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Separation and Quantification of Component Monosaccharides of Cold Water-Soluble Polysaccharides from *Ephedra sinica* by MECC with Photodiode Array Detector

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1. Introduction

The *Ephedra* plant, or "Mahuang" of traditional Chinese medicine, is one of the oldest medicinal plants known to mankind. More than 45 species of *Ephedra* plants exist and are indigenous to regions of Asia, North, Central and South America and Europe. Mahuang contains ephedrine alkaloids as their principal components, which are primarily localized in the aerial parts of the plant [1]. In recent years, many herbs used in popular medicine have been reported to contain polysaccharides with a great variety of biological activities and the polysaccharides are also demonstrated to be one of the main bioactive constituents of *Ephedra* plant except for a series of ephedrine alkaloids [2-4]. For these reasons, great interest arose on the reliable analytical methods of the Mahuang polysaccharides, which can be used for exploring the new functional products with polysaccharides due to its pharmacological importance and application in the pharmaceutical industry. Immunosuppressive effects of acidic polysaccharides from the stems of *E. sinica* have been demonstrated by carbon clearance test, delayed type hypersensitivity reaction and humoral immune response *in vivo* [2].

The commonly used separation techniques for carbohydrate analysis are gas chromatography (GC), high-performance liquid chromatography (HPLC), and capillary electrophoresis (CE) [5-7]. GC is a very classical method to analyze monosaccharide compositions of polysaccharides. Neutral monosaccharides are derivatized by silylation or acetylation before analysis, whereas acidic monosaccharides such as glucuronic acid and galacturonic acid can not be derivatized at all [8]. The uronic acid contents can be calculated by the difference between before and after carboxyl reduction of polysaccharides [9, 10]. So it is very laborious to calculate the uronic acid contents by GC method. Most of the HPLC and CE techniques are often used labeling with either fluorescence or UV tags for enhanced detection because these native carbohydrates are lack of chromophores or fluorophores in the structure. 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 [11-16]. CE seems to possess several

advantages over HPLC by offering higher separation efficiencies, yielding shorter analysis time, requiring small sample amounts, and consuming lower amounts of expensive reagents and solvents. It has the potential to become an important analytical separation tool for carbohydrate determination. Capillary zone electrophoresis (CZE) has been developed and successfully applied to analyze and quantify the aldoses and uronic acids [11-16]. However, few reports were proposed using MECC for the separation of the aldoses and uronic acids. The present paper is specifically concerned with the simultaneous separation of the 8 monosaccharides (aldoses and uronic acids) possibly found in natural herbs using pre-column PMP derivatization MECC and UV detection at 254 nm. Furthermore, the developed MECC method was applied to the quantitative analysis of component monosaccharides in the cold water-soluble polysaccharides from *E. sinica*.

2. Materials and methods

2.1 Rragents and standards

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 sulfuric acid (H_2SO_4) were purchased from Sigma (St. Louis, USA). Trifluoroacetic acid (TFA) was obtained from Merck (Darmstadt, Germany). 1-Phenyl-3-methyl-5-pyrazolone (PMP), purchased from Beijing Reagent Plant (Beijing, China), was recrystallized twice from chromatographic grade methanol before use. Throughout the study deionised water was used, prepared by a Milli-Q water system (Millipore, MA, USA). The pH value of the electrolyte solution was measured with a Sartorius PB-20 pH meter with Sartorius pH/ATC electrode (Sartorius, CO, Germany) calibrated with commercial buffers of pH 7.00, 10.00, and 12.0 (Titrisol, Merck kGaA, Germany). All other chemicals were of the highest grade available.

2.2 Plant material

The dry stems of *E. sinica* were collected in March 2007 from Datong of Shanxi Province, China and identified by Prof. Zhenyue Wang of Heilongjiang University of Chinese Medicine. The voucher specimen (20070016) was deposited at Herbarium of Heilongjiang University of Chinese Medicine, Harbin, P. R. China.

2.3 Extraction of polysaccharides from *E. sinica*

The dry stems of *E. sinica* were ground to powders, and submitted to extractions as follows: dry powders (1.0 kg) were extracted 3 times with 10 vol of 95% EtOH under reflux for 3 h each time to remove lipids. The residue was dried in air and then extracted 3 times with 10 vol of distilled water for 24 h (each time) at 4 °C. The combined aqueous extracts were filtered, concentrated 10-fold, and 95% EtOH added to final concentration of 80%. The precipitate was dissolved in 600 mL of water and deproteinated 15 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 the cold water soluble polysaccharide (8.5 g) was collected by centrifugation (3000 rpm, 10 min, 20 °C).

2.4 MECC equipment and conditions

The analysis of PMP-labeled monosaccharides was carried out on a P/ACE MDQ capillary electrophoresis instrument (Beckman Coulter, Fullerton, CA, USA). An integrated P/ACE 32 Karat Station (software version 4.0) was used to perform the data collection and to control the operational variables of the system. Separation was carried out in an unmodified fused silica capillary (48.5 cm × 50 μm i.d., effective length 40 cm). Both the capillary and samples were thermostatted to 25 °C. The samples were injected with a pressure of 0.5 psi for 5 s. The separation voltage was raised linearly within 0.2 min from 0 to 20 kV. Detection was done with direct UV monitoring using a photodiode array detector at wavelength 254 nm.

A new capillary from Yongnian Optical Fiber Factory (Hebei Province, China) was activated by washing consecutively with each of 0.1 M phosphoric acid (15 min), water (10 min), 0.1M sodium hydroxide (15 min), and water (10 min). At the beginning of each working day, the capillary was prewashed with 0.1 M phosphoric acid (2 min), water (2 min), 0.1 M sodium hydroxide (2 min), water (2 min) and running buffer (2 min), respectively. Between analyses the capillaries were consecutively rinsed with 0.1 M sodium hydroxide (1 min), water (1 min) and running buffer (1 min).

2.5 Complete acid hydrolysis

20 mg 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 being cooled to room temperature, the reaction mixture was centrifugated at 1000 rpm for 5 min. The supernatant was collected and dried under a reduced pressure. The hydrolyzed and dried sample solutions are added with 1 ml distilled water and then ready for the following experiments.

2.6 Derivatization procedure

PMP derivatization of monosaccharides was carried out as described previously with proper modification [11-17]. 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 0.3 M aqueous NaOH (100 μl) were added to each. Each mixture was allowed to react for 30 min at 70 °C water bath, then cooled to room temperature and neutralized with 100 μl of 0.3 M HCl. The resulting solution was performed on liquid-liquid extraction with same volume of isoamyl acetate (two times) and chloroform (one time), 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 0.45 μm membrane and diluted with water before MECC analysis.

2.7 Method validation

The regression equations were calculated in the form of y = a x + b, where y was the peak areas and x was the concentration of analytes. The signal-to-noise of 3:1 and 10:1 were used to establish LOD and LOQ, respectively. The measurement of intra- and inter-day variability was utilized to determine the precision of this newly developed method. The intraday variation was determined by analyzing the same mixed standard water solution for

five times within 1 day. While for inter-day variability test, the solution was examined in triplicate for consecutive 3 days. Stability of sample solution was tested at 0, 4, 8, 12, 24 and 48 h within 2 days. All solutions were kept at 4 °C before analysis. The analytes showed stable in water solution (RSD < 3.45%) at 4 °C during the tested period.

3. Results and discussion

3.1 Method development

We can only label carbohydrates according to Fig. 1 [16], usually yields neutral sugar derivatives which become negatively charged in aqueous basic solutions due to the partial dissociation of the enolic hydroxyl group of the PMP tag. In this study, the method development was achieved by optimizing background electrolyte pH, SDS and borax concentration of the buffer, applied voltage and capillary temperature.

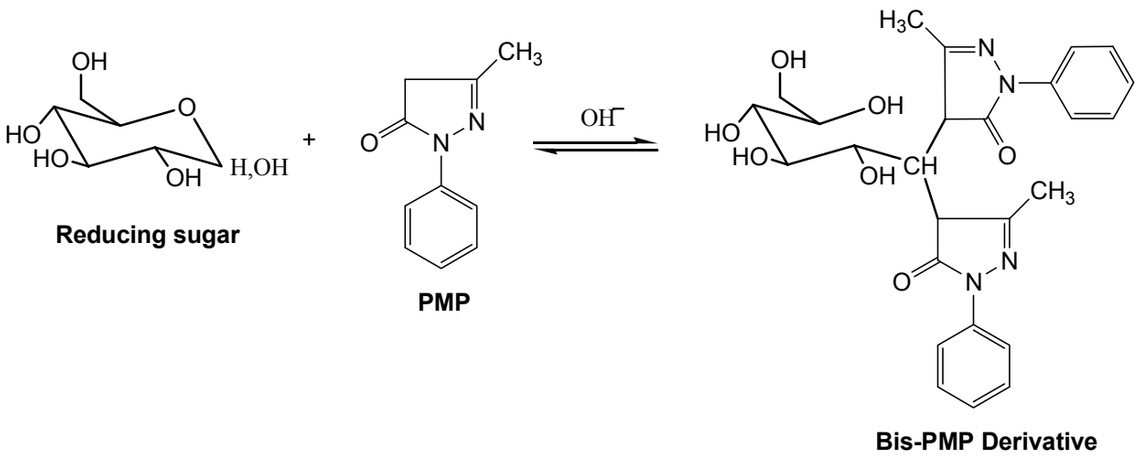

Fig. 1. Illustration of condensation reaction with PMP

3.1.1 Effect of pH

The effect of pH on the separation was also investigated in the pH range from 9.7 to 10.75 using borate buffer solutions as background electrolytes. Fig. 2 is comparison of four pH electrolytes in the analysis of 8 carbohydrates, from which the separation conditions are revealed more clearly. The result reveals that the pH of buffer had great influence on the resolutions and the migration time. Supposing an appropriate pH (9.70) chosen, ara and glc have been co-eluted as one peak. Only man, gal, glcUA and galUA have the high resolutions. If a higher pH (10.0 or 10.23) is adopted, there are poor resolutions between ara and glc. And if we use pH 10.75, good symmetry and the resolution for each analyte was achieved. Thus pH 10.75 was chosen as the optimal running buffer condition.

3.1.2 Effect of buffer and SDS concentration

The separation of PMP-labeled carbohydrates was very sensitive to borate buffer concentrations and SDS concentration in MECC. In this study, borate concentration in the range of 20–33 mM was evaluated (Fig. 3). The results indicated that the migration time of PMP-labeled carbohydrates generally increased with a gradual increasing of buffer

concentrations. Taking the shorter run-time and good resolution into consideration, 25 mM borate buffer solution was selected and the maximum resolution was obtained.

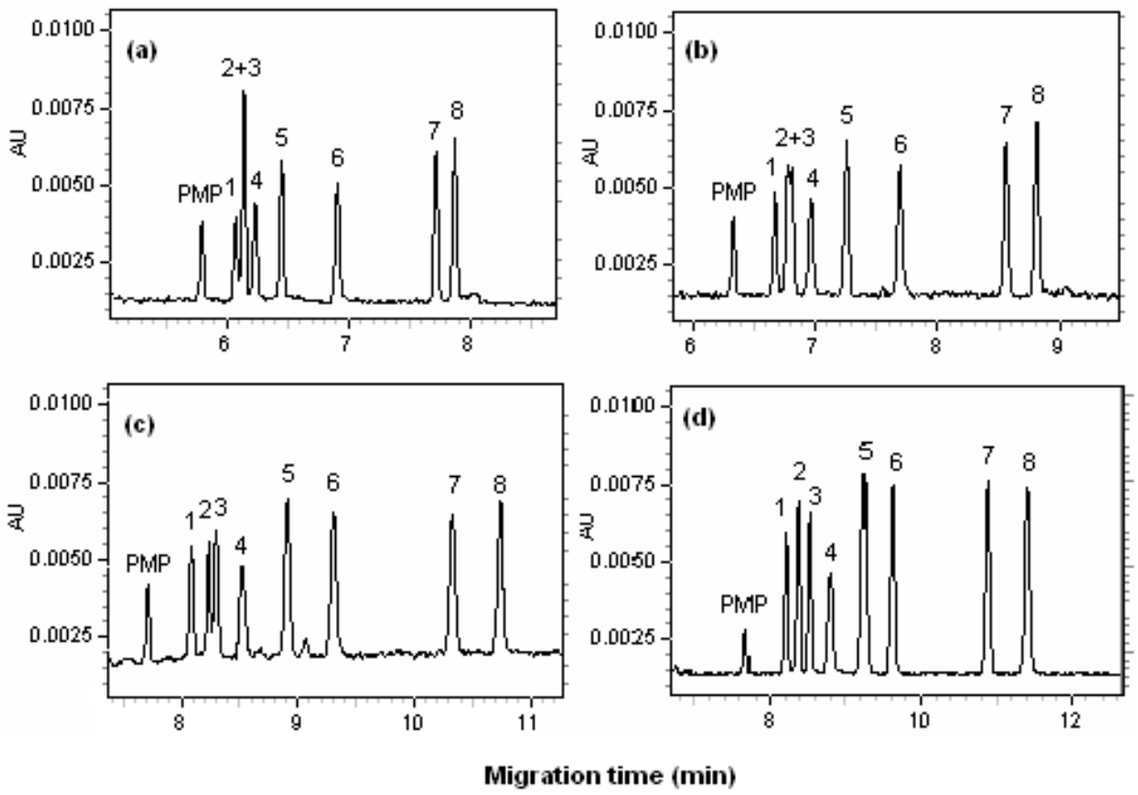

Fig. 2. Comparison of electropherograms of PMP derivatives of 8 standard monosaccharides with 25 mM borate buffer and 30 mM SDS at four pH electrolytes, 1 mM each. Peak identities: 1, xyl; 2, ara; 3 glc; 4 rha; 5, man; 6, gal; 7, glcUA; 8, galUA. Separation conditions: applied voltage, +20 kV; Separation conditions: +25 kV; detection, 254 nm direct mode; injection pressure, 0.5 psi for 5 s; capillary, fused-silica 40.0/48.5 cm (*L*det/*L*tot); separation temperature, 25 °C. The pH of borate buffer: (a) pH 9.70; (b) pH 10.00; (c) pH 10.23; (d) pH 10.75

Different concentrations of SDS (25, 30 and 35 mM) at pH 10.75 and 25 mM borate buffer on the separation of analytes were studied (Fig. 4). It was found that Xyl and Ara were not well separated at 20 mM SDS with poor resolution, but best resolution and highest theory plates were achieved at 30 mM SDS. Increasing SDS concentration, however, remarkably increased the migration time of all analytes. Therefore, 30 mM SDS was chosen as the optimal SDS concentration thus it was chosen for the further experiments.

3.1.3 Effect of capillary temperature and voltage

The temperature of the analysis may sometimes be important in MECC, as fluctuations in the temperature may affect the viscosity of the running buffer, leading to higher analyte electrophoretic mobilities and shorter analysis time. The temperature changes can also affect

the pH of the buffer. In this case, 25 °C is most optimal. In addition, the effects of three voltage values (10-28 kV) on separation of the analytes also were studied. The results showed that the good resolution and acceptable migration time were achieved at 25 kV.

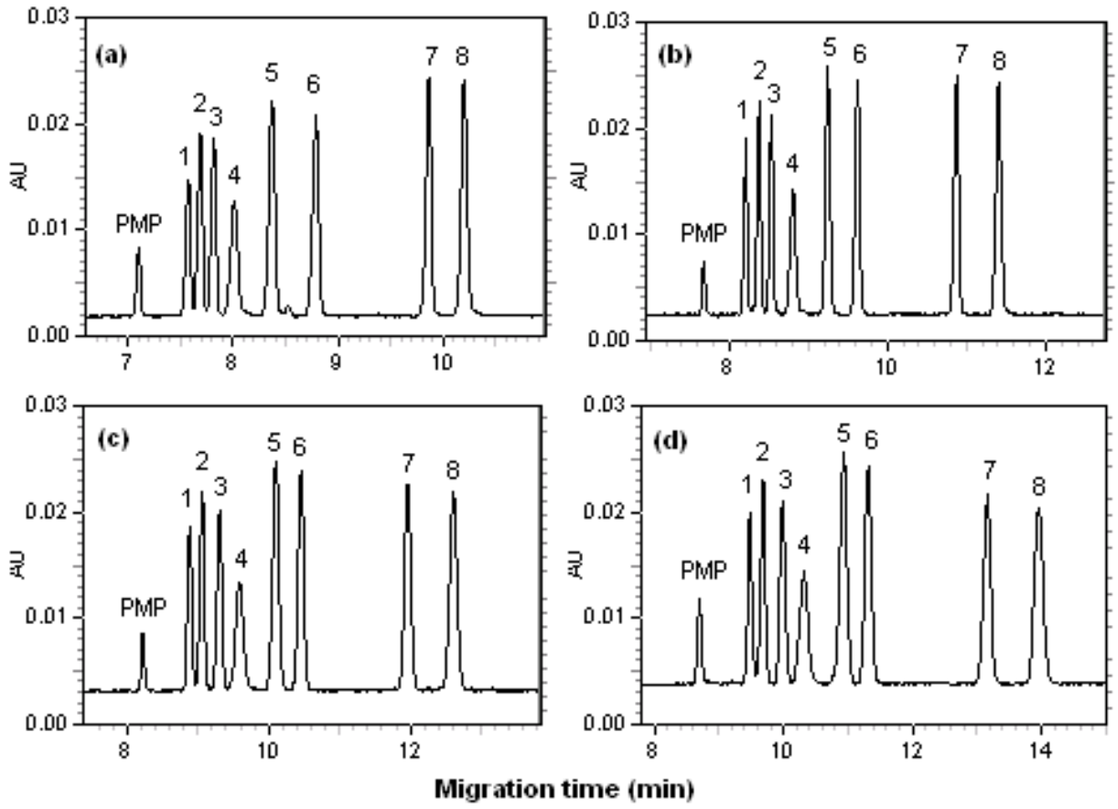

Fig. 3. Comparison of electropherograms of PMP derivatives of 8 standard monosaccharides at four buffer concentration electrolytes, 1 mM each. Borate concentration: (a) 20 mM; (b) 25 mM; (c) 27 mM; (c) 30 mM. Peak identities and other analytical conditions were in Fig. 2.

To achieve optimal separation, the operation at 20 mM of borax and 30 mM SDS at pH 10.75, capillary temperature 25 °C and applied voltage 20 kV, a complete baseline resolution for carbohydrate derivatives can be achieved within the shortest time. Under the proposed conditions, the separation of nine PMP-labeled carbohydrates is achieved within 12 min. The separation of standard mixture consisting of 8 PMP-labeled carbohydrates is shown in Fig. 5A.

3.2 Validation of the method developed

The MECC method was validated in terms of linearity, reproducibility, limit of detection (LOD) and precision. The linearity was verified by the analysis of six points in the range of 37.5 - 600.00 µM, and the linear regression parameters of the calibration curves were shown in Table 1. As a consequence, the good linearity (correlation coefficient $R^2 > 0.9993$) between y (peak area ratio 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 0.5 psi for 5 s of gradational dilutions of a standard mixture

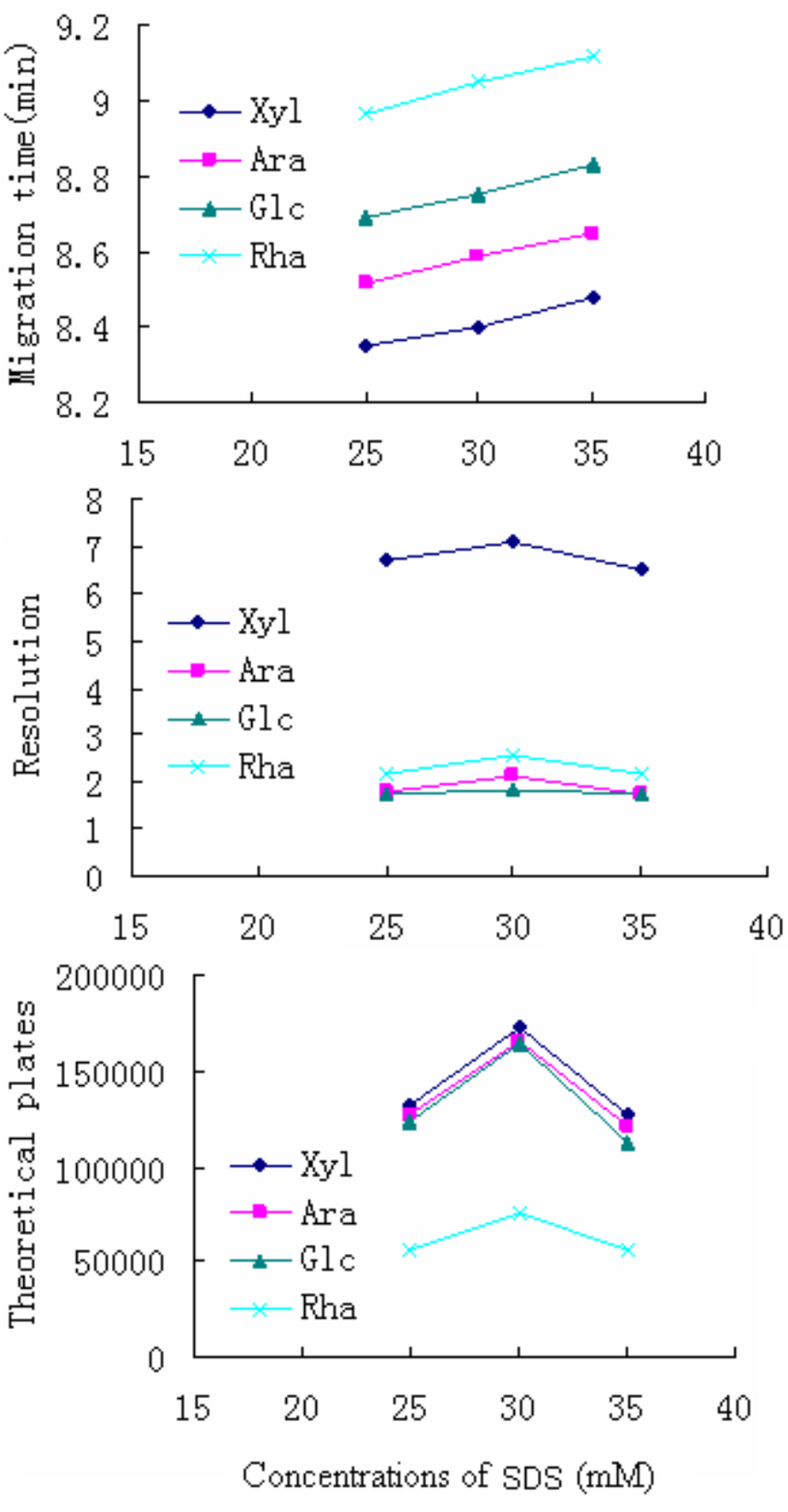

Fig. 4. Effects of SDS on the migration time, resolution and theory plates.

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 10.5 to 11.7 μM (Table 1), indicating that the sensitivity of the method was satisfactory.

Carbohydrats[a]	Regression equation (y = a + b x)[b]		correlation coefficient	LOD[c] (μM)	LOQ[d] (μM)
	a	b			
Xyl	-0.2106	0.0134	0.9998	10.5	33.6
Ara	-0.2681	0.0172	0.9994	11.2	35.8
Glc	-0.1576	0.0078	0.9993	11.4	36.4
Rha	-0.1196	0.0086	0.9997	11.7	37.5
Man	-0.1049	0.0203	0.9995	10.9	34.8
Gal	-0.26	0.0225	0.9995	11.3	36.0
GalUA	-0.4612	0.0232	0.9992	10.8	34.6

[a] Quantitated with a calibration curve at = 254 nm
[b] The y and x are peak area ratio of the analytes to internal standard (GlcUA) and concentration of the analytes (37.5-600μM), respectively
[c] LOD corresponds to concentrations giving a signal-to-noise ratio of 3.
[d] LOQ corresponds to concentrations giving a signal-to-noise ratio of 10.

Table 1. Linearity of CE method of different carbohydrate

Carbohydrats	Intra-day precision (RSD%, n = 5)		Inter-day precision (RSD%, n = 3)	
	Retention time	Peak area	Retention time	Peak area
Xyl	1.25	2.38	1.36	3.21
Ara	2.03	2.97	1.58	3.56
Glc	1.69	1.97	2.21	4.03
Rha	2.31	2.46	1.99	3.69
Man	2.09	2.36	2.16	2.79
Gal	1.95	2.57	1.94	2.88
GlcUA	1.85	2.77	2.37	4.35
GalUA	1.38	2.89	1.96	4.27

Table 2. Precision of the retention time and peak area of analytes in the present method

Moreover, method precision was also determined by measuring repeatability (intra-day variability) and intermediate precision (inter-day variability) of retention time and peak area for each tested monosaccharide. The precision of method was calculated as the coefficient of variation (RSD) for five successive injections of each tested monosaccharide at the concentration of 1 mM and the results were summarized in Table 3. The results showed that the intra-day reproducibility (RSD values) were less than 2.31% for the migration time and 2.97% for the peak areas, and the interday RSD values were less than 2.37% for the migration time and 4.35% for the peak areas, indicating that the method precision was satisfactory.

Component	added amount (μM)	found amount (μM)	recovery(%)[a] means ± SD	RSD (%)[b]
Ara	270	270.2	100.15 ± 4.03	4.02
Glc	62	61.7	99.51 ± 3.63	3.65
Rha	35	35.4	105.13 ± 2.79	2.76
Man	25	24.6	98.45 ± 2.86	2.91
Gal	53	53.5	103.97 ± 3.51	3.48
GalUA	30	29.7	97.28 ± 3.73	3.83

a Recovery (%) = [(mean of measured concentration−spiked concentration)/spiked concentration]×100.
b RSD (%) = (SD/mean)×100

Table 3. Recoveries of six monosaccharides in sample analysis (n= 6)

3.3 Analysis of the cold water-soluble polysaccharide extract from E. sinica

This experiment was designed to develop a rapid, repeatable and accurate MECC method for the quantification of the component carbohydrates in the cold water-soluble polysaccharide extract from *E. sinica*. In order to evaluate the applicability of the proposed method, the polysaccharide was hydrolyzed with 2M TFA, and PMP-labeled as described in the experimental section and finally, the released monosaccharide derivatives were analyzed by the described MECC method under the optimized conditions using GlcUA as internal standard. Fig. 5B shows a typical chromatogram of the cold water-soluble polysaccharide sample. As can be seen, the PMP derivatives of the component monosaccharides released from the Mahuang polysaccharide sample could be still baseline separated and the component monosaccharides could be identified by comparing with the chromatogram of the mixture of standard monosaccharides (Fig. 5A). The results showed that the cold water-soluble polysaccharide extract from *E. sinica* was a typical heteropolysaccharide and was composed of arabinose, glucose, rhamnose, mannose, galactose and galacturonic acid in the molar rate of 4.36:1.43:1.00:0.55:0.41:0.85:0.45, and their corresponding mole percentages were 57.24%, 13.12%, 7.16%, 5.40%, 11.17%, and 5.87% (mol%), respectively. It was clear that the predominantly composition monosaccharides in the cold water-soluble polysaccharide extract from *E. sinica* were arabinose up to 57.24% (mol%) of total carbohydrates, and only 5.87% of total carbohydrates 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 sample detected, and the resulting spiked sample was subjected to the entire analytical sequence. Each solute was spiked at a close concentration with 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 showed that the recoveries of all the monosaccharides ranged between 97.28% and 105.13% and the RSD values were within 2.76–4.02% (Table 3). Such results further demonstrated that this method is precise and practical for the analysis of polysaccharide samples from *E. sinica*.

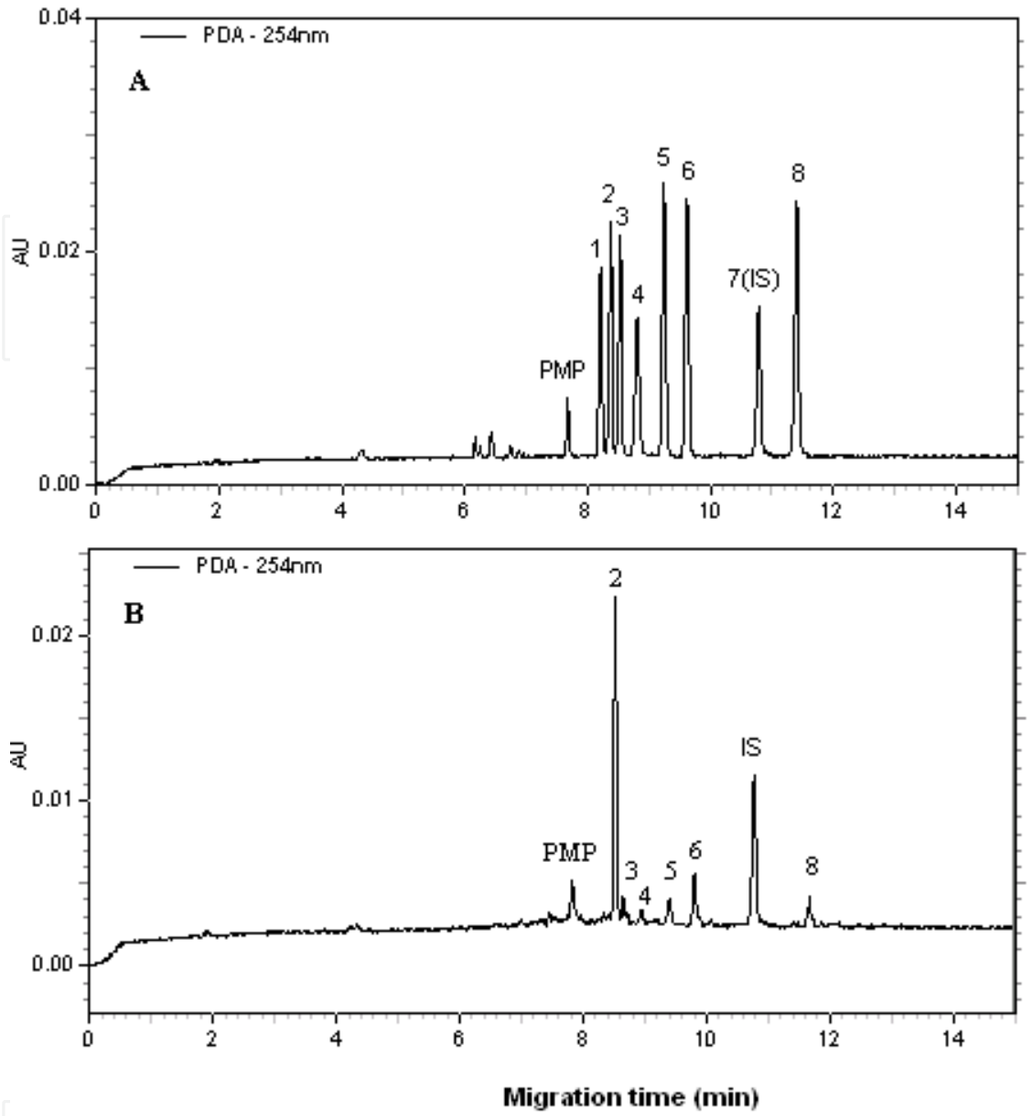

Fig. 5. Electropherograms of PMP derivatives of standard monosaccharides (A) and the sample (B). Peak identities and other analytical conditions were in Fig. 2.

4. Conclusions

In recent years, the monosaccharides PMP derivatives mentioned above were successfully separated by CZE and HPLC although the analysis was accomplished for a long time [11-16]. However, in this study, as shown in Fig. 5A, various PMP derivatives of monosaccharides were shown to separate quite nicely in MECC within 12 min and seven composition monosaccharides were successfully identified and the assay results were satisfactory. The separation is based on the differential distribution of the PMP derivatives between the aqueous mobile phase and the micellar phase. The applicability of the SDS micellar system was extended to the identification and quantitation of monosaccharides obtained from carbohydrate hydrolyzates from polysaccharide. The shape of the peaks was

very sharp and it was clear that a substantial improvement in the separation of neutral sugar and uronic acid derivatives was obtained by MECC method, and thus, the analytical cost is lower and is also eco-friendly for its non-consumption of organic solvents as compared with other analytical techniques.

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During the recent years, traditional Chinese medicine (TCM) has attracted the attention of researchers all over the world. It is looked upon not only as a bright pearl, but also a treasure house of ancient Chinese culture. Nowadays, TCM has become a subject area with high potential and the possibility for original innovation. This book titled Recent Advances in Theories and Practice of Chinese Medicine provides an authoritative and cutting-edge insight into TCM research, including its basic theories, diagnostic approach, current clinical applications, latest advances, and more. It discusses many often neglected important issues, such as the theory of TCM property, and how to carry out TCM research in the direction of TCM property theory using modern scientific technology. The authors of this book comprise an international group of recognized researchers who possess abundant clinical knowledge and research background due to their years of practicing TCM. Hopefully, this book will help our readers gain a deeper understanding of the unique characteristics of Chinese medicine.

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