A novel and rapid HPGPC-based strategy for quality control of saccharide-dominant herbal materials: Dendrobium officinale, a case study

Jun Xu
Hong Kong Baptist University

Song-Lin Li
Jiangsu Province Academy of Traditional Chinese Medicine

Rui-Qi Yue
Hong Kong Baptist University

Chun-Hay Ko
The Chinese University of Hong Kong

Jiang-Miao Hu
Chinese Academy of Sciences

See next page for additional authors

This document is the authors’ final version of the published article.
Link to published article: http://dx.doi.org/10.1007/s00216-014-8060-9

APA Citation

This Journal Article is brought to you for free and open access by HKBU Institutional Repository. It has been accepted for inclusion in HKBU Staff Publication by an authorized administrator of HKBU Institutional Repository. For more information, please contact repository@hkbu.edu.hk.
Authors
Jun Xu, Song-Lin Li, Rui-Qi Yue, Chun-Hay Ko, Jiang-Miao Hu, Jing Liu, Hing-Man Ho, Tao Yi, Zhong-Zhen Zhao, Jun Zhou, Ping-Chung Leung, Hu-Biao Chen, and Quan-Bin Han

This journal article is available at HKBU Institutional Repository: https://repository.hkbu.edu.hk/hkbu_staff_publication/2601
A novel and rapid HPGPC-based strategy for quality control of saccharide-dominant herbal materials: *Dendrobium officinale*, a case study

Jun Xu¹, Song-Lin Li², Rui-Qi Yue¹, Chun-Hay Ko³, Jiang-Miao Hu⁴, Jing Liu¹,
Hing-Man Ho¹, Tao Yi¹, Zhong-Zhen Zhao¹, Jun Zhou⁴, Ping-Chung Leung³, Hu-Biao Chen*¹, Quan-Bin Han*¹

¹School of Chinese Medicine, Hong Kong Baptist University, Hong Kong
²Department of Pharmaceutical Analysis and Metabolomics, Jiangsu Branch of China Academy of Chinese Medical Sciences and Jiangsu Province Academy of Traditional Chinese Medicine, Nanjing, China
³State Key Laboratory of Phytochemistry and Plant Resources in West China, Institute of Chinese Medicine, The Chinese University of Hong Kong, Shatin, N. T., Hong Kong SAR, China
⁴State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, Yunnan, China

*Corresponding authors:

Q.B. Han
7 Baptist University Road, School of Chinese Medicine, Hong Kong Baptist University, Kowloon Tong, Hong Kong SAR, China
Tel: 00852-34112906 / Fax: 00852-34112461
E-mail: simonhan@hkbu.edu.hk

H.B. Chen
7 Baptist University Road, School of Chinese Medicine, Hong Kong Baptist University, Kowloon Tong, Hong Kong SAR, China
Tel: 00852-34112060 / Fax: 00852-34112461
E-mail: hbchen@hkbu.edu.hk
Abstract:
Qualitative and quantitative characterization of natural saccharides, especially polysaccharides, in herb materials remains a challenge due to their complicated structures and high macro-molecular masses. Currently available methods involve time-consuming and complicated operations, and present poor specificity. Here, a novel and rapid HPGPC-based approach is described for quality assessment of saccharide-dominant herbal materials by simultaneously qualitative and quantitative analysis of saccharide components. *Dendrobium officinale*, one of the rarest tonic herbs worldwide, was employed as the model herb in this study. First, a HPGPC (high performance gel permeation chromatography) fingerprint based on the molecular weight distribution of its carbohydrate components was established for qualitative identification of *D. officinale*. Then, HPGPC-guided dominant holistic polysaccharide marker was separated using ultra-filtration followed by HPGPC determination for quantitative evaluation of *D. officinale*. The experimental results suggest that this method is more efficient, stable and convenient compared with the currently available methods for authentication and quality evaluation of *D. officinale*, and we expect the method will have similar advantages when used for quality control of other saccharide-dominant herbal materials and products.

Keywords: HPGPC fingerprints, Saccharide-dominant herbal materials, Holistic polysaccharide marker, Quality evaluation, *Dendrobium officinale*
1. Introduction

Qualitative and quantitative characterization of natural saccharides, especially polysaccharides, in herb materials remains a challenge due to their complicated structures and macro-molecular masses [1]. Generally, isolation and purification followed by complete structural characterization, namely homogeneity and molecular weight determination, compositional monosaccharide analysis, glucosidic linkage type confirmation and then repetitive structural unit speculation, is the most reliable approach to quality evaluation of polysaccharides and oligosaccharides in herbal materials [2]. However, it is widely acknowledged that the methodologies involved are extremely complicated, difficult and time-consuming [3-6] and therefore not suitable for routine analysis. In addition to the comprehensive approach described above, there are two kinds of analytical methods, namely, total sugar content determination and sugar composition analysis that have been widely employing for quality control of carbohydrates in herbal materials [7, 8]. But these two methods have serious defects. Total sugar content determination by colorimetric method has very poor specificity and is thus inapplicable for qualitative purposes [9, 10]. Sugar composition analysis entails complicated operations, like acid hydrolysis and derivation, followed by qualitative and quantitative determination of sugar derivatives by chromatographic approach. In this latter method, the experimental results are always affected by multiple factors in the operating procedure, e.g. the temperature, reaction time and acid concentration of acid hydrolysis, and are therefore significantly variable [11-14]. Furthermore, more importantly, the method might be one-sided since
it could not reflect the original existence of polysaccharides and oligosaccharides before acid hydrolysis [15]. Nevertheless, data on the saccharide components is of increasing importance to quality control of herbal pharmaceuticals and even to clinical research. Therefore, a simple, efficient, quick and reliable method for identifying and quantifying carbohydrate components in herbal samples is urgently needed.

High performance gel permeation chromatography (HPGPC), a type of size exclusion chromatography that separates analytes on the basis of molecular size, is designed for analytical and preparative separation of synthesized water-soluble polymers, oligomers and biological substances such as polysaccharides, nucleic acids, proteins, peptides [16], etc. In research on herbal materials, HPGPC is widely employed for purity and molecular weight determination of purified polysaccharides or oligosaccharides by characterizing peak symmetry and calculating with established retention time-molecular weight standard curve, respectively [17, 18]. To the best of our knowledge, however, HPGPC has never been used for both qualitative authentication and direct quantitative determination of carbohydrates in herbal materials.

Dendrobii Officinalis Caulis, called *Tiepi Shihu* in Chinese, which is derived from dried stems of *Dendrobium officinale* Kimura et Migo, is traditionally recognized as the best *Dendrobium* herb for tonic purposes, such as benefiting the stomach, supplementing body fluids and strengthening immunity [19]. Nowadays, due to
extremely scarce wild resources and increasing demand, it has become one of the most expensive herbs in herbal markets worldwide, particularly in Southeast Asia. For selling in herbal markets, the stems of *D. officinale* are always heated, twisted into a spiral or spring form, and finally dried; in this form, it is commonly known as *Tiepi Fengdou* (Supplementary Fig. 2). The uncharacteristic appearance and high price of *Tiepi Fengdou* has led to the occurrence of adulterants, confused species, and counterfeits [20]. Authentication and quality evaluation of *Tiepi Fengdou* is therefore crucial for ensuring the safety and efficacy of this valuable herbal tonic.

Continuous efforts have been made for quality control of *D. officinale* based on qualitative and/or quantitative characterization, but the methods used are far from satisfactory. This herb has a unique chemical profile, in which saccharides account for up to 70%, along with some small molecules, such as bibenzyls and phenanthrenes [21-23], etc. Quality evaluation focusing on small molecules [24, 25] has failed to efficiently distinguish *D. officinale* from other *Dendrobium* species. And in these studies, the investigated constituents were less than 0.21% of the whole herb material. In other words, more than 99% components in these samples were uncontrollable by these methods. Given their dominant contents and proved bioactivities, carbohydrates are a more natural and logical target for quality control of *D. officinale*. Nevertheless, as mentioned above, quality control of carbohydrates in *D. officinale*, just like other saccharide-dominant herbal materials, has been stymied by a methodological bottleneck [24].
In this study, taking *D. officinale* as a model herb, we developed a novel, rapid, and reliable HPGPC method for determination and quantification of carbohydrates in herbal materials. First, a compartmental HPGPC fingerprint based on molecular weight distribution was established for qualitative identification of saccharide components of *D. officinale*. Subsequently, the dominant polysaccharide peak in the GPC chromatogram was separated and then regarded as the unique holistic chemical marker for subsequent quantitative evaluation of *D. officinale* (Supplementary Fig. 1A). For the purpose of comparison, the conventional method, sugar composition analysis (Supplementary Fig. 1B), was also performed.

2. Experimental

2.1 Materials and chemicals

The commercial *Tiepi Fengdou* samples were purchased from different locations in China. The authentic *Tiepi Fengdou* samples, and other fourteen *Dendrobium* species samples, namely *D. fimbriatum*, *D. nobile*, *D. chrysotoxum*, *D. thyrsiflorum*, *D. chrysanthum*, *D. aurantiacum*, *D. crepidatum*, *D. densiflorum*, *D. williamsonii*, *D. aphyllum*, *D. pendulum*, *D. primulinum*, *D. trigonopus* and *D. chameleon* (Supplementary Fig. 2), were kindly provided by several certificated production areas in mainland China and were authenticated by Dr. Chen Hubiao (Table 1). The voucher specimens were deposited at School of Chinese Medicine, Hong Kong Baptist University, Hong Kong.
Acetonitrile and ammonium acetate for HPLC analysis were purchased from Merck (Darmstadt, Germany). Deionized water was prepared by Millipore Milli Q-Plus system (Millipore, Bedford, MA, USA). Trifluoroacetic acid (TFA) used for acid hydrolysis of carbohydrates was from Riedel-de Haën (Honeywell International Inc., Germany). 1-Phenyl-3-methyl-5-pyrazolone (PMP) for monosaccharide derivatization was bought from Sigma (St. Louis, MO, USA). The reference substances, D-galacturonic acid monohydrate (GalA), D-glucuronic acid (GlcA), L-arabinose (Ara), D-mannose (Man), D-galactose (Gal), D-glucose (Glc), L-rhamnose monohydrate (Rha), D-fucose (Fuc) and D-ribose (Rib), and a series of Dextrans with different molecular weights (1 kDa; 5 kDa; 12 kDa; 25 kDa; 50 kDa; 80 kDa; 150 kDa; 270 kDa; 410 kDa and 670 kDa) were purchased from Sigma (St. Louis, MO, USA).

2.2 HPGPC analysis

2.2.1 Water extraction

Dried sample powder (0.10 g) was extracted with water at 100°C (5 mL × 1 h × 2 times). The extracted solutions were centrifuged at 4000×g for 10 min and the supernatants were then combined for further analysis.

2.2.2 HPGPC conditions

The prepared water extracts of Dendrobium samples were directly analyzed using
HPGPC performed on an Agilent 1100 series HPLC-DAD system (Agilent Technologies, Palo Alto, CA) coupled with evaporative light scattering detector (ELSD). The separation was achieved on a two tandem TSK GMPW XL columns (300 mm × 7.8 mm i.d., 10 μm) system operated at 40°C. Ammonium acetate aqueous solution (20 mM) was used as mobile phase at a flow rate of 0.6 mL/min. The signal from ELSD was transmitted to an Agilent Chemstation for processing through an Agilent 35900E interface. The parameters of ELSD were set as follows: the drift tube temperature was 120 °C; nebulizer nitrogen gas flow rate was at 3.2 L/min; impact off mode. An aliquot of 20 μL solution was injected for analysis. UV detection wavelengths were set at 260 and 280 nm.

Aqueous stock solutions of dextrans (2 mg/mL) with different molecular weights (1 kDa; 5 kDa; 12 kDa; 25 kDa; 50 kDa; 80 kDa; 150 kDa; 270 kDa; 410 kDa and 670 kDa) and glucose were injected into the HPGPC using the same conditions as for the construction of the molecular weight-retention time calibration curve by plotting logarithm of the peak area versus the retention time of each analyte.

2.2.3 HPGPC-guided chemical marker separation

The dominant peak (named DOP) in the HPGPC fingerprints of Tiepi Fengdou, representing most of the polysaccharides in Tiepi Fengdou, were separated using ultra centrifugal filters [molecular weight cut-off (MWCO)= 10 kDa] (Millipore, Billerica, MA). In detail, each water extract of D. officinale (15 mL) was transferred into an ultra centrifugal filter tube and then centrifuged at 4000×g eight times (15 min each).
Finally, the remains were re-dissolved in 15 mL water and then freeze-dried for further analysis.

2.2.5 HPGPC quantitative method validation

The HPGPC method for quantitative analysis of DOP was validated in terms of linearity, sensitivity, precision, accuracy and stability.

Aqueous stock solution of DOP was diluted to appropriate concentrations for the construction of a calibration curve. Six concentrations of the solution were analyzed in triplicate, and the calibration curve was constructed by plotting logarithms of the peak areas versus logarithms of the DOP concentrations. The stock solutions were diluted to a series of appropriate concentrations with aqueous solutions, and an aliquot of each of the diluted solutions was injected into HPGPC for analysis. Intra- and inter-day variations were chosen to determine the precision of the developed assay.

For intra-day variability test, the *Tiepi Fengdou* sample (ATF-03) was extracted and analyzed for six replicates within one day, while for inter-day variability test, the same sample was examined in duplicates for three consecutive days. Variations in the logarithms of the peak areas were expressed by the RSDs of the data. The spike recovery test was used to evaluate the accuracy of the method. About 0.05 g of *Dendrobium* sample (ATF-03) with known contents of DOP was weighed, and different amounts (high, middle and low level) of DOP were spiked, then extracted and analyzed in triplicate. The spike recoveries were calculated by the following equation: Spike recovery (%) = (total amount detected-amount original) / amount
spiked×100 %. The stability test was performed by analyzing the sample (ATF-03) extract over periods of 2 h, 4 h, 6 h, 8 h, 12 h, and 24 h. The RSD for the logarithm of the DOP peak areas was taken as the measures of stability.

2.3 Sugar composition analysis

2.3.1 Acid hydrolysis of water extracts

The prepared water extract (Section 2.2.1) solution (0.50 mL) was mixed with 2.50 mL of trifluoroacetic acid (TFA) (final concentration 2 M) solution in a screw-cap vial, and hydrolyzed for 2 h at 120 °C. After cooling, the hydrolysate was evaporated at 55°C on a rotary evaporator until dry. Then 1 mL aqueous solution was added to dissolve the hydrolysate, and the precipitate was removed after centrifugation (15700 ×g, 5 min), the supernatant was then subjected to PMP derivatization.

2.3.2 PMP derivatization of monosaccharides

The sugar derivatization was performed according to previous report [26] with modifications. Briefly, the acid hydrolysate (100 μL) was mixed with the same volume of ammonia water and 0.5 M PMP methanolic solution (200 μL). The mixture was allowed to react at 70°C for 30 min and then cooled to room temperature. Afterwards, 100 μL glacial acetic acid and 500 μL chloroform were successively added to neutralize the reaction solution and remove the excess PMP reagents, respectively. After vigorous shaking followed by centrifugation at 15700 ×g for 5 min, the organic phase was discarded. The operation was performed five times, and finally
the aqueous layer was diluted 10 times and filtered through a 0.22 μm syringe filter (Agilent Technologies, USA) before LC-DAD analysis. A standard solution, containing 7 monosaccharides (Rha, Ara, Fuc, Man, Glu, Gal and Rib) and 2 uronic acids (GlcA and GalA), was also treated as mentioned above.

2.3.3 HPLC analysis

Analysis of PMP derivatives of released monosaccharides in *Dendrobium* aqueous extracts after acid hydrolysis was performed on an Agilent 1100 series HPLC-DAD system (Agilent Technologies, Palo Alto, CA) which was equipped with a vacuum degasser, a binary pump, an autosampler and a diode array detector. Samples (5 μL) were injected onto a Grace Alltima™ C18 column-W (250 mm × 4.6 mm i.d., 5 μm) operated at 30°C. The separation was achieved using gradient elution with 100 mM ammonium acetate aqueous solution (A) (pH=5.58) and acetonitrile (B) at a flow rate of 1.0 mL/min: 0~5 min, 17~20 % B; 5~30 min, 20~28 % B; 30-35 min, 28 % B. UV detection wavelength was set at 245 nm.

The HPLC method for quantitative analysis of the compositional monosaccharides was validated with regard to linearity, sensitivity, precision, accuracy and stability.

3 Results and discussion

3.1 Methodology optimization

3.1.1 Optimization of water extraction

Extraction conditions were optimized in order to achieve complete extraction of
carbohydrates. The saccharides in the sample materials could be completely extracted at 100°C after two times (1h each time) since no sugar was detected by sulfuric acid-phenol method in the subsequent third extraction.

3.1.2 Optimization of HPGPC analysis

In order to furthest separate the carbohydrates in the investigated *Dendrobium* samples, the HPGPC columns used in this study were optimized. Previously, the TSK G3000PWXL gel column was tested, but the results showed that some of the macro-molecular polysaccharides (>60 kDa) were not retained on the columns and instead were eluted immediately due to the limited molecular weight range capacity (~60 kDa) of these columns [27]. Finally, the other kind of SEC column with greater molecular weight range (~7, 000 kDa), TSK GMPWXL column, was employed in this study and two TSK GMPWXL columns were connected in series for improving the resolution. Under the optimized conditions, polysaccharides, oligosaccharides and monosaccharides in the *Dendrobium* samples could be roughly separated (calculated by the established molecular weight-retention time calibration curve) (Fig. 1).
Fig. 1 HPGPC chromatograms of water extracts from authentic *Tiepi Fengdou* samples, other *Dendrobium* species (A) and commercial *Tiepi Fengdou* samples (B). The sample codes are the same as in Table 1.

Additionally, the method for separation of DOP from the water extracts of *Tiepi Fengdou* samples was also optimized. Ultra centrifugal filters [molecular weight cut-off (MWCO) = 10 kDa] were used because the molecular weights of DOP were larger than 10 kDa and the other part with smaller molecular weights (< 10 kDa) (seen in Section 3.4) could be completely cut off by centrifugation (Fig. 2A). The centrifugal time was confirmed with the aid of HPGPC monitoring till no peak with the molecular weight less than 10 kDa was detected in the remains.
Fig. 2 Ultra-filtration for preparation of the holistic polysaccharides marker (peak 1, DOP) (A) and the statistical results for the description of Tiepi Fengdou HPGPC fingerprints (B). The sample codes are the same as in Table 1.

3.1.3 Optimization of sugar composition analysis

The concentration of TFA, the reaction time and temperature of acid hydrolysis were optimized. The total amount of derivative monosaccharides produced from water
extracts of *Tiepi Fengdou* samples by acid hydrolysis was used as the evaluation maker for optimization. The results (data not shown) suggested that 2 M TFA should be selected for acid hydrolysis and that the reaction was most efficient at 120°C for 2 h. In the PMP derivation, ammonia water, instead of the conventionally used sodium hydroxide, was selected because it could be easily removed by acetic acid. The ammonium acetate generated by this process was identical with the mobile phase of HPLC assay and therefore had no negative effect either on the HPLC column or the analysis. Different types of reversed-phase columns were tested for the HPLC analysis and the Grace Alltima™ C18 column-W was finally selected on account of its excellent separation efficiency and repeatability.

### 3.2 Method validation for quantitative analysis

Method validation for HPGPC quantitative analysis was performed. The linearity data indicated good relationship between concentrations and logarithm peak areas of the separated DOP within the test ranges ($R^2 = 0.9990$). And the overall RSDs of intra- and inter-day variations were not more than, severally, 1.64 % and 3.85 %. The established method also had acceptable accuracy with spike recovery of 95.69-107.89 % for the analytes (RSDs < 6.23 %). As to stability test, the RSDs of the peak areas detected within 24 h were lower than 1.43 %. All these results indicated that the established quantitative HPGPC method was linear, sensitive, precise, accurate, and stable enough for determination of DOP in the *Dendrobium* sample. Besides, as to HPLC analysis for compositional monosaccharide determination, the
method validation results were also acceptable (data not shown).

3.3 Sugar composition analysis

The compositional monosaccharides in the water extracts of investigated Dendrobium samples were determined using the established HPLC method. The typical chromatograms and calculated contents of the released monosaccharides after acid hydrolysis in water extracts of all Dendrobium samples were summarized in Fig. 3 and Table 1, respectively, and further confirmed the obtained results in previous reports [24]. The analysis demonstrated that sugar composition exhibited highly qualitative consistence in all Dendrobium samples. There were mainly two compositional monosaccharides, Man and Glc, in the water extracts of the Dendrobium samples. In addition, the quantitative results of the two compositional monosaccharides in some of the samples were also similar. It has been clearly proved that sugar composition analysis was poor in selectivity and therefore might hinder the authentication and quality evaluation of D. officinale. Besides, the experimental procedure was so complicated that the quantitative results could be influenced by multiple factors (Most RSD values of the quantitative results are close to 5%) (Table 1).
Table 1 Quantitative results of carbohydrate components in the investigated *Dendrobium* samples (mg/g) based on sugar composition analysis and HPGPC analysis

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Harvesting/Purchasing time</th>
<th>Locality</th>
<th>Sugar composition analysis</th>
<th>HPGPC quantitative analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Water extracts</td>
<td>DOP</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>D-mannose</td>
<td>D-glucose</td>
</tr>
<tr>
<td>Authentic <em>Tiepi Fengdou</em> samples</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATF-01</td>
<td>Oct., 2011</td>
<td>Guangxi, China</td>
<td>431.55 a) (4.97) b)</td>
<td>73.42 (3.57)</td>
</tr>
<tr>
<td>ATF-02</td>
<td>May, 2012</td>
<td>Anhui, China</td>
<td>399.30 (4.44)</td>
<td>76.52 (1.00)</td>
</tr>
<tr>
<td>ATF-03</td>
<td>Mar., 2012</td>
<td>Yunnan, China</td>
<td>413.27 (4.39)</td>
<td>77.86 (4.63)</td>
</tr>
<tr>
<td>ATF-04</td>
<td>Jul., 2012</td>
<td>Zhejiang, China</td>
<td>458.40 (4.81)</td>
<td>63.79 (3.29)</td>
</tr>
<tr>
<td>ATF-05</td>
<td>Oct., 2011</td>
<td>Guangxi, China</td>
<td>418.96 (1.73)</td>
<td>63.55 (2.18)</td>
</tr>
<tr>
<td>ATF-06</td>
<td>Oct., 2011</td>
<td>Guangxi, China</td>
<td>388.19 (1.57)</td>
<td>105.72 (5.09)</td>
</tr>
<tr>
<td>ATF-07</td>
<td>Jul., 2012</td>
<td>Zhejiang, China</td>
<td>414.72 (4.89)</td>
<td>67.31 (2.46)</td>
</tr>
<tr>
<td>ATF-08</td>
<td>Mar., 2012</td>
<td>Yunnan, China</td>
<td>410.71 (3.31)</td>
<td>62.22 (4.98)</td>
</tr>
<tr>
<td>ATF-09</td>
<td>Jul., 2012</td>
<td>Zhejiang, China</td>
<td>419.53 (3.13)</td>
<td>83.12 (3.84)</td>
</tr>
<tr>
<td>ATF-10</td>
<td>Mar., 2012</td>
<td>Yunnan, China</td>
<td>424.55 (2.10)</td>
<td>65.38 (5.38)</td>
</tr>
<tr>
<td>Commercial <em>Tiepi Fengdou</em> samples</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTF-01</td>
<td>Apr., 2012</td>
<td>Hong Kong</td>
<td>404.38 (3.88)</td>
<td>75.97 (4.39)</td>
</tr>
<tr>
<td>CTF-02</td>
<td>Apr., 2012</td>
<td>Hong Kong</td>
<td>289.86 (5.01)</td>
<td>93.82 (4.22)</td>
</tr>
<tr>
<td>CTF-03</td>
<td>May, 2012</td>
<td>Guangzhou, China</td>
<td>460.07 (4.31)</td>
<td>117.23 (2.40)</td>
</tr>
<tr>
<td>CTF-04</td>
<td>Apr., 2012</td>
<td>Hong Kong</td>
<td>468.69 (3.92)</td>
<td>78.86 (3.19)</td>
</tr>
<tr>
<td>CTF-05</td>
<td>Apr., 2012</td>
<td>Hong Kong</td>
<td>338.72 (4.79)</td>
<td>70.71 (2.13)</td>
</tr>
<tr>
<td>Date</td>
<td>Location</td>
<td>Species</td>
<td>Yunnan, China</td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>--------------</td>
<td>---------------</td>
<td>---------------</td>
<td></td>
</tr>
<tr>
<td>CTF-06 Jun., 2012</td>
<td>Anhui, China</td>
<td>D. nobile</td>
<td>46.66 (4.01)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>D. thyrsiflorum</td>
<td>189.98 (4.74)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>D. chrysanthum</td>
<td>104.26 (3.38)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>D. aurantiacum</td>
<td>93.04 (4.12)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>D. crepidatum</td>
<td>107.91 (4.86)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>D. densiflorum</td>
<td>139.09 (2.33)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>D. williamsonii</td>
<td>78.54 (4.63)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>D. aphyllum</td>
<td>118.99 (3.33)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>D. pendulum</td>
<td>121.45 (4.69)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>D. primulinum</td>
<td>32.64 (4.21)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>D. fimbriatum</td>
<td>123.43 (4.43)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>D. chrysotoxum</td>
<td>135.13 (4.25)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>D. trigonopus</td>
<td>60.74 (4.08)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>D. chameleon</td>
<td>96.16 (3.20)</td>
<td></td>
</tr>
</tbody>
</table>
a) The data was present as average of triplicate determination;

b) The RSD value of triplicate quantitative results (%).
Fig. 3 HPLC chromatograms of compositional monosaccharide analysis in different *Dendrobium* samples. (A) Mixed monosaccharide standards; (B) Authentic and commercial *Tiepi Fengdou* samples and other *Dendrobium* species samples. The sample codes are the same as in Table 1.

### 3.4 HPGPC analysis

Total *Dendrobium* samples were qualitatively analyzed by HPGPC. First, water extracts of ten batches of authentic *Tiepi Fengdou* samples (Table 1) were analyzed and compared using HPGPC-DAD-ELSD, in which UV 260 nm and 280 nm were selected for monitoring saccharide-conjugated nucleic acids and/or peptides, and the
major peaks had no obvious absorbance under the investigated conditions (data not shown). As shown in Fig. 1A, the GPC chromatograms of the 10 batches of *Tiepi Fengdou* samples were strikingly similar. According to the overlapped GPC chromatograms, five peaks, namely peak 1 (DOP, RT: 23.39±1.06 min; MW: 276.23-877.66 kDa), 2 (RT: 29.73±0.31 min; MW: 5.58-9.34 kDa), 3 (RT: 30.94±0.16 min; MW: 2.16-2.90 kDa), 4 (RT: 32.40±0.49 min; MW: 0.34-0.98 kDa) and 5 (RT: 33.93±0.51 min; MW: 0.05-0.18 kDa) (calculated by the established molecular weight-retention time calibration curve), appeared in these *Tiepi Fengdou* samples based on their molecular distribution (Fig. 1) with peak 1 (DOP) was dominant. The results could preliminarily demonstrate the quality of the ten batches of authentic *Tiepi Fengdou* samples presented consistently and the chromatograms could be regarded as the HPGPC fingerprints for qualitative identification of *Tiepi Fengdou*.

When fourteen other commonly used *Dendrobium* species samples were also analyzed, their GPC chromatograms differed from that of *D. officinale* with regard to not only molecular size range but also the peak pattern (Fig. 1A). In particular, peak 1 (DOP), which was most prominent in samples of *D. officinale*, was hardly detected in most of these samples. This was true even for *D. nobile*, one of the most frequently used substitutes of *D. officinale*. These results clearly demonstrate that the carbohydrates in *D. officinale* are characteristic and dissimilar with those in other confused species.
Finally, thirteen batches of commercial *Tiepi Fengdou* samples were investigated. Their GPC chromatograms are summarized in Fig. 1B. It can be seen that they are similar to authentic samples and distinctively different from other *Dendrobium* species samples. However, based on the chromatograms, even though the molecular weight ranges of carbohydrates in these commercial samples were similar with the authentic ones, their distributions were inconsistent. For example, in the chromatograms of some commercial samples (CTF-11, 12 and 13), peak 1 (DOP) were sharply decreased while peak 5 was dominant compared with those of authentic *Tiepi Fengdou* samples. The HPGPC analysis results could partly illustrate that all these commercial samples might be derived from *D. officinale* plants but just their quality were not well controlled.

To sum up, in the HPGPC fingerprints, the dominant role of peak 1 (DOP) in the non-monosaccharide (polysaccharide and oligosaccharide) parts (peak 1, 2, 3 and 4) was the most distinctive trait of the authentic and commercial *Tiepi Fengdou* samples and this peak pattern consistently distinguished *D. officinale* from other species. So the area of peak 1 (DOP) was selected as the underlying variable and a correlation coefficient (F) for all the samples was obtained by the following equation:

$$F = \frac{A_1 - \sum_{k=2}^{4} A_k}{\sum_{k=1}^{4} A_k},$$

where A means the area of the corresponding peak, to represent the HPGPC fingerprint characteristics and further evaluate the quality of *Tiepi Fengdou* (Fig. 2B).
Theoretically, the more dominant peak 1 in four peaks, the closer F approaches 1. As shown in Fig. 2B, the F values of ten authentic Tiepi Fengdou samples are stable and very close to 1.0 while those of the commercial samples fluctuated between 0.5-1.0. However, the values of the other species samples were far from 0.5-1.0 and even dropped to -1.0. The curve on the graph clearly shows that two numerical intervals, F→1.0 and 0.5<F<1.0, could be regarded as the judgement standards for superior quality and authenticity of Tiepi Fengdou samples, respectively.

The above obtained results of HPGPC analysis should be more reasonable than those of sugar composition analysis. For example, carbohydrate components in CTF-10 and DT should be qualitatively and quantitatively consistent in view of their similar monosaccharide compositions (Table 1). But, based on HPGPC chromatograms, they were definitely different (Fig. 1). As known, sugar composition analysis is built on breakdown of original existential state of polysaccharides and oligosaccharides in herbal materials. So it is easy to understand that there is no necessary relationship between compositional monosaccharides and holistic chemical properties, such as molecular weight, of carbohydrate components, which are very important for carbohydrate-based quality control of herbal materials. It could be therefore concluded that sugar composition analysis method seemed to be unreasonable and might confuse the quality control of Tiepi Fengdou. Conversely, HPGPC fingerprint, which does not need any sample pretreatment and is therefore much more convenient than sugar composition analysis, could provide characteristics of component carbohydrates in terms of molecular weight distribution. After all, as a
fingerprint method, the use of this HPGPC marker needs to be tested by more real samples.

3.5 HPGPC quantitative analysis

In addition to identification, the highly consistent GPC chromatograms of ten batches of authentic Tiepi Fengdou samples also suggested that the dominant polysaccharide peak (peak 1, DOP) could be used for quantitative quality control of Tiepi Fengdou. Thus, in this study, DOP in ten batches of authentic Tiepi Fengdou samples was purified (Section 3.1.2) (Fig. 2A) and the qualitative consistency of ten separated peaks was then further confirmed in terms of their highly similar compositional monosaccharides (peak area ratios of Man and Glc are 5.37~6.17). After that, the obtained DOP was used as the holistic polysaccharide marker for quantitative determination of all investigated Tiepi Fengdou samples, and the method was validated (Section 3.2). The results, summarized in Table 1, illustrated that the contents of DOP in ten batches of authentic Tiepi Fengdou samples were close and all over 30%, while those in commercial samples varied greatly. This verifies the unstable quality of commercial Tiepi Fengdou.

Though multiple defects mentioned above, sugar composition analysis is still a presently well-accepted, and also most reliable method for carbohydrate content determination in natural products [1]. To verify the accuracy of our HPGPC quantitative method, DOP in all the D. officinale samples was separated and then
subjected to sugar composition analysis as described above (Section 2.3). The quantitative results are listed in Table 1 and statistically compared (Supplementary Fig. 3). It can be expressly seen that the sets of data derived from these two methods are close without significant differences (Supplementary Fig. 3), further confirming that the novel HPGPC approach is accurate and credible for quantitative determination of polysaccharides in herbal materials. The results are the same but the HPGPC-based quantitative method is much more convenient, stable (RSD less than 2.75%, Table 1) and therefore efficient than sugar composition analysis, which is generally recognized as a tedious method susceptible to error.

4 Concluding remarks

In this study, taking D. officinale as a model herb, a novel and rapid HPGPC-based method was developed for quality control of saccharide-dominant herbal materials by simultaneously qualitative and quantitative characterization of saccharide components. The concepts of HPGPC fingerprint and holistic polysaccharide marker were firstly proposed, and HPGPC was also firstly employed for direct quantitation of natural polysaccharides. The experimental results indicated that the newly-established method was more efficient, stable and convenient in the authentication and quality evaluation of D. officinale, compared to the current methods. We expect the method will have similar advantages when used for other saccharide-dominant herbal materials and products.
Acknowledgements

This study was funded by Hong Kong Baptist University (FRG2/11-12/048, FRG1/12-13/018, FRG2/12-13/006, MPCF-001-2014/2015, and RC-start up grant).
References: