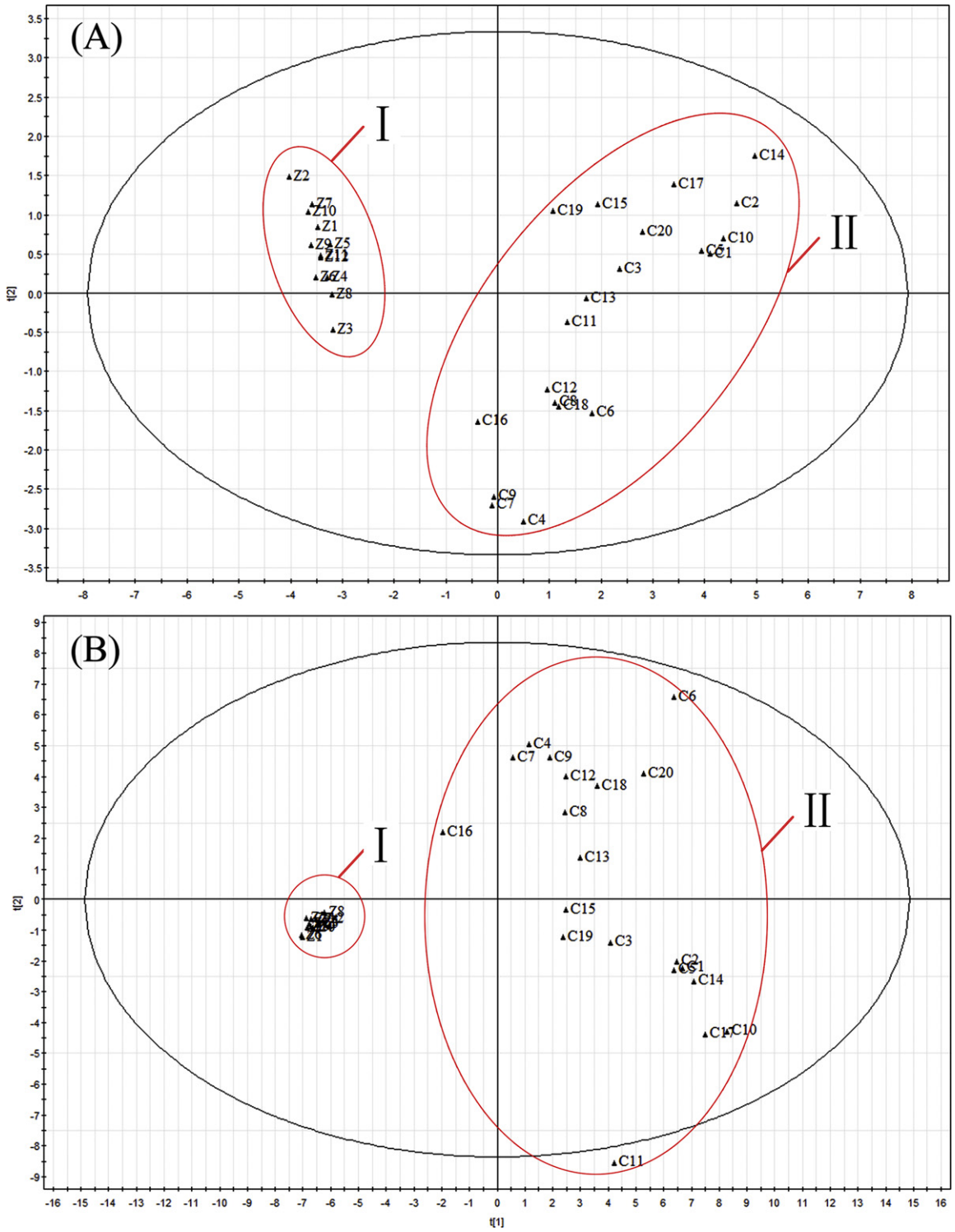



**Fig. 7.** Scatter plot of PCA of 32 batches of *Ganoderma* on the first two principle components. (A) with content of triterpenes and polysaccharides; (B) with area of visual peaks in chromatograms, after alignment.

better separation with resolution bigger than 1.5 for most of the main peaks. Solid phase extraction was also applied, mainly for smoother base line and column protection. In addition, the established methods for analysis of triterpenes and polysaccharides could also be used in quality control of *G. lucidum* and *G. sinense*.

Results of triterpene determination with UPLC and RRLC-ESI-$MS^n$ were comparable and *G. sinense* was chemically different from *G. lucidum*, for the lack of significant triterpenoids commonly existed in *G. lucidum*. Further pharmacological research could be designed for the comparison of their biological effect. Meanwhile, results of $\log \varepsilon$ indicated that peak areas could not replace contents in data analysis, since ganoderenic acids showed much more intense UV absorbance.

The method established for polysaccharides could also be used in quality control. Difference of polysaccharides was not as obvious as that of triterpenes. Sort of monosaccharide and total content of

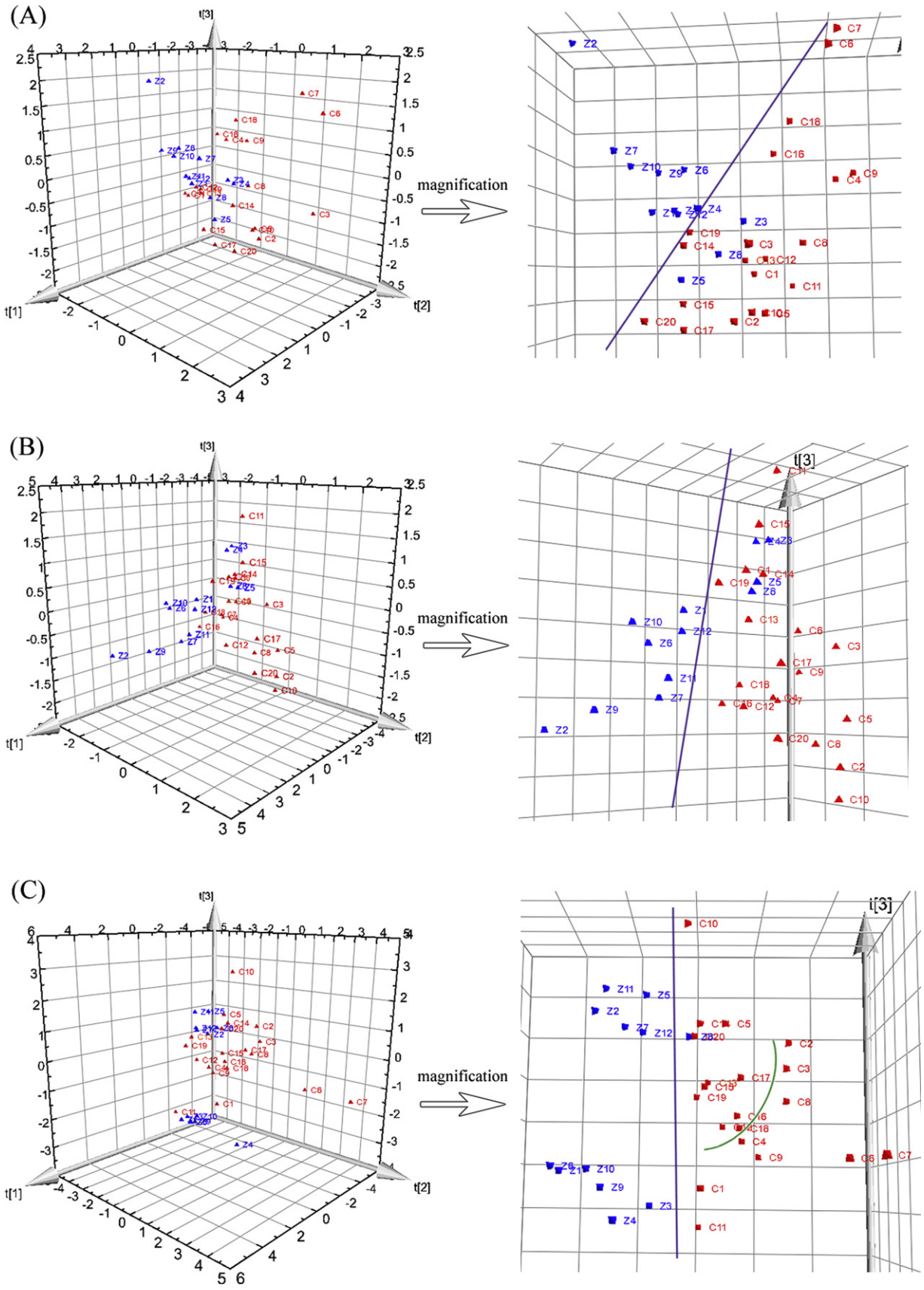



**Fig. 8.** 3D plot of PCA of *Ganoderma* on the first three principle components. (A) with content of monosaccharide components in polysaccharides; (B) with ratio of each monosaccharide in total content; (C) with area of peaks in chromatograms.

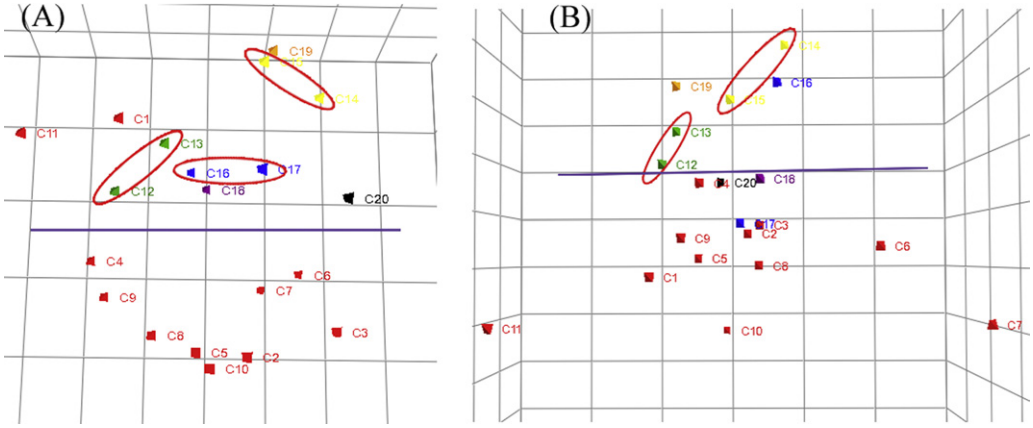



**Fig. 9.** 3D plot of *G. lucidum* from different provinces on the first three principle components after magnification. (A) with content of monosaccharide components in polysaccharides and (B) with area of peaks in chromatograms, after alignment; dots of G. lucidum were shown in different color according to 7 different provinces.

main components were similar, but the ratio of each monosaccharide was different.

PCA was applied in the analysis because the comparison was multivariable. Simultaneous PCA of different types of components and combination of separate fingerprints were tried and found to be feasible. PCA is based on the content of main components and responses of all visual peaks in chromatograms were also performed in comparison.

PCA based on content seems to be more reliable because of the following reasons: firstly, these components were pre-selected and all their information was desired; secondly, the data of content were accurately obtained from replicated samples; thirdly, simultaneous PCA of content was easier, and data alignment was not necessary, thus the result was relatively objective.

However, PCA of peak response of all visual peaks provided the global information and was efficient in outlier organization. Alignment was necessary especially when the chromatographic condition was not stable. The effect of alignment could be clearly observed through similarity analysis. For fingerprints of polysaccharides, the similarity (C1 as the reference) increased from 0.012–0.941 to 0.917–0.997, because aqueous buffer solution contained in mobile phase and resulted in column aging and retention time shifting. For triterpenes, with relatively stable chromatographic condition, effect of alignment was not so obvious (0.801–0.991 to 0.804–0.991). PCA of data before alignment also showed the effect in Fig. S5 and outlier could not be observed compared with Fig. 8B. However, selection of alignment methods was objective.

Beside the successful distinction of two species, PCA was also tried in discrimination of geographical origins. The result was meaningful despite not to the satisfaction. That could be explained by several reasons. Firstly, geographical origin might not be the only factor influencing contents of triterpenes and polysaccharides. For example, *G. lucidum* strains might be different, some species were cultivated for spore production, and some were used for polysaccharides enrichment. But it was hard to obtain such information from the herbal market. Secondly, other active components in *Ganoderma* exist except for triterpenes and polysaccharides (sterols for example), thus information of extracts from other solvents might be useful. Thirdly, for PCA of content, not all triterpenes in *Ganoderma* were taken into consideration. For example, GEA and GK could not be separated with ACQUITY UPLC® HSS T3 column and could not be determined. Finally, more samples should be collected and studied with established methods.

## 5. Conclusions

In this study, through successful investigation of two types of bioactive components in 32 batches of commercial *Ganoderma* samples with multiple-optimized chromatographic and spectroscopic methods, chemical differences were revealed. They were different not only in the types of chemical components but also their contents. The most apparent distinction was the lack of common triterpenes in *G. sinense*. Difference of polysaccharides contained in *G. lucidum* and *G. sinense* could not be directly observed but was revealed by PCA analysis. Therefore, it was unwise for TCM practitioners and manufactories not pointing out species of *Ganoderma* in TCM preparation. This study also led to a way for geographical discrimination, although it was not perfect and further study was needed.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.12.017.

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