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Fast classification and compositional analysis of polysaccharides from TCMs by ultra-performance liquid chromatography coupled with multivariate analysis

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ABSTRACT

A reversed-phase ultra-performance liquid chromatographic (UPLC) method is described for the simultaneous determination of aldoses and uronic acids. The separation was carried out on a UPLC™ HSS T3 column (100 mm × 2.1 mm, 1.7 μm) using precolumn derivatization with 1-phenyl-3-methyl-5-pyrazolone (PMP) and UV detection at 250 nm, and 9 PMP derivatives of mannose, ribose, rhamnose, glucuronic acid, galacturonic acid, glucose, xylose, galactose and arabinose were baseline separated within 5.5 min. Furthermore, the described method was successfully applied to the quantitative analysis of component monosaccharides in polysaccharides extracted from traditional Chinese medicines (TCMs). The results demonstrated the proposed UPLC method was precise and practical for the analysis of TCM polysaccharides. Finally, principal component analysis (PCA) and linear discriminant analysis (LDA) were used to classify and differentiate 30 polysaccharide samples from five TCMs to provide the basis for guiding reasonable use of TCM polysaccharides and controlling their quality better.

1. Introduction

The use of traditional Chinese medicines (TCMs) has a history of several thousands of years and has developed into a unique holistic health care system for the prevention, diagnosis and treatment of diseases (Wang et al., 2009). Recently, water-soluble polysaccharides from TCMs are a class of macromolecules that have attracted a great deal of attention because of their broad spectrum of therapeutic properties and relatively low toxicity (Chen, Zhang, & Zhang, 2007; Fang & Ding, 2007; Schepetkin & Quinn, 2006). It has been reported that the polysaccharides from TCMs have many pharmaceutical effects such as immunomodulatory, immunosuppressive, anti-inflammatory, anti-ulcer, and antioxidant activities (Duan, Chen, Dong, Ding, & Fang, 2010; Inngjerdingen et al., 2007; Luo et al., 2010; Ovodova et al., 2009; Schepetkin & Quinn, 2006; Yamada et al., 1991). However, quality control of TCM polysaccharides is a challenge because it is complex, difficult and time consuming to obtain structural information (Huang, Jin, Zhang, Cheung, & Kennedy, 2007; Sun et al., 2010). Meanwhile, few reports focused on the discrimination of plant polysaccharide origins, which are crucial for quality control of polysaccharides from TCMs (Guan & Li, 2010; Stefan et al., 2009).

Though enzymatic digestion has been proven to be valuable for identification of polysaccharides, it is very difficult to be popularized in a common laboratory due to its high price and laboriousness. However, the monosaccharide profile of polysaccharides is usually determined by chromatographical analysis of the monosaccharides released after acid hydrolysis with either trifluoroacetic acid (TFA), hydrochloric acid (HCl), or sulfuric acid (H_2SO_4) in some cases (Arnous & Meyer, 2009; De Ruiter, Schols, Voragen, & Rombouts, 1992; Meseguer, Boix, Para, & Aguilar, 1999). Though monosaccharide profiles are possible to provide the available knowledge of different plant polysaccharides, they are rarely used to discriminate the different types of plant polysaccharides (Arnous & Meyer, 2009). Actually if some characteristic peaks in batches of monosaccharide profiles show a quite stable appearance under the same condition, they can be used to discriminate different TCM polysaccharide origins even if a lower amount of monosaccharides can be destroyed during the acid hydrolysis process. So monosaccharide profiles from acid hydrolysis are crucial for quality control of polysaccharides from TCMs according to their monosaccharide species and quantities.

The commonly used separation techniques for carbohydrate analysis are gas chromatography (GC), high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE), respectively according to different separation model and detection method. The GC method has good sensitivity and separation efficiency as a popular tool, however, the laborious sample preparation required before the analysis is a drawback (Chen, Xie, Wang,

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Nie, & Chang, 2009; Guo, Xie, Yan, & Wan, 2006; Perry, MacLean, Patrauchan, & Vinogradov, 2007). The most commonly used analytical technique for carbohydrates is HPLC with refractive index detector (RID). Although HPLC-RID method is simple, it has several demerits. The RID is lack of sensitivity and selectivity. When mobile phase or separation column is changed, the method requires some hours until the baseline is stabilized. As an alternative technique, high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) has been popular owing to its ability to analyze carbohydrates without derivatization (Townsend, 1995). However, this method is limited by unstable baselines, loss of sensitivity owing to noise and use of high pH and high salt concentration (Anumula & Taylor, 1991). Except for HPAEC-PAD, most of the LC and CE techniques often use labeling with either fluorescence or UV tags for enhanced detection because these native carbohydrates generally have low intrinsic UV spectral activity (Chen et al., 2009; Daotian & Roger, 1995; Lv et al., 2009). The reagent 1-phenyl-3-methyl-5-pyrazolone (PMP) is one of the popular labels that react with the reducing carbohydrate under mild conditions, requiring no acid catalyst and causing no desialylation and isomerization (Andersen, Bjergegaard, Mller, Srensen, & Srensen, 2003; Daotian & Roger, 1995; Honda et al., 1989; Honda, Suzuki, & Taga, 2003; Lv et al., 2009; Wang & Fang, 2004; Zhang, Xu, Zhang, Zhang, & Zhang, 2003). Since carbohydrates encompass a number of homologues with very similar structures, carbohydrate analysis inevitably requires high-resolution separation and determination techniques. Recently, a novel HPLC technology named ultra-performance liquid chromatography (UPLC) has been developed. By utilizing a new generation of HPLC columns packed with small hybrid material particles (diameter of 1.7 μm) and operating at a much higher column pressure (up to 15,000 psi) than HPLC, UPLC performs much better in terms of resolution, sensitivity and separation efficiency with a significant reduction in sample analysis time and mobile phase solvent consumption (Li, Zhang, Xu, & Fang, 2009). UPLC is becoming increasingly recognized as an important separation technique. Up to now, there has been no report on the quantitative analysis of the monosaccharide composition of polysaccharides using UPLC. Therefore, it is necessary to develop a UPLC method for separation and quantification of component monosaccharides of TCM polysaccharides.

The present paper is specifically concerned with the simultaneous separation of the 9 monosaccharides (aldoses and uronic acids) possibly found in TCMs using UPLC with pre-column PMP derivatization and UV detection at 250 nm. Furthermore, this study was undertaken to discriminate and predict plant polysaccharides from *Ephedra sinica*, *Sanguisorba officinalis*, *Forsythia suspensa*, *Physalis alkekengi* and *Achyranthes bidentata* by using monosaccharide profile data coupled with principal component analysis (PCA) and linear discriminant analysis (LDA), which can provide more comprehensive insights and guidance for the discrimination of TCM polysaccharides.

2. Experimental

2.1. Materials and reagents

The stems of *E. sinica* (Mahuang), fruits of *F. suspensa* (Lianqiao), roots of *A. bidentata* (Niuxi), persistent calyx of *P. alkekengi* (Suanjiang) and roots of *S. officinalis* (Diyu) were collected in May 2009 from Harbin Medical Market and identified by Prof. Zhenyue Wang of Heilongjiang University of Chinese Medicine. All voucher specimens were deposited at Herbarium of Heilongjiang University of Chinese Medicine, Harbin, PR China.

D-Mannose (Man), L-rhamnose (Rha), D-glucose (Glc), D-galactose (Gal), L-arabinose (Ara), D-xylose (Xyl), D-glucuronic acid (GlcUA), D-galacturonic acid (GalUA) and D-ribose (Rib), were purchased from Sigma (St. Louis, USA). Trifluoroacetic acid (TFA) was obtained from Merck (Darmstadt, Germany). Water was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA). 1-Phenyl-3-methyl-5-pyrazolone (PMP), purchased from Beijing Reagent Plant (Beijing, China), and was recrystallized twice from chromatographic grade methanol before use. All other chemicals were of the highest grade available.

2.2. Preparation of polysaccharides from TCMs

The powders of medical materials (200 g) were immersed with 20 folds volume of deionized water and refluxed at 100 °C for 2 h. The filtrate of the obtained extract was condensed in vacuo to a syrup (ca. 300 mL) and precipitated with five volumes of 95% ethanol (approximate 1500 mL). The crude polysaccharide part was precipitated from the alcoholic liquor during its subsequent standing at 4 °C overnight. The precipitate was collected after centrifugation and then washed sequentially with smaller amounts of ethanol, acetone, and ether, respectively. The solid was dissolved in 600 mL of water and deproteinated 5 times with 200 mL of 5:1 chloroform-*n*-butanol as described by Staub (1965). The resulting aqueous fraction was extensively dialyzed (cut-off M_w 3500 Da) against tap water for 48 h and distilled water for 48 h and precipitated again by adding a 5-fold volume of ethanol. After centrifugation, the precipitate was washed with anhydrous ethanol and then dissolved in water and lyophilised to yield each crude polysaccharide was collected by centrifugation (3000 rpm, 10 min, 20 °C). Total carbohydrate contents in polysaccharide samples were determined by phenol–sulfuric acid colorimetric method using glucose as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1958). Proteins in the polysaccharides were detected by the methods of Lowry (Bensadoun & Weinstein, 1976) and UV absorption on a TU-1800PC spectrophotometer (Beijing Purkinje General Instrument Co., Ltd., China).

2.3. Acid hydrolysis of the polysaccharide

A 10 mg aliquot of polysaccharide sample was dissolved in 2 mL of 2 M TFA in an ampoule (5 mL). The ampoule was sealed under a nitrogen atmosphere and kept in boiling water bath to hydrolyze the polysaccharide into component monosaccharides for 10 h. After the ampoule was cooled to room temperature, the reaction mixture was centrifugated at 1000 rpm for 5 min. The supernatant was collected and 1.5 mL of methanol was added into it for the reaction mixture to be evaporated to dryness under a reduced pressure. Then the same amount of methanol was again added and dried by the same method as above, and the procedure was repeated thrice for TFA to be removed. The hydrolyzed and dried sample solutions are added with 2 mL distilled water and then ready for the following experiments. Of course, same procedures were repeated for the various single-factor acid hydrolysis tests.

2.4. Derivatization with PMP reagent

PMP derivatization of monosaccharides was carried out as described previously with proper modification (Andersen et al., 2003; Daotian & Roger, 1995; Honda et al., 2003; Lv et al., 2009; Zhang et al., 2003). 200 μL of individual standard monosaccharide, or mix standard monosaccharide solutions, or the hydrolyzed polysaccharide samples were placed in the 2.0 mL centrifuge tubes, respectively, then 0.5 M methanol solution (100 μL) of PMP and 200 μL of ammonia were added to each. Ribose as an internal standard was added to each sample before the derivatization. Each mixture was allowed to react for 30 min in a 70 °C water bath, then cooled to room temperature and neutralized with 200 μL of formic

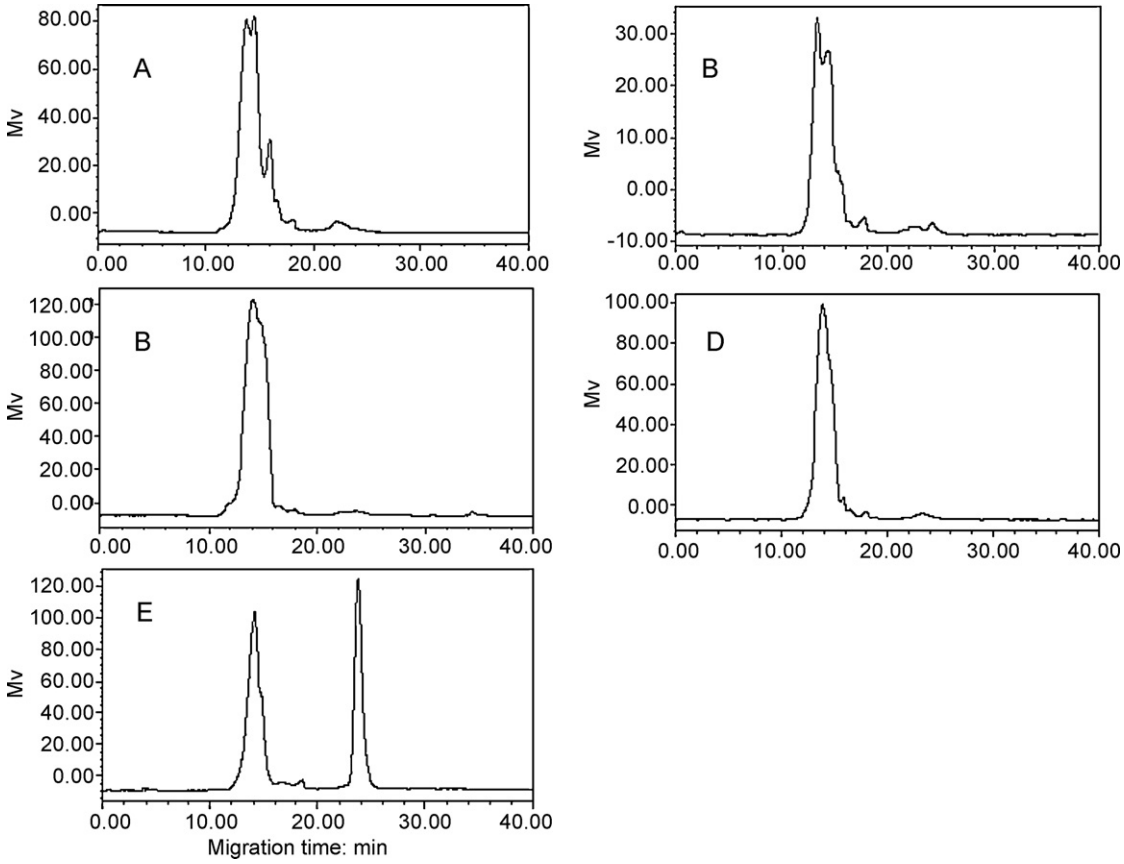

Fig. 1. HPSEC-ELSD profiles of polysaccharides by Shodex sugar KS-805 column. (A) Mahuang polysaccharides; (B) Diyu polysaccharides; (C) Lianqiao polysaccharides; (D) Suanjiang polysaccharides; (E) Niuxi polysaccharides.

acid. The resulting solution (0.9 mL) was separated by liquid–liquid extraction using a volume of isoamyl acetate (two times, 0.9 mL) and chloroform (one time, 0.9 mL), respectively. After being shaken vigorously and centrifuged, the organic phase was carefully discarded to remove the excess reagents. Then the aqueous layer was filtered through a 0.22 μm membrane and diluted with water before UPLC and HPLC analysis.

2.5. HPLC apparatus and conditions

The analyses were performed using a Shimadzu 2010 liquid chromatography system. The separation was carried out on a Shiseido Capcell Pak C_{18} UG 120 (4.6 mm × 150 mm, 5 μm). The isocratic elution program was used with a mobile phase of 0.1 M phosphate buffer (KH_2PO_4–NaOH, pH 6.8)–acetonitrile (84:16). The flow rate was set at 0.8 mL/min and the injection volume was 10 μL with column temperature of 30 °C and detection wavelength of 250 nm.

2.6. UPLC apparatus and conditions

These analyses were performed on Waters Acquity UPLC system coupled with a photodiode array detection method. An Acquity UPLC™ HSS T3 (100 mm × 2.1 mm, 1.7 μm), also from Waters, was used. The column temperature was maintained at 40 °C with detection wavelength of 250 nm. The binary system phases were: A (20 mM ammonium formate) and B (acetonitrile), with a flow rate of 0.4 mL/min, giving a maximum back pressure of 9500 psi, which is within the capabilities of the UPLC. The injection volume was 3 μL. The gradient program was used as follows: initial 0–2 min, linear change from A/B (88:12, v/v) to A/B (84:16, v/v); 2–5 min, linear change to A/B (83:17, v/v); 5–5.1 min, linear change to A/B (88:12, v/v); 5.1–6 min, equilibrated with A/B (88:12, v/v).

2.7. High performance size exclusion chromatography (HPSEC)-ELSD analysis

These analyses were performed on high performance liquid chromatography (HPLC) and evaporative light scattering detector (ELSD), using Waters 2695 HPLC and Alltech ELSD 2000 (Grace Davision Division, Discovery Sciences, Alltech Chromatography, Deerfield, IL, USA). The separation was carried out on a Shodex sugar KS-805 column (8.0 mm × 300 mm, 17 μm) coupled with a Shodex KS-G guard column (6 mm × 50 mm, 7 μm). The isocratic elution was employed using water with 0.5 mL/min at 30 °C and the injection volume was 10 μL. While the drift tube temperature for ELSD was set at 116 °C, the nitrogen flow rate was 3.3 L/min for the determination of polysaccharides.

2.8. Data treatment

SPSS 16.0 program (SPSS Inc., USA) was used for principal component analysis and linear discriminant analysis, respectively.

3. Results and discussion

3.1. Extraction of polysaccharides from TCMs

Such drugs as the stems of *E. sinica* (Mahuang), fruits of *F. suspensa* (Lianqiao), roots of *A. bidentata* (Niuxi), persistent calyx of *P. alkekengi* (Suanjiang) and roots of *S. officinalis* (Diyu) are well-known TCMs, which have also been officially listed in the Chi-

nese Pharmacopoeia for a long time. Polysaccharide extracts from Mahuang, Niuxi and Suanjiang have been elucidated to possess important bioactivity such as immunosuppressive effects, antitumor, hypoglycemic activity, and free radicals scavenging activity (Cheng et al., 2008; Kuang, Xia, Yang, Wang, & Wang, 2011; Tong, Liang, & Wang, 2008). Therefore, it is very essential to develop a simple, reliable and accurate method for the quality control of active polysaccharides from different TCMs.

Thirty batches of polysaccharide samples from the five TCMs were prepared according to the same procedure of the experimental part. Water-soluble polysaccharides are usually obtained by hot-water extraction and alcohol precipitation. Low molecular weight compounds especially monosaccharides incorporated in alcohol precipitate were removed by dialysis against tap water for 48 h and distilled water for 48 h and washed with 95% ethanol to avoid interference during the analysis of saccharides in acidic hydrolysates of polysaccharides. In addition, UV 260 nm and 280 nm were also selected for detection of conjugated nucleic acid or peptide. But only a few low peaks could be detected in the samples. The Lowry method further confirmed that all plant polysaccharides contained minor amounts of protein (2.87–6.35%, w/w). Total carbohydrate contents in Mahuang, Diyu, Lianqiao, Suanjiang and Niuxi polysaccharide samples were more than 87.4% (w/w) by phenol–sulfuric acid method. Firstly, we tried to use HPSEC-ELSD profiles for quality controls of polysaccharide samples from TCMs. The results showed that all polysaccharides samples except ones from Niuxi could not be distinguished just based on their HPSEC-ELSD profiles due to nearly the same retention time and peak shapes (Fig. 1). Therefore, acidic hydrolysates of polysaccharides should be further investigated, which could provide more detailed information.

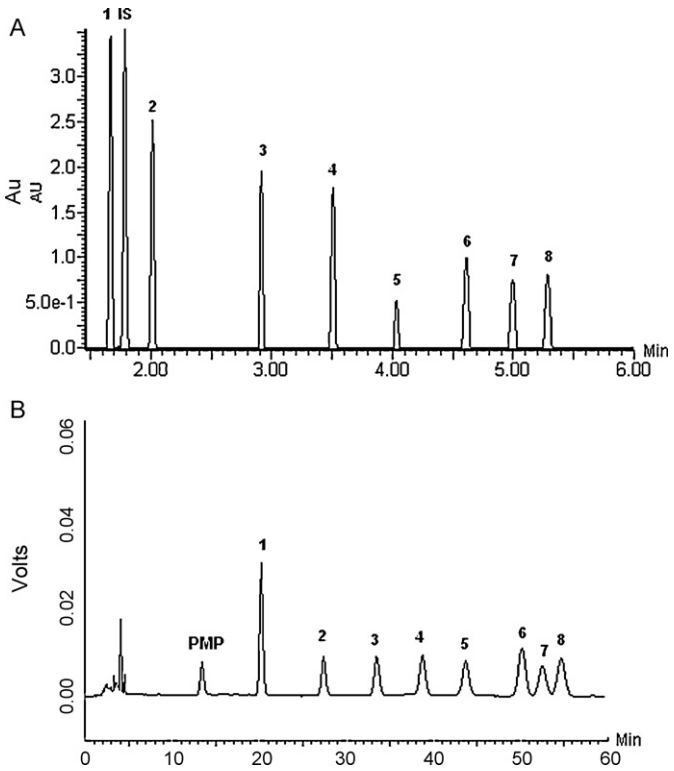

Fig. 2. The UPLC (A) and HPLC (B) chromatograms of PMP derivatives of 8 standard monosaccharides. Peaks: 1, mannose; 2, rhamnose; 3, glucuronic acid; 4, galacturonic acid; 5, glucose; 6, xylose; 7, galactose; 8, arabinose; IS, ribose.

3.2. Quantification and validation

3.2.1. Optimization of chromatographic conditions

The chromatographic conditions were optimized to obtain chromatograms with a good resolution of adjacent peaks within a short analysis time.

In these studies, two different C18 columns, both from Waters, were tested. One was an Acquity UPLC™ BEH 100 mm × 2.1 mm, with 1.7 μm particle size, and the other was an Acquity UPLC™ HSS T3 100 mm × 2.1 mm with 1.7 μm particle size. The HSS T3 column has been proved to obtain a good chromatographic separation. Therefore, the Acquity UPLC™ HSS T3 column was chosen.

To obtain chromatograms with good resolution, acetonitrile–water and methanol–water were investigated and the former was chosen. Various additives of formic acid, acetic acid (both 0.1% and 0.2%, v/v) and ammonium formate (20 mM) were investigated, and 20 mM ammonium formate gave the best separation and peak shape. Therefore, acetonitrile–20 mM ammonium formate aqueous was chosen as mobile phase for gradient elution.

There was no sharp effect upon changing the column temperature (25–40 °C) on either the peak symmetry or the resolution of the eluted peaks, but only a marked increase in the retention time and so the column temperature of 40 °C was used. The effect of different flow rates (0.2–0.6 mL/min) has also been examined. Tailing and poor peaks symmetry appeared at lower values of the flow rate, so 0.4 mL/min was selected as the optimum flow rate. Under these optimum conditions, all the studied aldoses and uronic acids were well separated from each other (Fig. 2A). The peaks were identified in the order of mannose, ribose, rhamnose, glucuronic acid, galacturonic acid, glucose, xylose, galactose and arabinose by comparing the retention time of the unknown peaks with that of the standards under the same conditions.

3.2.2. Validation of the method developed

The UPLC method was validated in terms of linearity, reproducibility, limit of detection (LOD) and precision. It is difficult to be precise between sample preparation procedures. Hence, internal standard is always used to overcome this problem. According

Table 1
Linearity of UPLC method of different carbohydrates.

Carbohydrates[a]	Regression equation[b], $y = ax + b$		R^2	Linear range (μM)	LOQ (μM)	LOD (μM)
	a	b				
Man	0.0732	+0.087	0.9999	4.43–443.00	3.57	1.02
Rha	0.0647	−0.0292	1.0000	4.43–443.00	3.43	0.98
GlcUA	0.0629	−0.0613	1.0000	4.43–443.00	2.91	0.83
GalUA	0.077	−0.1670	0.9982	4.43–443.00	3.68	1.05
Glc	0.0429	−0.0316	1.0000	8.86–443.00	3.92	1.12
Xyl	0.0745	−0.0574	0.9999	8.86–443.00	5.03	1.58
Gal	0.0682	−0.0399	0.9999	8.86–443.00	3.68	1.05
Ara	0.0727	−0.0369	1.0000	8.86–443.00	4.31	1.23

[a] Quantitated with a calibration curve at 250 nm.
[b] Area ratio equation, where y was the area ration between analytes and internal standard and x was the concentration (μM) of analytes.

Table 2
Retention time (t_R) repeatability, area repeatability and recovery of 8 carbohydrates by UPLC.

Carbohydrates	Recovery	Intra-day (n = 5)				Inter-day (n = 3)			
	Mean[a] ± SD	t_R ± SD	RSD (%)[b]	Area ± SD	RSD(%)[b]	t_R ± SD	RSD (%)[b]	Area ± SD	RSD (%)[b]
Man	99.34 ± 3.49	1.67 ± 0.0055	0.33	238,876 ± 156.2	0.07	1.69 ± 0.0346	2.05	240,150 ± 1697.5	0.71
Rha	100.56 ± 4.03	2.01 ± 0.0000	0.00	204,104 ± 224.7	0.11	2.04 ± 0.0436	2.14	205,410 ± 1681.8	0.82
GlcUA	96.00 ± 3.57	2.93 ± 0.0000	0.00	202,897 ± 1624.5	0.80	2.95 ± 0.0436	1.48	200,727 ± 2818.1	1.40
GalUA	97.43 ± 2.48	3.53 ± 0.0052	0.15	248,749 ± 6050.2	2.43	3.55 ± 0.0529	1.49	247,116 ± 6166.9	2.50
Glc	107.77 ± 2.35	4.04 ± 0.0055	0.14	133,700 ± 136.7	0.10	4.08 ± 0.0839	2.06	134,224 ± 1143.1	0.85
Xyl	99.59 ± 2.63	4.62 ± 0.0052	0.11	245,067 ± 2214.9	0.90	4.67 ± 0.0954	2.04	251,747 ± 4593.1	1.82
Gal	103.83 ± 3.30	5.01 ± 0.0063	0.13	331,919 ± 6330.2	1.91	5.06 ± 0.0964	1.91	326,002 ± 9675.3	2.97
Ara	101.98 ± 3.89	5.30 ± 0.0052	0.10	243,768 ± 2895.1	1.19	5.35 ± 0.0872	1.63	244,747 ± 734.8	0.30

[a] Mean value for three different concentrations.
[b] RSD (%) = (SD/mean) × 100.

to our experimental results, ribose, which can be well separated with our analytes but not found in Mahuang, Lianqiao, Niuxi, Suanjiang and Diyu polysaccharide samples, was selected as an internal standard. The linearity was verified by the analysis of six points in the range of 4.43–443.00 μM for mannose, rhamnose, glucuronic acid and galacturonic acid, and 8.86–443.00 μM for glucose, xylose, galactose and arabinose, and the linear regression parameters of the calibration curves were shown in Table 1. As a consequence, the good linearity (correlation coefficient $R^2 > 0.9982$) between y (peak area ratios of the analytes with internal standard) and x (concentration of the standards) was achieved in the tested range. Furthermore, LOD of each tested analyte was obtained by injecting 3 μL of gradational dilutions of a standard mixture derivatized as mentioned above in the derivatization procedure, followed by the comparison of peak height with baseline noise level and a signal-to-noise ratio (S/N) of 3 assigned the detection limit. The results showed that the LOD of the monosaccharides was in the range from 0.83 to 1.58 μM (Table 1) indicating that the sensitivity of the method was satisfactory.

Moreover, the precision of method was also determined by measuring relative standard deviations (RSD) for five successive injections of each tested monosaccharide at the concentration of 20 μM and the results were summarized in Table 2. The results showed that the intra-day reproducibility (RSD values) were less than 0.3% for the migration time and 2.4% for the peak areas, and the interday RSD values were less than 1.1% for the migration time and 3.0% for the peak areas, indicating that the method precision was satisfactory.

3.2.3. Comparison between UPLC and HPLC

By comparing the data obtained and chromatograms generated from the UPLC and HPLC analyses, the advantages of the UPLC method can be summarized as follows (Fig. 2): with UPLC the running time (5.5 min) (Fig. 2A) was about ten times shorter than that with HPLC (60 min) (Fig. 2B). The combination of the shorter running time with a smaller flow rate of 0.4 mL/min reduced the solvent consumption to only 2.2 mL, whereas solvent usage for a single run in HPLC was 48 mL. The lower limit of quantification (LOQ) was defined as the lowest compound concentration that could be determined with a signal-to-noise ratio of 10. In view of the results presented in Table 3, all 8 monosaccharides present a lower LOD and LOQ in UPLC than in HPLC. In respect of repeatability, RSD values in terms of retention time and peak area (inter- and intra-day) were better in UPLC than in HPLC (Table 3). Therefore, UPLC performs much better in terms of resolution, sensitivity, repeatability and is also eco-friendly for its less consumption of organic solvents as compared with HPLC. Finally, we can confirm that the UPLC method is sufficiently selective and sensitive to be applied to analyze chemical composition of TCM polysaccharides.

3.2.4. Optimization of acid hydrolysis procedures

Trifluoroacetic acid (TFA), an effective acid in degrading polysaccharides, was chosen to hydrolyze TCM polysaccharides into component monosaccharides (De Ruiter et al., 1992; Lv et al., 2009). To further confirm the influence of acid hydrolysis for TCM polysaccharides, taking Suanjiang polysaccharides for an example, the hydrolysis results with different molarity acid (1.0, 2.0, and 3.0 M TFA) at 100 °C for different hydrolysis time were characterized by UPLC with pre-column PMP derivatization for the determination of the liberation rate of the neutral and acidic sugars. As depicted in Fig. 3A, although the release of acidic sugars from Suanjiang polysaccharides was considerably accelerated with the increase of TFA concentration, the hydrolysis with 3.0 M TFA gave the highest recovery of free acidic sugars for 6 h, followed with 2.0 M TFA for 10 h, and with 1.0 M TFA for 12 h in decreasing order. However, for neutral sugars, the hydrolysis with 2.0 M TFA gave the highest recovery for 10 h, followed with 1.0 M TFA for 12 h, and 3.0 M TFA for 6 h in decreasing order (Fig. 3B). The results indicated that for polysaccharides complete hydrolysis with 1.0 M TFA cannot be achieved while most neutral and acidic sugars have been destroyed with 3.0 M TFA. Meanwhile, as shown in Fig. 3C, the release of neutral and acidic sugars was significantly dependent on the hydrolysis temperature and went through a peak (100 °C and 120 °C) and then decreased. At a temperature

Table 3
Comparison between UPLC and HPLC methods.

Carbohydrates	t_R (min)		LOD (μM)		LOQ (μM)		t_R RSD% (intra-day)		t_R RSD% (inter-day)		Area RSD% (intra-day)		Area RSD% (inter-day)	
	UPLC	HPLC	UPLC	HPLC	UPLC	HPLC	UPLC	HPLC	UPLC	HPLC	UPLC	HPLC	UPLC	HPLC
Man	1.67	16.52	1.02	1.28	3.57	4.48	0.3	0.8	2.0	2.3	0.1	1.4	0.7	1.3
Rha	2.02	22.40	0.98	1.52	3.43	5.32	0.0	0.6	2.1	2.9	0.1	0.9	0.8	1.6
GlcUA	2.92	27.47	0.83	0.97	2.91	3.40	0.0	1.0	1.5	1.7	0.8	1.6	1.4	2.5
GalUA	3.51	31.87	1.05	1.27	3.68	4.45	0.1	0.6	1.5	2.2	2.4	2.9	2.5	3.4
Glc	4.03	35.96	1.12	1.63	3.92	5.71	0.1	1.0	2.1	3.6	0.1	1.3	0.9	3.5
Xyl	4.61	41.28	1.58	1.75	5.53	6.13	0.1	0.5	2.0	3.2	0.9	3.7	1.8	2.2
Gal	5.00	43.23	1.05	1.45	3.68	5.08	0.1	1.1	1.9	2.4	1.9	2.3	3.0	4.4
Ara	5.29	45.01	1.23	1.73	4.31	6.06	0.1	0.8	1.6	1.5	1.2	1.8	0.3	3.3

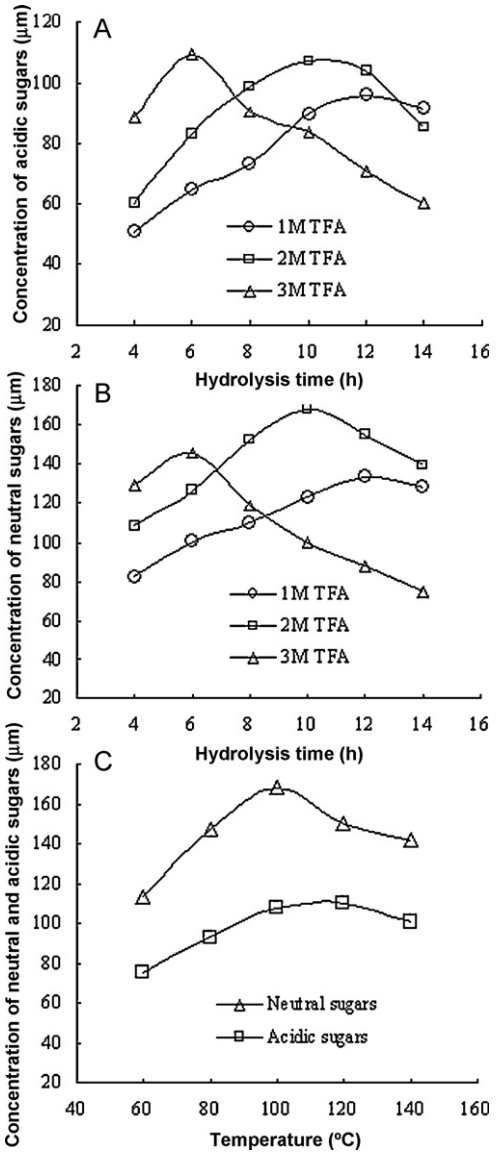

Fig. 3. Concentration of acidic sugars (A) and neutral sugars (B) (μM) released from Suanjiang polysaccharides subjected to treatment with 1.0, 2.0, and 3.0 M TFA at 100 °C versus different hydrolysis time; (C) concentration of neutral and acidic sugars (μM) with 2.0 M TFA at 10 h versus different hydrolysis temperatures. Hydrolysis procedure and UPLC condition are as described in Section 2.

Table 4
Concentration of eight sugars (μM) based on five different plant polysaccharides.

Carbohydrates	Concentration (μM)					
	S1	S2	S3	S4	S5	S6
a						
Man	15.08	14.21	14.32	14.58	14.50	15.12
Rha	22.18	21.01	21.16	21.6	21.18	22.36
GlcUA	8.56	7.99	7.86	7.74	7.69	8.56
GalUA	120.19	109.95	109.8	109.12	112.02	110.39
Glc	36.24	31.86	30.34	30.68	31.02	35.27
Xyl	18.67	20.23	19.9	19.77	20.82	18.45
Gal	60.34	53.50	52.29	53.40	54.31	55.56
Ara	174.47	171.67	167.76	167.34	170.47	168.38
b						
Man	6.05	6.62	6.67	7.15	6.85	7.57
Rha	4.77	5.04	5.99	6.08	5.84	6.56
GlcUA	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
GalUA	57.58	51.47	53.05	65.44	55.83	50.39
Glc	165.56	189.71	170.24	178.55	183.56	192.30
Xyl	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Gal	23.85	20.43	22.66	24.45	23.50	25.29
Ara	36.61	31.51	34.02	30.08	32.65	33.17
c						
Man	13.96	12.92	13.96	14.04	13.84	14.12
Rha	39.55	40.35	38.72	39.74	41.74	40.56
GlcUA	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
GalUA	109.83	108.72	111.9	113.01	112.62	112.57
Glc	45.02	46.70	45.11	44.29	43.26	45.86
Xyl	19.75	20.12	21.62	19.37	20.36	20.48
Gal	55.63	43.72	56.66	56.27	55.72	55.80
Ara	38.35	37.98	38.47	39.24	38.06	38.20
d						
Man	14.74	14.80	15.48	11.39	13.33	14.37
Rha	13.68	13.85	13.96	10.95	12.87	12.35
GlcUA	4.06	4.50	5.19	5.10	5.03	4.97
GalUA	103.32	97.25	103.19	92.31	99.26	95.54
Glc	17.32	15.98	15.76	16.04	16.57	15.89
Xyl	19.01	19.01	18.72	17.09	18.46	19.27
Gal	51.71	51.79	46.51	47.07	48.64	50.77
Ara	51.50	51.51	50.45	46.61	49.35	48.78
e						
Man	3.14	4.34	4.35	2.03	1.82	3.36
Rha	7.75	8.85	7.88	8.78	6.59	6.96
GlcUA	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
GalUA	99.76	91.73	93.35	90.84	93.08	97.80
Glc	13.39	14.10	16.07	15.24	17.34	15.37
Xyl	5.38	6.24	6.92	6.27	5.31	7.51
Gal	17.88	19.16	20.48	19.61	18.92	19.26
Ara	60.18	68.71	79.62	69.26	65.08	78.56

N.D.: not detected. a, Mahuang polysaccharides; b, Diyu polysaccharides; c, Lianqiao polysaccharides; d, Suanjiang polysaccharides; e, Niuxi polysaccharides.

of 120 °C, it is a very obvious phenomenon that the destruction rate of neutral sugars is much bigger than the release rate of acidic sugars. Considering destruction rate and release rate of both neutral and acidic sugars, the optimal acid hydrolysis concentration, time and temperature were set at 2.0 M TFA, 10 h and 100 °C to accomplish a good compromise. When hydrolysis mixtures were further analyzed by HPSEC-ELSD, almost all polysaccharide peaks disappear with concomitant increases in several peaks whose retention time were identical with monosaccharides rather than oligosaccharide peaks. Besides, we verified mass balance of total carbohydrates and uronic acids between before and after acid hydrolysis under optimal hydrolysis conditions by phenol–sulfuric method (Dubois et al., 1958) and *m*-hydroxydiphenyl-sulfuric acid method (Blumenkrantz & Asboe-Hansen, 1973). The results showed that only a few contents being 5.2% (w/w) of total carbohydrate and 2.3% (w/w) of uronic acids were lost during the hydrolytic steps for Suanjiang polysaccharides and strongly suggested that most TCM polysaccharide samples could be essentially hydrolyzed completely using 2 M TFA in 100 °C for 10 h.

3.2.5. Application to the analysis of real samples

This experiment was designed to develop a rapid, repeatable and accurate analysis method for the quantification of the component monosaccharides in the TCM polysaccharides up to 30 batches of samples from Mahuang, Diyu, Lianqiao, Suanjiang and Niuxi. In order to evaluate the applicability of the proposed method, each polysaccharide sample was hydrolyzed with TFA, dried and PMP-labeled as described in Section 2 and finally, the released monosaccharide derivatives were analyzed by the described UPLC method under the optimized conditions using ribose as internal standard. Fig. 4 shows a typical chromatogram of five TCM polysaccharide samples and the detected contents were listed in Table 4. As can be seen, the PMP derivatives of the component monosaccharides released from all polysaccharide samples could still be

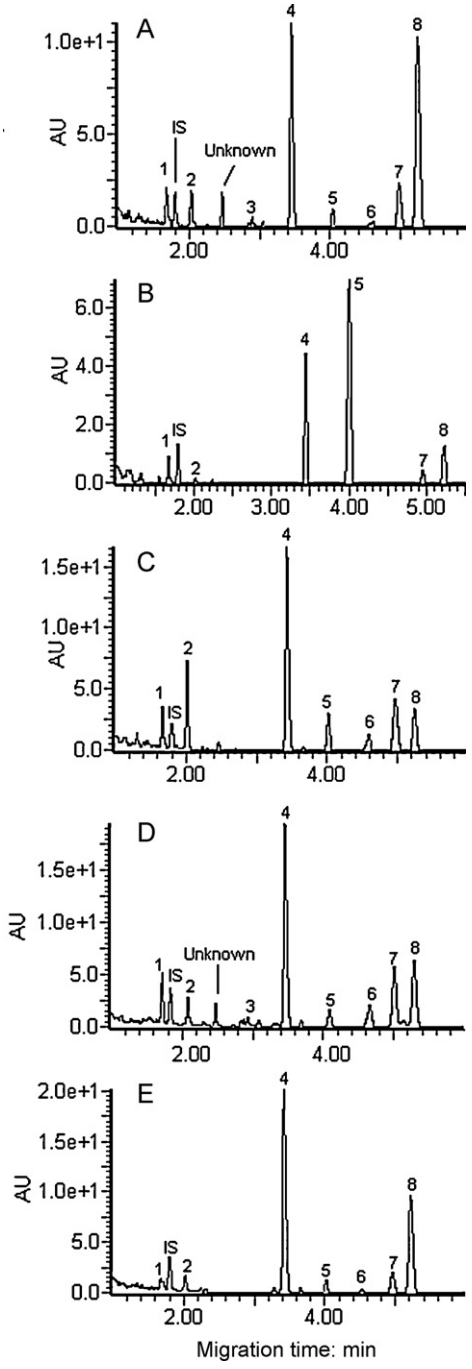

Fig. 4. UPLC chromatograms of PMP derivatized hydrolysate of polysaccharides from five TCMs. (A) Mahuang polysaccharides; (B) Diyu polysaccharides; (C) Lianqiao polysaccharides; (D) Suanjiang polysaccharides; (E) Niuxi polysaccharides. Peak identities were described in Fig. 2.

baseline separated and the component monosaccharides could be identified by comparing with the chromatogram of the mixture of standard monosaccharides (Fig. 2A). The results showed that all polysaccharide samples from five TCMs were typical acidic heteropolysaccharides and consisted of mannose, rhamnose, and/or glucuronic acid, galacturonic acid, glucose, and/or xylose, galactose and arabinose, and their corresponding molar contents were different from each other. It was clear that the predominant composition monosaccharides in Mahuang and Diyu polysaccharide samples were arabinose and glucose. However, the predominant composition monosaccharide in Lianqiao, Suanjiang and Niuxi polysaccharide samples was galacturonic acid.

Furthermore, recovery experiments were performed in order to investigate the accuracy of the method. Known amounts of each monosaccharide solute were added to the Mahuang polysaccharide samples detected, and the resulting spiked sample was subjected to the entire analytical sequence. Each solute was spiked at a concentration close to the sample and recoveries were calculated based on the difference between the total amount determined in the spiked samples and the amount observed in the non-spiked samples. All analyses were carried out in triplicate. The results show that the recoveries of all the eight monosaccharides ranged between 96.00% and 107.77% (Table 2) and the RSD values fell within 2.18–4.01%. Such results further demonstrated that this method is precise and practical for the analysis of polysaccharide samples from TCMs.

3.3. Discrimination of polysaccharides based on multivariate statistical analysis

3.3.1. Principal component analysis (PCA)

The determination by eye of trends between 30 polysaccharide samples from five TCMs can be extremely difficult and time consuming. PCA is a widely used unsupervised method. It relates a data matrix containing independent variables from samples (Lu et al., 2008). By performing PCA on this monosaccharide profile data, trends can be visualized and key marker compounds related to different polysaccharide samples from TCMs can be determined. A two-component score plot of UPLC data was utilized to depict general variations of polysaccharide samples among the different TCMs. The clear separation of different polysaccharide samples was observed in the PCA score plot (Fig. 5A), where each coordinate represents a sample. The difference between these polysaccharide samples is a result of them coming from different TCMs.

The loading plot was utilized to identify the differential monosaccharides accountable for the separation among different groups (Fig. 5B). Clearly, two of the variables (Ara and GlcUA) are associated with Mahuang polysaccharides (group a), which are also in accordance with the fact that the contents of arabinose and glucuronic acid are much higher in Mahuang polysaccharide samples than other polysaccharide samples. So arabinose and glucuronic acid can be considered to be marker components for Mahuang polysaccharides to differentiate other TCM polysaccharide samples. Moreover, the variable x_5 (Glc) was characterized by negative values of both principal components 1 and 2. Meanwhile, it exhibited a positive correlation to the group b (Diyu polysaccharides) and negative correlation to the remains and therefore can be used to discriminate Diyu polysaccharide samples from other TCM polysaccharides.

3.3.2. Linear discriminant analysis (LDA)

LDA could be used to build a predictive model of the group membership based on observed characteristics of each case. This procedure generates a discriminant function (or, for more than two groups, a set of discriminant functions) based on linear combinations of the predictor variables that provide the best discrimination among the groups. The functions were generated from the samples with known membership; the functions could then be applied to new cases with measurements for the predictor variables but with unknown group membership. Here, we collected 30 polysaccharide samples from five different TCMs as training samples to establish discriminant model (function) of Mahuang, Diyu, Lianqiao, Suanjiang and Niuxi polysaccharide samples. As a result, all training samples could be classified into five groups (Fig. 5C). The analysis of canonical discriminant functions using LDA was consis-

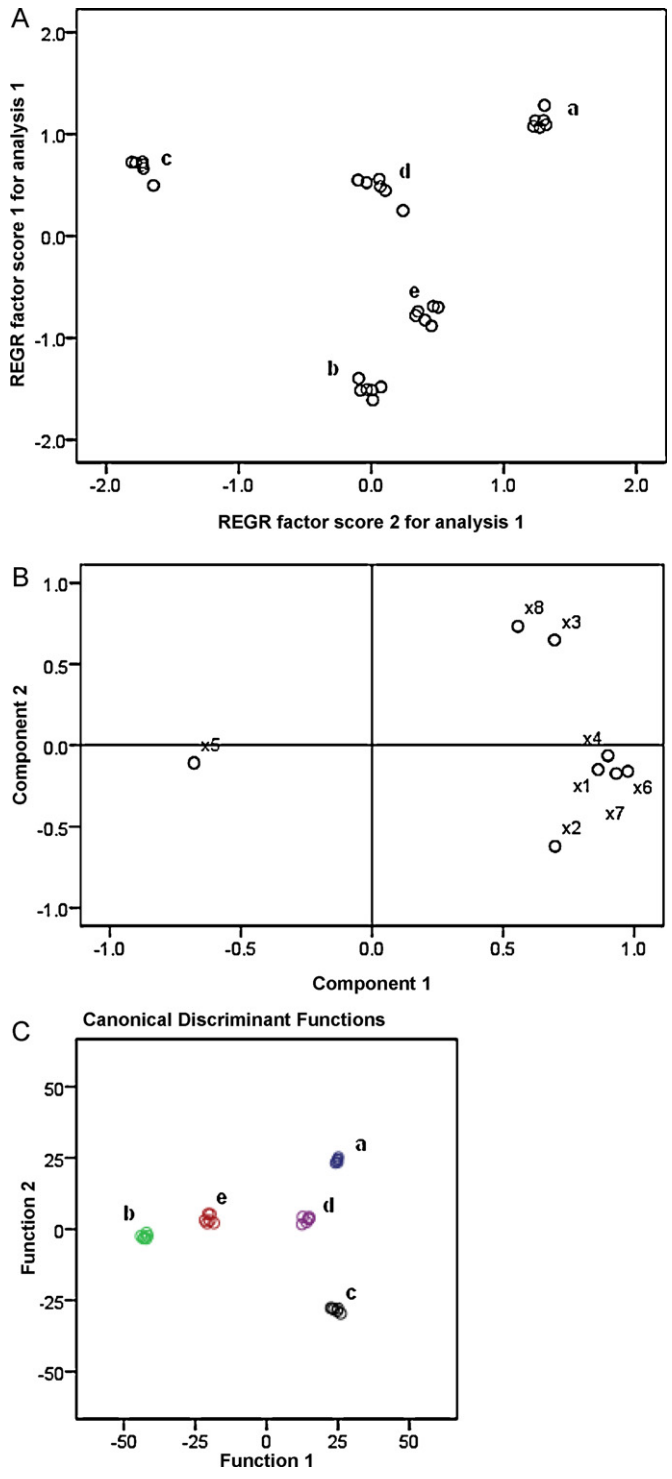

Fig. 5. (A) Score plots from PCA; (B) loading plots from PCA; (C) comprehensive scatter diagram of LDA discriminant functions. a, Mahuang polysaccharides; b, Diyu polysaccharides; c, Lianqiao polysaccharides; d, Suanjiang polysaccharides; e, Niuxi polysaccharides. x_1, Man; x_2, Rha; x_3, GlcUA; x_4, GalUA; x_5, Glc; x_6, Xyl; x_7, Gal; x_8, Ara.

tent with that of PCA (Fig. 5A). All the variables were of value to the establishment of discriminant function. The five discriminant functions from five different TCMs were as follows:

$$y_1 = -60.993x_1 + 49.852x_2 + 200.496x_3 + 7.335x_4 + 3.204x_5 + 77.934x_6 + 3.631x_7 + 7.397x_8 - 2.858 \times 10^3$$

$$y_2 = -18.656x_1 + 2.897x_2 - 32.495x_3 + 6.624x_4 + 9.230x_5 - 2.345x_6 - 0.253x_7 + 3.960x_8 - 1.024 \times 10^3$$

$$y_3 = -79.102x_1 + 82.365x_2 + 101.809x_3 + 7.593x_4 + 3.715x_5 + 93.802x_6 + 7.514x_7 - 2.864x_8 - 2.713 \times 10^3$$

$$y_4 = -32.996x_1 + 27.238x_2 + 170.242x_3 + 5.731x_4 + 1.678x_5 + 84.476x_6 + 5.603x_7 - 3.624x_8 - 1.485 \times 10^3$$

$$y_5 = -33.667x_1 + 18.143x_2 + 0.230x_3 + 7.744x_4 + 2.879x_5 + 13.179x_6 - 0.678x_7 + 5.483x_8 - 633.914 \times 10^3$$

where y_1 denotes samples from Mahuang, y_2 denotes samples from Diyu, y_3 denotes samples from Lianqiao, y_4 denotes samples from Suanjiang, y_5 denotes samples from Niuxi, and x_i denotes the variables.

From the discriminant functions, it can be seen that all 8 variables were used to generate the functions. These 8 variables denoted the molar contents of mannose, rhamnose, glucuronic acid, galacturonic acid, glucose, xylose, galactose and arabinose, respectively. When assigning an unknown sample, the molar content values of the 8 variables can be put into the four functions and the sample belongs to the group where the calculated value of the function is the highest. According to discriminant functions y_1–y_5, 100% of original grouped cases were correctly classified.

In practice, any one of the two statistical methods could accomplish the identification process, but the combination of representative UPLC monosaccharide profiles with PCA and LDA provided a more reliable and comprehensive result. It was concluded that the established method was considered to be suitable for monosaccharide profile analysis to identify plant polysaccharide origins.

4. Conclusion

This study coupled UPLC with chemometrics for both qualitative and quantitative analysis of monosaccharide components in the evaluation of the origins of TCM polysaccharides with high sample throughput. Compared with conventional HPLC, this method offered shorter analysis time in one tenth of the time required for the conventional system and allowed the detection of higher numbers of chemical constituents in plant matrices.

This newly established method was validated to be sensitive, precise and accurate, and has been successfully applied to the quantitative determination of eight monosaccharide components in Mahuang, Diyu, Lianqiao, Suanjiang and Niuxi polysaccharide samples. PCA was used to objectively screen marker components for the determination of trends within similar samples from different TCM polysaccharide samples. LDA could be used to build a predictive model of the group membership based on observed monosaccharide profile data of each polysaccharide sample. The proposed method can also be used for the standardization and differentiation of large numbers of similar polysaccharide samples of TCM plants.

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