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Isolation and quantitative determination of inulin-type oligosaccharides in roots of *Morinda officinalis*

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ABSTRACT

Inulin-type oligosaccharides with different degree of polymerization (DP) were isolated from the traditional Chinese medicine: *Morina officinalis* by size-exclusion chromatography, and their purities were determined by HPLC–ELSD equipped with cyclodextrin-bond column. Through analysis, the purities of obtained inulin-type oligosaccharides were higher more than 98% by one-step process. The structures of inulin-type oligosaccharides were confirmed by a combination of NMR, MS as well as comparison with already existing NMR data. Using D-fructose and the isolated inulin-type oligosaccharides as standards, a determination method of monosaccharide and inulin-type oligosaccharides in *Morinda officinalis* was first developed and validated. The validated method was successfully applied to analyze monosaccharide and oligosaccharides in three types of roots of *M. officinalis* and provided a new basis of assessment on quality of *M. officinalis*.

1. Introduction

Morinda officinalis (family Rubiaceae, Bajitian in Chinese) is a vine that mainly grows in humid areas of southeast China, such as Guangdong, Fujian and Guangxi Province. The extracts of its roots (Radix Morindae Officinalis) have been used to treat a wide range of symptoms, including depression, inflammation and osteoporosis in China since ancient times (Choi et al., 2005; Kim et al., 2005; Li, Zheng, Yang, Yi, & Zhi, 2003; Li et al., 2004; Zhang, Yuan, Yang, Luo, & Zhao, 2002; Zhu, Wang, Zhang, Pei, & Fen, 2008). Many compounds were found in M. officinalis, such as carbohydrate constituents, anthraquinone, iridoid lactone, iridoid glucoside, β-sitosterol and rotungenic (Yoshikawa, Yamaguchi, Nishisaka, Yamahara, & Murakami, 1995).

Carbohydrate constituents, accounting for 49.79–58.25% of dry weight (Lin, Xu, Wang, & Feng, 1992), are significant compounds of roots of *M. officinalis*. Using chemical and spectroscopic methods, the primary structures of four inulin-type oligosaccharides were identified (Cui et al., 1995). Inulin is a natural storage carbohydrate, which is mainly found in plants of the Asteraceae family. It is a mixture of oligo- and/or polysaccharides composed of fructose unit chains (linked by β-(2 → 1)-D-fructosyl-fructose bonds) of various length, and terminated by a single glucose unit (linked by an α-D-glucopyranosoyl bond). As non-digestible carbohydrate containing naturally occurring fructooligosaccharides, inulin possesses some characteristics of dietary fibre, and as such is of particular interest for its metabolic properties (Laparra, Tako, Glahn, & Miller, 2008). Besides its health benefits, inulin is also considered to have prebiotic properties such as the ability to stimulate probiotic bacteria (López-Molina et al., 2005) without adversely affecting flavor (Akın, Akın, & Kırmacı, 2007; Buriti, Cardarelli, Filisetti, & Saad, 2007). Therefore, quantification of inulin is important for farming and food industry.

Due to their ubiquity in nature and significant roles in industry, the separation and characterization of inulin-type oligosaccharides still gain attention in recent years. A variety of chromatographic methods including anion exchange chromatography (Corradini et al., 2004; Marsilio et al., 2000; Ronkart et al., 2007) and size-exclusion chromatography (Ronkart et al., 2007) were developed to analyze inulin. So far, only GF_{2-4} has been commercialized, so quantitative analysis of inulin in plants, food and medicine is still difficult, owing to the absence of standards of inulin with higher DP.

The present paper was to develop a method to obtain inulin-type oligosaccharides (DP < 10) from the traditional Chinese medicine: *M. officinalis*. The structures of inulin-type oligosaccharides were identified by a combination of NMR and MS. Their purities and contents in *M. officinalis* were determined by HPLC equipped with cyclodextrin-bond column and evaporative light scattering detector (ELSD).

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2. Experimental

2.1. Materials

The roots of *M. officinalis* collected in autumn were purchased from the farmer in Yongding County, Fujian Province, China in 2007 and identified by Professor Mingyue Zhao (Zhengzhou Tobacco Research Institute of CNTC, Zhengzhou). There were three types of roots in our lab, that is, roots of type I (6 growth years, with cob), roots of type II (10 growth years, with cob), roots of type III (10 growth years, without cob). The voucher specimens (ZTRI 07001, 07002, 07003) were deposited in the Laboratory of Flavor & Fragrance, Zhengzhou Tobacco Research Institute of CNTC. DEAE Sepharose fastflow (GE Healthcare, Sweden) and Bio-gel P-2 gel, extra fine (Bio-rad, America) were all obtained from H&E Co. Ltd. (Beijing, China). The column of cyclobond I 2000 (250 mm × 4.6 mm I.D., 5 μm) was from YMC. Co., Ltd. (Kyoto, Japan). Oasis HLB cartridges (6 ml, 150 mg) containing [poly-(divinybenzene-co-N-vinypyrrolidone)] were purchased from Waters Corporation (Miford, MA, USA). All other reagents were analytical grade. Ultra pure water was prepared with a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Extraction and purification of oligosaccharides

The dried roots of *M. officinalis* (20.0 g) were powered (40 meshes) and extracted with 95% ethanol (400 ml) for 1.5 h at 100 °C. After filtered, the extracts were concentrated to 20 ml under reduced pressure at 50 °C. The residue was mixed with 20 ml water, and then added to a column (40 cm × 3.5 cm) of DEAE Sepharose fastflow previously equilibrated with the NaCl solution (1 M). Elution was carried out with ultra pure water at a flow rate of 2.5 ml/min. Fractions were collected (2 ml/tube), and sugar content was monitored by the phenol-sulfuric acid method at 490 nm. The collected fractions containing sugar were dialyzed and lyophilized to give light-yellow oligosaccharide mixture.

2.3. Isolation of oligosaccharides with different DP

Water solution of oligosaccharides mixture (17%, w/v) was fractionated on a column (100 cm × 2.6 cm) of Bio-gel P-2 gel, and elution was carried out with ultra pure water at flow rates of 0.4, 0.2 ml/min. Fractions were collected (2 ml/tube), and sugar content was monitored by the phenol-sulfuric acid method at 490 nm. Fractions of the top of same peak were collected and lyophilized to give white powder. Consequently, one monosaccharide mixture and eight inulin-type oligosaccharides were obtained through the procedure above. The purities of isolated oligosaccharides with different DP were detected by Agilent 1200 HPLC system (Aglient, USA) equipped with an evaporative light scattering detector (ESLD), 2000ES (Alltech, USA).

2.4. Structure identification of monosaccharide and oligosaccharides

The powders of the monosaccharide and oligosaccharide obtained through size-exclusion chromatography (Bio-gel P-2 gel) were dissolved in D_2O respectively. NMR spectra were recorded on a Bruker DRX-400 spectrometer operating at 400.132 MHz for ^1H NMR and 100.612 MHz for ^{13}C NMR. The acquisition parameters of 1D NMR and 2D NMR experiments employed were as follows: (1) ^1H NMR, TD (time domain data) = 65536, AQ (acquisition time) = 3.98 s, NS (number of scans) = 16, SWH (spectral width) = 8223.68 Hz, D1 (relaxation delay between successive scans/transients) = 1.00 s, LB (exponential line broadening applied prior to Fourier transformation) = 0.3 Hz. (2) ^{13}C NMR, TD = 65536, AQ = 1.36 s, NS = 64, SWH = 24038.46 Hz, D1 = 2.00 s, LB = 1.0 Hz. (3) ^1H–^1H COSY (correlated spectroscopy), TD = 2048, AQ = 0.30 s, NS = 4, SWH = 3742 Hz, D1 = 1.50 s. (4) ^1H–^{13}C HSQC (heteronuclear single quantum coherence), TD = 1024, AQ = 0.30 s, NS = 2, SWH = 1692.62 Hz, D1 = 1.50 s. The inverse-gated decoupling ^{13}C NMR was used to determinate the ratio of monosaccharide in the sample of monosaccharide mixture. The following acquisition parameters were employed in this technology: TD = 131072, AQ = 2.72 s, NS = 5120, SWH = 24038.46 Hz, D1 = 7.50 s, LB = 1.00 Hz.

The ESI–MS experiments were performed by a Finnigan LTQ linear ion trap mass spectromerter (Thermo Electron Corporation, San Jose, CA, USA) equipped with an ESI source. For ESI–MS analysis, the powder of monosaccharide mixture and oligosaccharides obtained were dissolved in methanol (10 mg/l) respectively and were tested under both positive and negative ion mode. The ESI–MS conditions were as follows: sheath gas, N_2 30 arbitrary units; auxiliary gas, N_2 10 arbitrary units; sweep gas, N_2 10 arbitrary units; spray voltage, 3.93 kV.

2.5. Quantitative determination of monosaccharide and inulin-type oligosaccharides in roots of M. officinalis

2.5.1. Sample processing

About 0.5 g dried powder of raw *M. officinalis* was sonicated in 50 ml water for 30 min. The extracts were centrifuged for 20 min at 10,000 rpm/min. An aliquot of 4 ml supernatant fluid centrifuged was transferred to HLB cartridge. The HLB cartridge was preconditioned by initial passage of 5 ml methanol, followed by 5 ml water. After the sample (4 ml) was eluted through HLB cartridge, a 2 ml aliquot of water was loaded and eluted twice. Because the monosaccharide and oligosaccharide were polar compounds, they were not absorbed on sorbents of cartridge. The eluates containing monosaccharide and oligosaccharide were collected and transferred it to a 10 ml volumetric flask. Ultra pure water was added to make up the volume. All the solvents and samples were passed through HLB cartridges by gravity.

2.5.2. Standard solutions

The standard stock solution of the mixture of fructose and inulin-type oligosaccharides was prepared by dissolving accurately weighted portions of the standards in MeOH, then transferring it to a 10 ml volumetric flask, and finally adding MeOH to make up the volume. The concentrations of fructose and inulin-type oligosaccharides were 2.23 mg/ml for fructose, 2.36 mg/ml for sucrose, 0.81 mg/ml for trisaccharide, 2.29 mg/ml for tetrasaccharide, 2.17 mg/ml for pentasaccharide, 2.16 mg/ml for hexasaccharide, 2.22 mg/ml for heptasaccharide, 2.05 mg/ml for octasaccharide and 1.83 mg/ml for enneasaccharide respectively. A series of working standard solutions with gradient concentration was obtained by diluting the mixed standard stock solution. All the standard stock solutions were stored in a refrigerator at 4 °C.

2.5.3. HPLC–ELSD analysis

An Agilent 1200 series liquid chromatography (LC) system equipped with a binary pump was connected with an Agilent G1329A autosample. Chromatographic separation was carried out at 25 °C using a column of cyclobond I 2000 (250 mm × 4.6 mm I.D., 5 μm). The mobile phase consisted of acetonitrile and water (v/v, 73:27) with flow rate of 1.0 ml/min. Detector was an Alltech 2000ES (USA). The drift tube temperature for the ELSD was set at 82.2 °C with the nitrogen flow rate of 2.1 l/min. The injection volume was 5 μl.

2.5.4. Method validation

The method for quantitative analysis was validated to determine the linearity, sensitivity, precision and accuracy for each analyte.

Table 1
Calibration equations, correlation coefficients, linear ranges, LOD and LOQ of oligosaccharides (LOD: limit of detection; LOQ: limit of quantification).

DP	Calibration equation	Correlation coefficient (R^2)	Linear ranges (mg/ml)	LOD (μg/ml)	LOQ (μg/ml)
1	$y = 15302x - 1753.4$	0.9933	0.11–1.12	7.4	24.5
2	$y = 14793x - 1543.7$	0.9983	0.12–1.18	8.1	27.1
3	$y = 8059.6x - 187.2$	0.9988	0.04–0.41	2.1	7.2
4	$y = 11137x - 952.7$	0.9968	0.11–1.15	14.5	48.5
5	$y = 10521x - 772.05$	0.9981	0.11–1.09	14.8	49.3
6	$y = 10127x - 681.5$	0.9973	0.11–1.08	3.8	12.7
7	$y = 10310x - 964.58$	0.9950	0.11–1.11	9.4	31.4
8	$y = 9544.1x - 676.77$	0.9961	0.10–1.03	12.3	41.1
9	$y = 9199.9x - 582.11$	0.9987	0.09–0.92	8.3	27.7

Calibration curves were constructed using a range of concentration of working standard as showed Table 1. Each line was based on six concentrations of standard. The limits of detection (LOD) and quantification (LOQ) were estimated by baseline noise method with a signal 3 and 10 times higher than that of the baseline noise, respectively. The precision was determined with roots of type II in six times and reported as RSD%. Accuracy was determined with control samples prepared in triplicates at three different concentration levels, by spiking different amounts of reference standards to the power of roots with known concentration of oligosaccharide and inulin-type oligosaccharides. Then the control samples were treated according to the same procedure. The accuracy was evaluated as percentage recovery of the analytes found to the amount added.

3. Results and discussion

3.1. Extraction and isolation of oligosaccharides

The oligosaccharides of *M. offficinalis* were extracted with ethanol (95%). This is because polysaccharides and other macromolecules could not dissolve in high concentration of ethanol (95%) (Yu et al., 2007; Zhang, Ye, & Wang, 2010). However, some small molecules such as the pigment and amino acid were extracted simultaneously. When the extracts were added to a column (40 cm × 3.5 cm) of DEAE Sepharose fastflow previously equilibrated with the NaCl solution, some charged pigment and amino acid were absorbed on the column. Owing to free charge of oligosaccharides, they could not be absorbed by the gel of DEAE Sepharose fastflow and flowed out from the column with water. The yield of the oligosaccharide mixture from roots of *M. officinalis* was about 30.3% (w/w).

Then the oligosaccharide mixture was separated by size-exclusion chromatography (SEC). In the present study, flow rate was considered as an important factor that influenced the separation of oligosaccharides. Two flow rates (0.4 ml/min, 0.2 ml/min) were experimented. Through experiment, the flow rate of 0.2 ml/min was optimum flow rate to separate the oligosaccharides (DP < 10) (Fig. 1). When the flow rate was increased to 0.4 ml/min, only the oligosaccharides of DP < 6 could be separated completely. The tubes of top peak were collected and lyophilized to give white power. The purities of obtained oligosaccharides with different DP were detected by HPLC–ESLD, and their purities were more than 98% except for monosaccharide mixture.

3.2. Identification of monosaccharide mixture and oligosaccharides

NMR spectroscopy was used to perform the structural characterization of monosaccharide mixture and oligosaccharides isolated. ^{1}H and ^{13}C NMR spectra of monosaccharide mixture and inulin-type oligosaccharides are shown in Fig. 2.

From the ^{1}H and ^{13}C NMR spectra of monosaccharide mixture (DP = 1), it could be deduced that it was a mixture of different monosaccharide. In the ^{13}C NMR spectrum of monosaccharide mixture, two main regions were characterized: a low-frequency region between δ 60 and 84 ppm and a region between δ 90 and 105 ppm, in which five signals of anomeric carbons of monosaccharide were observed. In the ^{1}H NMR spectrum of monosaccharide mixture, two main regions were evident. The first region was at mid-low-frequency between δ 3.00 and 4.00 ppm, which was very rich in signals. The second region was between δ 4.40 and 5.20 ppm, in which there were two anomeric proton signals at δ 5.11 and 4.52 ppm. Through the above observation, it could be judged that

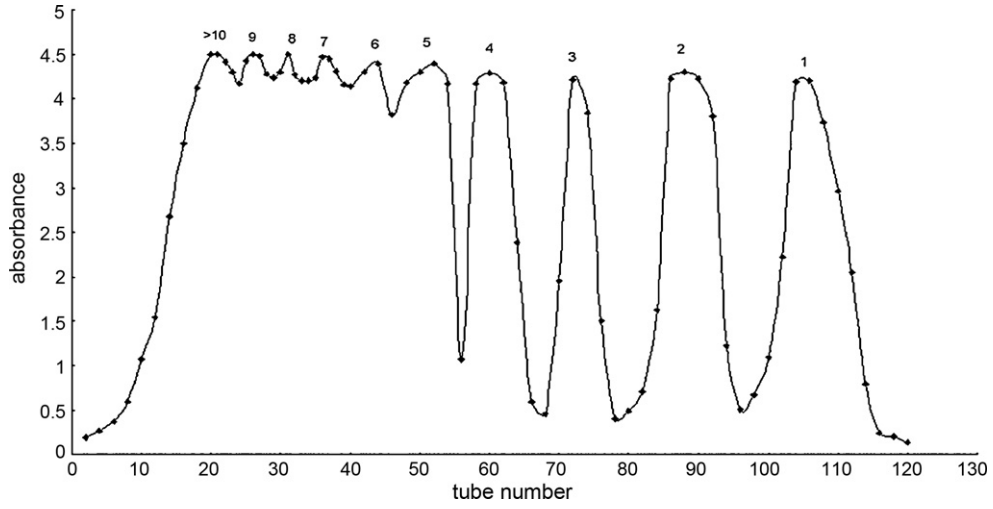

Fig. 1. Size-exclusion chromatography of oligosaccharides mixture. Flow rate: 0.2 ml/min (the numbers above peaks were the DP of oligosaccharides).

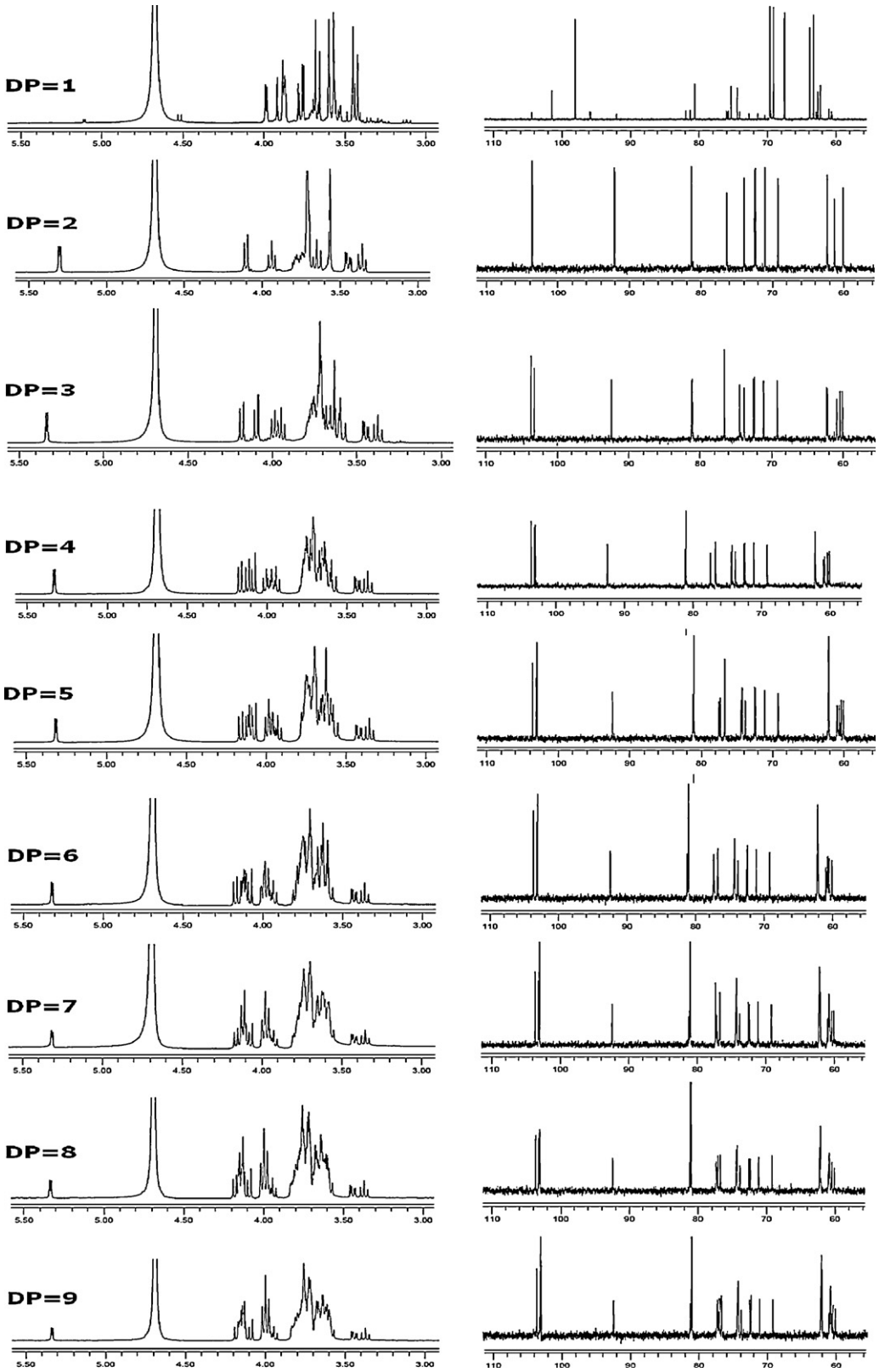

Fig. 2. The ^{1}H NMR and ^{13}C NMR spectra of oligosaccharides obtained from the roots of *M. officinals*.

there were five distinct isomeric forms of hexose in monosaccharide mixture, three of which were ketose. This was mainly because that there were no anomeric proton signals of ketose in anomeric region of ^{1}H NMR spectrum, owing to the absence of anomeric proton. Through the analysis above and comparison with the literature data (Duquesnoy, Castola, & Casanova, 2008; Lolli, Bertelli, Plessi, Sabatini, & Restani, 2008), the monosaccharide mixture was composed of two monosaccharides: glucose and fructose. The glucose existed in two forms: α-glycopyranose and β-glycopyranose, while fructose was in form of α-fructofuranose, β-fructofuranose and β-

Table 2
^{1}H and ^{13}C NMR spectral data of monosaccharide mixture and oligosaccharides using D_2O as solvent, δ in ppm.

DP	Monosaccharidesand residues	$\delta^{1}H/^{13}C$ (ppm)					
		H-1/C-1	H-2/C-2	H-3/C3	H-4/C-4	H-5/C-5	H-6/C-6
1	α-Glycopyranose	5.11 92.0	3.42 71.4	3.59 72.7	3.28 69.6	3.72 71.4	3.66 3.72 60.5
	β-Glycopyranose	4.52 95.9	3.14 74.1	3.37 75.7	3.29 70.4	3.35 75.9	3.58 3.71 61.0
	α-Fructofuranose	3.54 62.8	– 104.4	4.00 81.9	3.88 76.0	3.94 81.3	3.62 3.78 60.7
	β-Fructofuranose	3.45 62.6	– 101.4	4.00 75.3	4.00 74.4	3.70 80.6	3.56 3.69 62.3
	β-Fructopyranose	3.44 3.59 63.9	– 98.0	3.68 67.5	3.74 69.6	3.88 69.2	3.60 3.92 63.3
2	α-Glcp-(1→	5.29 92.1	3.44 71.0	3.65 72.5	3.36 69.2	3.88 72.3	3.81 60.1
	β-Fru*f*-(2→	3.57 61.3	– 103.6	4.10 76.4	3.94 73.9	3.74 81.3	3.71 62.3
3–9	α-Glcp-(1→	5.34 92.1	3.44 71.1	3.68 72.5	3.37 69.2	3.75 72.3	3.74 60.1
	→1-β-Fru*f*-(2→	3.57–3.83 60.5–61.0	– 103.0–103.6	4.01–4.18 76.7–77.4	3.93–4.01 73.8–74.4	3.74–3.77 81.0–81.2	3.72–3.83 61.0–62.3

fructopyranose. The assignments of the signals originating from each isomer in ^{1}H NMR and ^{13}C NMR spectra were summarized in Table 2. Using the integral intensity of the resonance signals of the anomeric carbons in the inverse-gated ^{13}C NMR spectrum, the ratio of α-glycopyranose, β-glycopyranose, α-fructofuranose, β-fructofuranose and β-fructopyranose in monosaccharide mixture was 0.28:0.60:1:4.02:12.17.

The ^{13}C NMR spectrum of disaccharide (DP = 2) showed 12 signals (Fig. 2). There were two signals (δ 92.1 and 103.6 ppm) in anomeric spectral region, and other signals were between δ 60 and 82 ppm. In the ^{1}H NMR of disaccharide (Fig. 2), the only signal (δ 5.29 ppm) was in anomeric spectral regions, other signals were between δ 3.30 and 4.20 ppm. Because there were 12 signals in the ^{13}C NMR spectrum of disaccharide, it was obviously that the disaccharide was composed of two hexose reisdues. From the only one signal in anomeric spectral regions, it could be deduced that one of the two hexose residues was ketose residue. Through analysis above and comparison with chemical shifts of disaccharide with that of the aforementioned monosaccharides, it was clear that the disaccharide had a glycopyranose residue with α form and a fructofuranose residue with β form. Furthermore, careful comparison of the NMR data with those of a known carbohydrate, sucrose (Kato et al., 2008; Thompson, Robrish, Pikis, Brust, & Lichtenthaler, 2001), revealed that the disaccharide was sucrose. The proton and carbon signals of disaccharide in ^{1}H and ^{13}C NMR spectra were assigned in Table 2.

When the DP of oligosaccharides (2 < DP < 10) was bigger than two, according to whether the signals were overlap or not, the signals of oligosaccharide were divided into two categories in the ^{13}C NMR spectra (Fig. 2). The C-atom signals at δ 92.1, 72.5, 72.3, 71.1, 69.2 and 60.1 ppm were classified one category, while the other signals (δ 103.0–103.6, 81.0–81.2, 76.7–77.4, 73.8–74.4, 61.0–62.3 and 60.5–61.0 ppm) was classified another category. The signals of the first category were originated from one aldohexose. The number of aldohexose in oligosaccharide was fixed, no matter how much the degree of polymerization of oligosaccharide. Due to the tiny difference of chemical shifts in each range of the second category, it could be confirmed that those signals were attributed to the same type of ketohexose. From Fig. 2, it could be observed that the ^{1}H NMR and ^{13}C NMR spectra of oligosaccharides (2 < DP < 10) were similar with those of sucrose except that there were more overlapped signals. It could be deduced that the oligosaccharide had same type monosaccharide residue: glycopyranose residue with α form and fructofuranose residue with β form. The difference between them was that the oligosaccharide had more fructofuranose residues with β form. Moreover, the NMR data of oligosaccharides (2 < DP < 10) were consistent with those of inulin which was composed of fructose unit chains (linked by β-(2 → 1)-D-fructosyl-fructose bonds) of various length, and terminated by a single glucose unit (linked by an α-D-glucopyranosoyl bond) (Bock et al., 1984; Bradbury & Jenkins, 1984; Cérantola et al., 2004; Thompson et al., 2001). Based on those findings, the oligosaccharides were elucidated as inulin-type oligosaccharides. Due to no overlap of the proton signals of H–C(3) (δ 4.01–4.18 ppm) and H–C(4) (δ 3.93–4.01 ppm) of β-fructofuranose residue in ^{1}H NMR spectrum, the DP of oligosaccharides could be rough estimated through the integral ratio of anomeric proton in glycopyranose residue to H–C(3) or H–C(4) in β-fructofuranose residue.

The isolated monosaccharide mixture and oligosaccharides were tested under positive and negative ESI conditions. In (−) ESI mode, the ions were at *m*/*z* 179, 341, 503, 665, 827, 989, 1151, 1313 and 1475 respectively. These ions detected were confirmed as $[M-H]^-$. It was because compound lost proton and formed $[M-H]^-$ easily in negative mode. In (+) ESI mode, compound was easy to form ion adduct. The most abundant ions were at *m*/*z* 203, 365, 527, 689, 851, 1013, 1175, 1337, 1499, so it was evident that these ions were ion adducts ($[M+Na]^+$). The MS characterization of oligosaccharide obtained was discussed in our earlier studies (Yang et al., 2010).

3.3. Quantitative determination of monosaccharide and oligosaccharides in M. officinalis

In order to study the acetonitrile concentration in the mobile phase on the separation effect of oligosaccharide, four mobile phases with different acetonitrile concentrations (67%, 70%, 73% and 75%, v/v) were tested using HPLC–ELSD. Fig. 3 showed the retention factor of oligosaccharides versus acetonitrile concentration in the mobile phase. The retention factors of oligosaccharides with different DP increased when the acetonitrile concentration increased from 67% to 75%. At a given mobile phase composition, the retention factor increased with the increase of DP. Later literature discussed the mechanism of oligosaccharide separation with cyclodextrin bonded phase detailedly (Berthod, Chang, Kullman, & Armstrong, 1998). The portioning interactions and hydrogen bonding interactions between the hydroxyl groups of the stationary phase and those of saccharide were the two possible mechanisms responsible for sugar retention. Because the inulin-type

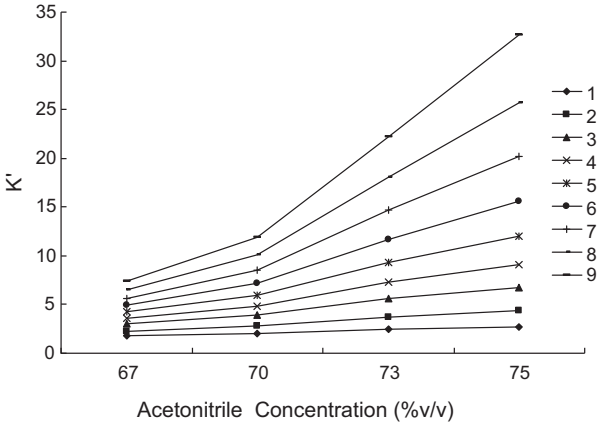

Fig. 3. Effect of acetonitrile concentration on retention factor (the numbers right the figure are the DP of oligosaccharide). Experimental conditions: cyclobond I 2000 (250 mm × 4.6 mm I.D., 5 μm); evaporative light scattering detector; flow rate: 1 ml/min; injection volume: 5 μl.

oligosaccharides of *M. officinalis* were linear oligosaccharide, the mechanism was also applicable in the separation of inulin-type oligosaccharides from *M. officinalis*. The separation of inulin-type oligosaccharides (DP < 10) was performed in 30 min with baseline resolution when the mobile phase contained 73% acetonitrile (Fig. 4A).

Contents of inulin-type oligosaccharides in roots of *M. officinalis* were determined by HPLC–ELSD equipped with cyclodextrin-bond column. The mobile phase consisted of acetonitrile and water (73:27, v/v). The standards of inulin-type oligosaccharides were isolated from the roots of *M. officinalis*. Because the types of monosaccharide in *M. officinalis* were fructose, D-fructose was used as the standard of monosaccharide. The chromatogram of standards (Fig. 4A) and sample (Fig. 4B) was showed in Fig. 4. The linearity of the method was evaluated from triplicate injection of a series of standard solutions. The results of calibration equation, calibration range, correlation coefficient, and LOD and LOQ were summarized in Table 1. From the table, all the calibrations showed good linear behavior within test ranges, with values of regression coefficient (R^2) values all above 0.99. The LOD was estimated using the criterion of S/N of 3. The LOD range of oligosaccharides was from 2.1 to 14.8 μg/ml. The LOQ, which was determined using the criterion of S/N ratio of 10, was 7.2–49.3 μg/ml.

Because inulin-type oligosaccharides were strong polar compounds, water was used as extracting solvent for oligosaccharide. Accordingly, as the inulin-type oligosaccharides are unstable, a relatively soft extraction method, sonication, was conducted. Through experiment, it was sufficient for sonication extraction for 30 min. Due to the presence of large amount of iridoid glucosides (Peng, Zhou, Ou, & Du, 2008) and other glucosides in the roots of *M. officinalis*, they could be extracted by water simultaneously and interfere the quantification of oligosaccharides. Therefore, the samples must be treated by the HLB cartridges to get rid of glucosides before the analysis of sample.

The optimized procedure was applied in the analysis of the roots of *M. officinalis* with 10 growth years. Relative standard deviations (RSDs) of the nine determined oligosaccharides are between 1.4% and 3.3% ($n = 6$). From the data listed in Table 3, the oligosaccharides recovery ranged from 94.6% to 108.2% ($n = 3$).

Three types roots of *M. officinalis*, that is, type I (6 growth years with cob), type II (10 growth years with cob) and type III (10 growth years without cob), were analyzed according to the procedure above. The contents of monosaccharide, inulin-type oligosaccharides and total oligosaccharides in each type roots of *M. officinalis* were shown in Table 4. For total oligosaccharide content in each type roots, the type I was higher than other type of roots. For the individual oligosaccharide content in each type roots, the content of trisaccharide was lower than that of other oligosaccharide. The content of individual oligosaccharide in type I was higher than those of type II except for that of sucrose (DP = 2). The content of total oligosaccharide in type III was almost the same as type I. For roots

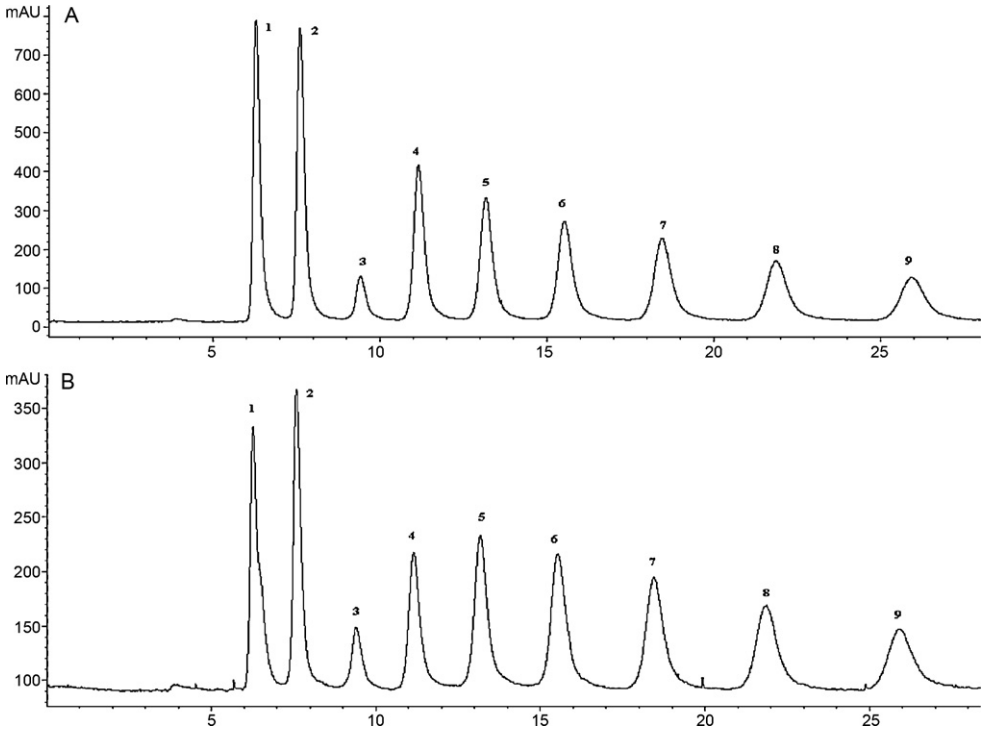

Fig. 4. HPLC–ELSD Chromatograms of (A) reference standard and (B) sample (the numbers above peaks are the DP of oligosaccharide). Column: cyclobond I 2000 (250 mm × 4.6 mm I.D.); detector: evaporative light scattering detector; flow rate: 1 ml/min; mobile phase: acetonitrile/water (v/v, 73:27).

Table 3
The precisions and recoveries of oligosaccharides in roots of *M. officinalis* (roots of type II).

DP	Contents (mg/g)	Precision ($n=6$) (%)	Added standard (mg/g)	Recovery ($n=3$) (%)
1	43.7	1.5	78.1	108.2
			44.6	101.2
			22.2	99.6
2	44.3	2.0	82.6	106.5
			47.2	99.5
			23.6	96.4
3	18.7	2.0	28.4	107.2
			16.2	102.5
			8.1	95.7
4	38.8	2.0	80.2	108.2
			45.8	100.4
			22.9	103.5
5	48.8	0.9	76.0	105.2
			43.4	98.2
			21.7	94.6
6	51.2	3.3	75.6	106.2
			43.2	98.5
			21.6	97.5
7	52.5	3.2	77.7	107.8
			44.4	99.8
			22.2	93.5
8	49.2	3.3	71.8	106.8
			41.0	100.9
			20.5	96.3
9	42.3	1.4	64.1	104.6
			36.6	97.5
			18.3	95.3

Table 4
Contents of oligosaccharide in three types of roots sample of *M. officinalis* (calculation is based on dry weight of roots of *M. officinalis*).

DP	Type I (mg/g)	Type II (mg/g)	Type III (mg/g)
1	55.9	43.7	63.5
2	24.1	44.3	35.1
3	23.0	18.7	20.2
4	42.5	38.8	39.9
5	55.8	48.8	52.2
6	62.8	51.2	54.5
7	59.6	52.5	54.2
8	54.6	49.2	50.4
9	47.5	42.3	46.9
Total contents	425.9	389.6	416.8

of type II, the reason for lower content of oligosaccharides might be that with the increase of growth years, the oligosaccharides were converted into the corresponding polysaccharides. This cause led to the result that total oligosaccharides content of roots of type II were lower than that of roots of type I.

4. Conclusions

Firstly, the oligosaccharides were isolated by column chromatography filled with polyacrylamide gel at flow rate of 0.2 ml/min. Secondly, the structures of oligosaccharides isolated were confirmed by a combination of NMR, MS as well as comparison with already existing data. NMR not only determines the DP of oligosaccharides, but also confirms the type of monosaccharide residue and the configuration of anomeric carbon atom. Thirdly, stable cyclodextrin-bond stationary phase columns were first introduced for oligosaccharide analysis by Armstrong and Jin in 1989. In the past few years it has been found that these cyclodextrin-bond columns are particularly useful for the separation of oligosaccharides. In the present paper, the inulin-type oligosaccharides in roots of *M. officinalis* were separated by cyclodextrin-bond column successfully, thus expanding the usage scope of cyclodextrin-bond column in oligosaccharide separation. Furthermore, a quantitative determination method of inulin-type oligosaccharide was developed using the cyclodextrin-bond column, and this method was successfully applied to the quantification of oligosaccharides in roots of *M. officinalis*.

The oligosaccharides are important compounds in roots of *M. officinalis*, but the roots of different growth year and different habitats vary considerably in contents of oligosaccharides. The quantification method in this experiment is stable and reproducible, which can distinguish the internal quality and provide the reference for quality control of *M. officinalis*.

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