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Development and Application of a Rapid and Efficient CZE Method Coupled with Correction Factors for Determination of Monosaccharide Composition of Acidic Hetero-polysaccharides from *Ephedra sinica*

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ABSTRACT:

Introduction – Ephedrine alkaloids cannot account for all the effects of *Ephedra sinica* and the polysaccharides are also demonstrated to be one of the main bioactive constituents of *E. sinica*. However, no work has been reported on the analysis of monosaccharide composition of purified polysaccharides isolated from the stem of *E. sinica*.

Objective – To develop a rapid and efficient capillary zone electrophoresis (CZE) method based on pre-column derivatisation with 1-phenyl-3-methyl-5-pyrazolone for the simultaneous determination of neutral and acidic sugars of purified polysaccharides from *E. sinica*.

Methodology – Three polysaccharides (ESP-A3, ESP-A4 and ESP-B4) were isolated and purified by ion exchange and gel-filtration chromatography from the stem of *E. sinica*. The effects of background electrolyte pH and concentration, applied voltage and temperature on the separation were investigated. Meanwhile, factors affecting the hydrolysis of ESP-B4 with sulphuric acid were investigated by changing the hydrolysis time, acid concentration and hydrolytic temperature to achieve complete hydrolysis. The standard curves coupled with correction factors were used to calculate molar ratios.

Results – The optimal CZE method coupled with correction factors was successfully applied to the determination of molar ratios of three purified polysaccharides and their corresponding partial acid hydrolysis products. ESP-A3, ESP-A4 and ESP-B4 were all typical acidic hetero-polysaccharides and consisted of xylose, arabinose, glucose, rhamnose, mannose, galactose, glucuronic acid and galacturonic acid, and their corresponding molar ratios were 6.8:7.5:1.0:14.0:13.7:22.3:10.2:3.8 for ESP-A3, 1.2:4.1:1.0:5.1:1.6:17.3:3.1:2.2 for ESP-A4, and 1.0:4.5:1.0:2.0:1.0:5.5:1.5:50.0 for ESP-B4.

Conclusion – The results provided scientific evidence for the further study of the structure and bioactivity of complex acidic *E. sinica* polysaccharides.

Keywords: capillary zone electrophoresis; polysaccharides; monosaccharide composition; molar ratios; correction factors; *Ephedra sinica*

Introduction

Botanical polysaccharides with multiple pharmacological activities have recently stimulated the interest of academia and the pharmaceutical industries (Hurtley *et al*., 2001). In fact, the roles of water-soluble polysaccharides from traditional Chinese medicines in biological processes have been studied with increasing attention over the past recent years because of their broad spectrum of therapeutic properties and relatively low toxicity (Schepetkin and Quinn, 2006; Chen *et al*., 2007; Fang and Ding, 2007). Indeed, immunomodulation, anti-tumour, antivirus, anticoagulant, hypoglycaemic, anti-complementary, anti-inflammatory and antioxidation bioactivities have been presented by many polysaccharides extracted from medicinal fungi and plants (Schepetkin and Quinn, 2006; Chen *et al*., 2007; Fang and Ding, 2007; Liu *et al*., 2007; Yu *et al*., 2007; Hsieh *et al*., 2008).

Ephedra sinica is a known traditional Chinese medicine that has been used for thousands of years to treat asthma, bronchitis, antitussive, central nervous system stimulant, anti-inflammatory and colds (Trujillo and Sorenson, 2003; Roman *et al*., 2004; Dunnick *et al*., 2007). A series of ephedrine alkaloids have for a long time been considered as the pharmacologically active ingredients of *E. sinica* for the treatment of various diseases and symptoms (Konno *et al*., 1985; Meng *et al*., 2007), but they cannot account for all the effects mentioned above and the water-soluble polysaccharides have also been demonstrated to be one

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of the main bioactive constituents of *E. sinica* (Konno *et al*., 1985; Cheng *et al*., 2001; Ding *et al*., 2006).

Accordingly, an understanding of the basic composition properties of polysaccharides from *E. sinica* could contribute to our understanding of their practical applications. Many international researches have suggested that polysaccharides of medicinal plants composed of glucan boost the immune system and have perfect anti-tumour activities (Yoon *et al*., 2008). In another report, researchers found that sulphation of the neutral (1 → 3)-linked D-galactan from lac tree afforded derivatives with good antioxidant capacities (Aguirre *et al*., 2009). In addition, more heteropolysaccharides have been isolated from herb plants with obvious bioactivities. For example, *Physalis alkekengi* polysaccharide consisting of Ara, Gal, Glc and GalA in the ratio of 2.6:3.6:2:1 can significantly reduce blood glucose levels and water intake, and increase the body weight in alloxan-induced diabetic mice (Tong *et al*., 2008). Therefore, investigation of the basic sugar composition of the polysaccharides is particularly necessary to better discover their functional properties for wide application in the pharmaceutical industries.

There are several methods currently used for the separation of carbohydrates, including thin-layer chromatography (TLC), gas chromatography (GC), high-performance liquid chromatography (HPLC) (Lee and Lin, 1996) and high-performance capillary electrophoresis (HPCE) (El Rassi, 1996). Although TLC method is simple and convenient, it is adequate only for qualitative analysis due to its poor separation efficiency and poor precision for quantitative purposes (Lee and Lin, 1996; Zhang *et al*., 2003). The GC method has good sensitivity and separation efficiency as a popular tool, however; the sample preparation procedure before the analysis is very labourious (Lee and Lin, 1996; Lv *et al*., 2009). Because of the inherent hydrophilic nature of carbohydrates, aqueous-based separation methods including HPLC and HPCE are very suitable for their analysis (El Rassi, 1996). Presently, HPLC is one of the most widely used and important separation methods for the analysis of carbohydrates. Various kinds of bonded phase and ion-exchange columns have been developed for the HPLC method (Honda, 1984). A major drawback of HPLC is its lack of separation efficiency. In addition, HPLC often needs a longer time for column equilibration and the cost of a specialised column is substantial. In this regard, HPCE seems to possess several advantages over HPLC by offering higher separation efficiencies, yielding shorter analysis time, requiring smaller sample amounts and consuming lower amounts of expensive reagents and solvents (Honda, 1996; Paulus and Klockow, 1996; El Rassi, 1999). However, a major difficulty that has to be surmounted for HPCE is the separation of complex carbohydrate samples. The lack of chromophores or fluorophores in the structure of monosaccharides limits their sensitive detection in HPCE (Plocek and Novotny, 1997; Boulis *et al*., 1998; Altria, 1999; Soga and Serwe, 2000; Yang *et al*., 2007; Rovio *et al*., 2007). Therefore, carbohydrates are generally tagged with a suitable chromophore or fluorophore to obtain highly sensitive detection. The reagent 1-phenyl-3-methyl-5-pyrazolone (PMP) is one of the popular labels and reacts with reducing carbohydrate under mild conditions, requiring no acid catalyst and causing no isomerisation (Honda *et al*., 1989, 2003; Daotian and Roger, 1995; Andersen *et al*., 2003; Zhang *et al*., 2003; Lv *et al*., 2009).

Although the principle of the complexation between borate and vicinal hydroxy groups in sugars to form negative complexes was widely used for the separation of carbohydrate compounds (Hoffstetter-Kuhn *et al*., 1991; Guttman, 1997; David *et al*., 2005), to the best of our knowledge rather limited monosaccharides were usually baseline or fast separated, which significantly affected the analytical accuracy or efficiency (Wang and Fang, 2004; Größl *et al*., 2005). Therefore, the improved separation of a series of monosaccharides needs to be further investigated in the presence of borate complexation. In this paper, a simple and rapid CZE method based on PMP derivatisation has been developed for simultaneous separation and determination of the neutral and acidic sugars. The effects of background electrolyte pH and concentration, applied voltage and temperature on the separation were investigated. Meanwhile, the factors affecting hydrolysis of *E. sinica* polysaccharide with sulphuric acid were also investigated by changing the hydrolysis time, acid concentration and hydrolytic temperature to achieve complete hydrolysis of the polysaccharide polymer. Finally, the developed optimised CZE method coupled with correction factors was successfully applied to the determination of monosaccharide composition of three purified polysaccharides from *E. sinica* and their corresponding partial acid hydrolysis products.

Experimental

Plant material

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

Reagents 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 sulphuric acid (H_2SO_4) were purchased from Sigma (St. Louis, MO, USA). Trifluoroacetic acid (TFA) was obtained from Merck (Darmstadt, Germany). 1-Phenyl-3-methyl-5-pyrazolone, purchased from Beijing Reagent Plant (Beijing, China), was recrystallised 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.

CZE 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), 0.1 M 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).

Isolation, purification and acid hydrolysis

Isolation and purification. The dry stems of *E. sinica* were ground to powder, and submitted to sequential extractions as follows: dry powders (1.0 kg) were extracted three times with 10 vols of 95% EtOH under reflux for 3 h each time to remove lipids. The residue was dried in air and then extracted three times with 10 vols 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 dialysed (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 crude polysaccharide A (8.5 g) collected by centrifugation (3000 rpm, 10 min, 20°C). A similar procedure was used with 10 vols of hot water (100°C, 3 h, three times) for above the residue after cold water extraction. The fraction obtained was labeled crude polysaccharide B (35.7 g).

Crude polysaccharide A (3.0 g) was dissolved in distilled water and passed through two series connected resin columns [Amberlite FPA90-Cl (Cl^- form) and Amberlite IRC-84 (H^+ form)] eluting with distilled water and 1.0 M NaCl to yield fractions Fr. A1 (1.2 g) and Fr. A2 (0.9 g), respectively. Fr. A1 (1.0 g) was chromatographed over DEAE-Sepharose F. F eluting with distilled water and 0.2 M NaCl to yield subfractions Fr. A1-1 (400.0 mg) and Fr. A1-2 (380.0 mg), respectively. Fr. A1-2 (380.0 mg) was further purified by gel-permeation chromatography on a high-resolution Sephacryl S-300 eluting with distilled water to afford ESP-A3 (310.0 mg). Fr. A2 (550.0 mg) was chromatographed over DEAE-Sepharose F.F eluting with 1.0 M NaCl to afford ESP-A4 (430.0 mg). Crude polysaccharide B (6.0 g) was dissolved in distilled water and passed through two series-connected resin columns [Amberlite FPA90-Cl (Cl^- form) and Amberlite IRC-84 (H^+ form)] eluting with distilled water and 1.0 M NaCl to yield fractions Fr. B1 (2.3 g) and Fr. B2 (2.2 g), respectively. Fr. B2 (1.0 g) was applied to a DEAE-cellulose 52 column with 1.0 M NaCl to afford ESP-B4 (880.0 mg). Polysaccharides in the eluted fractions were detected using the phenol–sulphuric acid method and all NaCl elute fractions in column chromatography were dialysed against distilled water for 48 h (cut-off M_w 3500 Da) and then freeze-dried.

Identification of purity of polysaccharides. Each polysaccharide (ESP-A3, ESP-A4 and ESP-B4) was dissolved in distilled water at a concentration of 5 mg/mL and analysed on a Waters 2695 HPLC system equipped with a quaternary solvent delivery system, a Waters 600 controller, two Waters 600 pumps, a 2695 autosampler, a Waters 2695 column oven and an Alltech ELSD 2000 detector. The separation was carried out on a Shodex sugar KS-805 column (8.0 × 300 mm, 17 μm) coupled with a Shodex KS-G guard column (6 × 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. The drift tube temperature for ELSD was set at 116°C and the nitrogen flow rate was 3.3 L/min for the determination of polysaccharides. Their purities were over 98% by HPLC analysis.

Complete acid hydrolysis of polysaccharides. Each polysaccharide sample (20 mg) was dissolved in 2 mL of 2.0 M H_2SO_4 in an ampoule (5 mL). The ampoule was sealed under a nitrogen atmosphere and kept at 110°C to hydrolyse the polysaccharide into component monosaccharides for 6 h, then cooled to room temperature and neutralised with 2 mL of 4.0 M NaOH. The reaction mixture was diluted to 5 mL with deionised water and was centrifugalised at 1000 rpm for 5 min. Then the supernatant was ready for the following experiments.

Partial acid hydrolysis of polysaccharides. Each polysaccharide sample (100 mg) was hydrolysed with 0.1 M TFA (3 mL) for 6 h at 100°C, and acid was removed in vacuum by addition of methanol repeatedly. The hydrolysis sample was dialysed with distilled water for 48 h in a dialysis sack (cut-off M_w 3500 Da), and then diluted the solution in the sack with ethanol. After hydrolysis, the precipitate (a) and supernatant in the sack (b) and the fraction out of sack (c) were dried, and then CZE analysis was carried out.

Derivatisation procedure

Preparation of standard solution. The individual standard monosaccharide (1.0 mM, each), and mix monosaccharide solution (1.0 mM) were prepared with deionised water. Working standard solutions were further obtained by appropriate dilution of the stock mix monosaccharide sample solutions with deionised water. All sample solutions were filtered through a 0.45 μm syringe filter and were degassed using an ultrasonic bath for 2 min prior to use. All the solutions prepared were stored in a refrigerator at +4°C.

Derivatisation procedure. PMP derivatisation of monosaccharides was carried out as described previously with proper modification (Honda *et al*., 1989, 2003; Daotian and Roger, 1995; Andersen *et al*., 2003; Zhang *et al*., 2003; Lv *et al*., 2009). Aliquots of 200 μL of individual standard monosaccharide, or mixed standard monosaccharide solutions, or the hydrolysed polysaccharide samples were placed in the 2.0 mL centrifuge tubes, 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 in a 70°C water bath, then cooled to room temperature and neutralised with 100 μL of 0.3 M HCl. The resulting solution was performed on liquid–liquid extraction with same volume of isoamyl acetate (twice) 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 HPCE analysis.

Method validation

The regression equations were calculated in the form of $y = ax + b$, where y was the peak areas and x was the concentration of analytes. The signal-to-noise ratios of 3:1 and 10:1 were used to establish LOD and LOQ, respectively. The measurement of intra- and inter-day variability was utilised to determine the precision of this newly developed method. The intraday variation was determined by analyzing the same mixed standard water solution five times within 1 day, while for inter-day variability test, the solution was examined in triplicate for a consecutive three 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 very stable in water solution (RSD 3.45%) at 4°C during the tested period.

Results and Discussion

CZE method development

We can only label carbohydrates as shown in Fig. 1, which 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 optimising background electrolyte pH and concentration, applied voltage and capillary temperature.

Effect of pH

The pH of background electrolyte is a very important parameter in capillary electrophoresis. It can affect the EOF rate and the degree of ionisation of analytes. Hence, the effect of running buffer pH (9.54, 10.02, 10.26) in 35 mM borate on the separation of monosaccharides was investigated. For GlcUA and GalUA, a

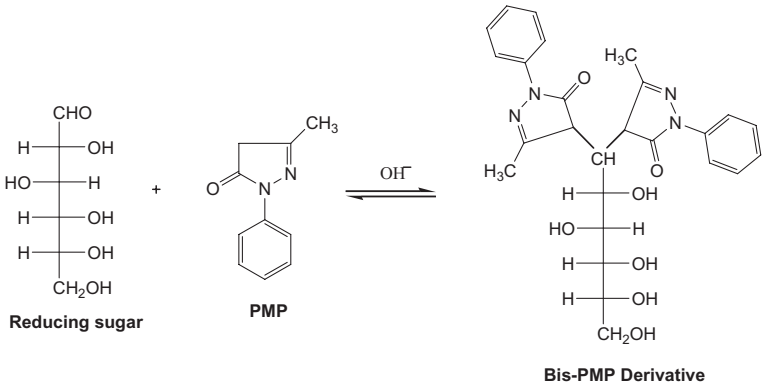

Figure 1. Illustration of condensation reaction with PMP.

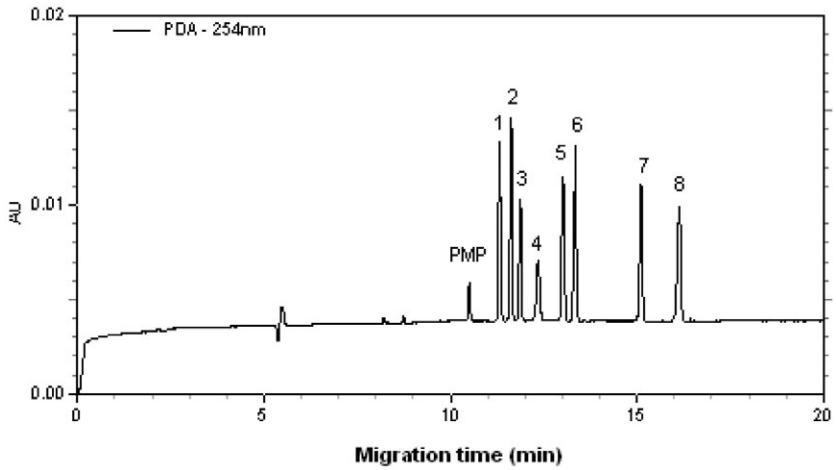

Figure 2. Electropherograms of PMP derivatives of eight standard monosaccharides at the optimal conditions including 35 mM borate at pH 10.02, capillary temperature 25°C and applied voltage 20 kV, 0.2 mM each. Peak identities: **1**, Xyl; **2**, Ara; **3**, Glc; **4**, Rha; **5**, Man; **6**, Gal; **7**, GlcUA; **8**, GalUA. 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.

steady baseline resolution could always be achieved at the investigated pH range whereas the separation of the neutral sugars showed relatively strong dependence on the pH as compared with the uronic acids. In particular, for Ara and Glc, assuming an appropriate pH (9.54) had been chosen, Ara and Glc co-eluted. If a higher pH (10.02) was adopted, good symmetry and resolution for each analyte were achieved. At pH 10.26, there was a longer analysis time for GalUA. Thus pH 10.02 was chosen as the optimal pH.

Effect of buffer concentration

The effect of different concentrations of borate (20, 35 and 50 mM) at pH 10.02 on the separation of analytes was studied. It was found that Xyl, Ara and Glc were not well separated at 20 mM of borate with poor resolution, but the best resolution and highest theory plates were achieved at 35 mM of borate. Increasing borate concentration, however, remarkably increased the migration time of all analytes. The result was in agreement with previous studies (Honda *et al*., 1989; Zhang *et al*., 2003). With a further increase in buffer concentration, a large amount of Joule heating affected separation efficiency. Taking both shorter runtime and separation efficiency into consideration, the borate buffer concentration was selected as 35 mM.

Selection of the capillary temperature and voltage

The temperature of the analysis may sometimes be important in CZE, 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 (18, 20, 22 kV) on separation of the analytes were also studied. The results showed that good resolution and acceptable migration time were achieved at 20 kV.

Under the proposed conditions including 35 mM borate at pH 10.02, capillary temperature 25°C and applied voltage 20 kV, a complete baseline resolution for carbohydrate derivatives was achieved within 16.5 min (Fig. 2).

Method validation

As shown in Table 1, all calibration curves showed good linear regression ($r \geq 0.9779$) within the test ranges. The LOD (S/N = 3) and the LOQ (S/N = 10) were less than 1.95 and 5.85 μM for all analytes, respectively. The measurement of intra- and inter-day variability was utilised to determine the precision of this newly developed method, which was investigated by the relative standard deviations (RSDs) of the migration time and peak area

Table 1. Linearity of CZE method of different carbohydrate

Carbohydrate[a]	Regression equation[b]	r	Linear range (μM)	LOD (μM)	LOQ (μM)
Xyl	$y = 267.68\,x - 709.79$	0.9966	5.00–400.00	1.40	4.20
Ara	$y = 321.64\,x + 332.25$	0.9988	5.00–400.00	1.09	3.27
Glc	$y = 238.74\,x - 251.21$	0.9793	5.00–400.00	1.63	4.89
Rha	$y = 175.04\,x - 1437.3$	0.9971	5.00–400.00	1.95	5.85
Man	$y = 342.59\,x + 316.33$	0.9779	5.00–400.00	1.22	3.66
Gal	$y = 387.90\,x + 1352$	0.9840	5.00–400.00	0.98	2.94
GlcUA	$y = 335.28\,x + 1614.6$	0.9980	5.00–400.00	1.47	4.41
GalUA	$y = 347.73\,x + 1597.5$	0.9953	5.00–400.00	1.12	3.36

[a] Quantitated with a calibration curve at = 254 nm.
[b] y and x stand for the peak area and the contentration (μM) of compounds, respectively.

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

Sugars	Intra-day precision (RSD%, $n = 5$)		Inter-day precision (RSD%, $n = 3$)	
	Retention time[a]	Peak area[b]	Retention time[a]	Peak area[b]
Xyl	1.25	3.68	1.75	4.17
Ara	1.03	2.84	2.08	3.46
Glc	1.39	2.82	1.29	4.45
Rha	2.07	2.93	2.61	4.79
Man	1.09	1.66	1.56	4.23
Gal	2.05	3.74	2.72	4.96
GlcUA	1.89	2.89	2.55	4.01

[a] Retention time ration between analytes and GalUA.
[b] Peak area ration between analytes and GalUA.

rations between analytes and GalUA. In order to overcome injection volume errors between injections, GalUA had to be chosen as the reference peak for the precision experiments. The overall intra- and inter-day variabilities of migration time rations were less than 2.72%, while the overall intra- and inter-day variabilities of peak area rations were less than 4.96% (Table 2).

Correction factors and molar ratios

The injection volumes in HPCE are typically small, 5–50 nL, and maintaining precision between injections is difficult. Hence, an internal standard is always used to overcome this problem. However, there are no less than eight monosaccharides in complex acidic hetero-polysaccharides, so it is not easy to find a suitable internal standard to overcome this problem. Here we used the standard curves coupled with relative correction factors for determination of monosaccharide composition (molar ratios) of acidic hetero-polysaccharides from *E. sinica* as follows. The correction factor is shown in the equation: $f_{i/n} = (m_i/A_i)/(m_n/A_n)$, where A_i and A_n are the values of the peak areas in the standard monosaccharide, respectively; m_i and m_n are the values of quantities (nmol) in the standard monosaccharide, respectively. The molar ratio value is shown in the equation: $R_{i/n} = f_{i/n} \times (A'_i/A'_n)$, where A'_i/A'_n is the ratio value of the corresponding peak areas in the tested samples and $f_{i/n}$ is the relative correction factor. However, $f_{i/n}$ should be a constant in both the standard monosaccharides and the tested samples for the monosaccharides i and n in the linearity range from the standard curves. Thus the standard curves have to play an important role in providing the linearity range for the tested sample solutions.

In the process of structural analysis of polysaccharides, the molar ratio of each monosaccharide in polysaccharides is one of the most important parameters (Roy *et al*., 2008; Tong *et al*., 2008). It is unnecessary to obtain the specific amount of each monosaccharide because researchers just want to know the molar ratios of each one (Dong *et al*., 2007; Yu *et al*., 2007; Hsieh *et al*., 2008; Xu *et al*., 2009). Therefore, the molar ratio can be calculated by the equation [$R_{i/n} = f_{i/n} \times (A'_i/A'_n)$] rather than specific amount of each sugar obtained. We believe that the former is more favourable in the elimination of systematic errors.

Optimisation of acid hydrolysis procedures

It is very difficult to achieve complete acidic hydrolysis of the polysaccharide polymers that contain uronic acid groups (Smidsrod *et al*., 1966; Ucar and Balaban, 2003). If they have more uronic acid groups, it will be more difficult to achieve complete hydrolysis. Therefore, optimisation of acidic hydrolysis conditions is necessary for the accurate analysis of each polysaccharide. In this study, sulphuric acid, an effective acid in degrading polysaccharides, was chosen to hydrolyse polysaccharides into their component monosaccharides (Kekos and Koukios, 1985; Yanishevskaya *et al*., 1987; Ucar and Balaban, 2003), and the factors affecting hydrolysis of ESP-B4 with sulphuric acid were investigated by changing the hydrolysis time, acid concentration, and

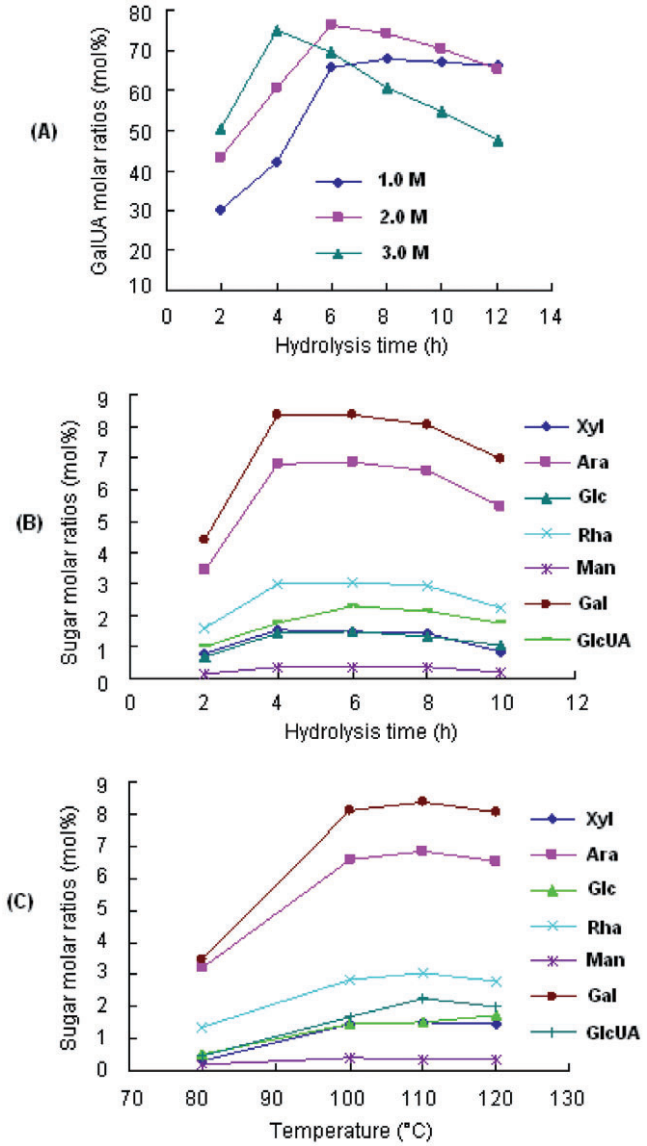

Figure 3. (A) Molar ratios of GalUA (mol%) released from ESP-B4 subjected to treatment with 1.0, 2.0, and 3.0 M H_2SO_4 at 110°C vs. different hydrolysis times; (B) molar ratios of seven sugar (mol%) released from ESP-B4 subjected to treatment with 2.0 M H_2SO_4 at 110°C vs. different hydrolysis times; (C) molar ratios of seven sugar (mol%) released from ESP-B4 subjected to treatment with 2.0 M H_2SO_4 at 6 h vs. different temperature. Hydrolysis procedure and CZE conditions are as described in Experimental section.

hydrolytic temperature to achieve complete hydrolysis of the polysaccharide polymer.

To further confirm the influence of acid hydrolysis, the hydrolysis results of ESP-B4 with different molarities of acid (1.0, 2.0, and 3.0 M H_2SO_4) at 110°C for different times were characterised by CZE coupled with correction factors for the determination of the liberation rate of the GalUA. As depicted in Fig. 3(A), although the release of GalUA from ESP-B4 was considerably accelerated with the increase in H_2SO_4 concentration, the hydrolysis with 2.0 M H_2SO_4 for 6 h gave the highest recovery of free GalUA, followed by 3.0 M H_2SO_4 for 4 h, and with 1.0 M H_2SO_4 for 8 h in decreasing order. When the hydrolysis time was further prolonged, the destruction rate of GalUA was greater than the release rate from

Table 3. CZE analysis results of three purified acidic heteropolysaccharides and their corresponding partial acid hydrolysis products

Fractions	Molar ratios							
	Xyl	Ara	Glc	Rha	Man	Gal	GlcUA	GalUA
ESP-A3	6.8	7.5	1.0	14.0	13.7	22.3	10.2	3.8
ESP-A4	1.2	4.1	1.0	5.1	1.6	17.3	3.1	2.2
ESP-B4	1.0	4.5	1.0	2.0	1.0	5.5	1.5	50.0
ESP-A3-a	4.3	3.6	—	4.8	8.9	7.7	4.2	1.0
ESP-A3-b	—	1.8	1.1	1.9	1.0	3.7	—	—
ESP-A3-c	—	12.6	2.0	7.1	1.0	15.7	—	5.1
ESP-A4-a	1.5	3.8	—	3.2	1.1	8.8	1.0	4.6
ESP-A4-b	1.2	5.5	1.0	4.3	3.7	32.4	3.5	1.1
ESP-A4-c	1.0	5.2	3.8	5.2	3.3	14.1	1.5	2.4
ESP-B4-a	3.1	7.3	1.0	3.7	1.4	11.6	1.9	71.1
ESP-B4-b	1.8	5.3	—	—	1.8	6.4	1.0	23.6
ESP-B4-c	—	1.9	—	—	—	1.0	—	17.2

[a] Precipitation in the sack.
[b] Supernatant in the sack.
[c] Fraction out of the sack.

ESP-B4, and such destruction was markedly accelerated at 3.0 M H_2SO_4. The results indicated that uronic acid groups in polysaccharide cannot achieve complete hydrolysis with 1.0 M H_2SO_4, while most sugar moieties are destroyed with 3.0 M H_2SO_4. Thus 2.0 M H_2SO_4 was chosen as the optimal acid hydrolysis concentration. When we fixed acid concentration as 2.0 M H_2SO_4; the other six neutral sugars and GlcUA also obtained better recoveries at the hydrolysis time of 6 h (Fig. 3B). In addition, as shown in Fig. 3(C), the release of most neutral sugars and GlcUA was significantly dependent on the hydrolysis temperature and went through a peak (110°C) and then decreased. Thus the optimal hydrolysis temperature and time were chosen as 110°C and 6 h.

Analysis of purified polysaccharides from *E. sinica*

ESP-A3, ESP-A4 and ESP-B4 were isolated and purified by ion exchange and gel-filtration chromatography. Determination of their chemical structures and biological activities is in progress. Here, in order to evaluate the applicability of the proposed method, the isolated polysaccharide was hydrolysed with 2 M H_2SO_4 for 6 h at 110°C, and PMP-labeled as described in the Experimental section. The released monosaccharide derivatives were analysed by the described CZE method under the optimised conditions. Figure 4 shows typical chromatograms of *E. sinica* polysaccharide samples and the detected contents are listed in Table 3. As can be seen, the PMP derivatives of the component monosaccharides released from ESP-A3, ESP-A4 and ESP-B4 could be still perfectly baseline separated from each other and the component monosaccharides could be identified by comparing with the chromatogram of the mixture of standard monosaccharides (Fig. 2). The results showed that ESP-A3, ESP-A4 and ESP-B4 were all typical acidic hetero-polysaccharides and were composed of Xyl, Ara, Glc, Rha, Man, Gal, GlcUA and GalUA in the molar ratios of 6.8:7.5:1.0:14.0:13.7:22.3:10.2:3.8 for ESP-A3, 1.2:4.1:1.0:5.1:1.6:17.3:3.1:2.2 for ESP-A4 and 1.0:4.5:1.0:2.0:1.0:5.5:1.5:50.0 for ESP-B4. Their corresponding molar percentages (mol%) were 8.6, 9.4, 1.3, 17.7, 17.3, 28.1, 12.9 and 4.9% for ESP-A3, 3.4, 11.5, 2.8, 14.3, 4.6, 48.6, 8.7 and 6.2% for

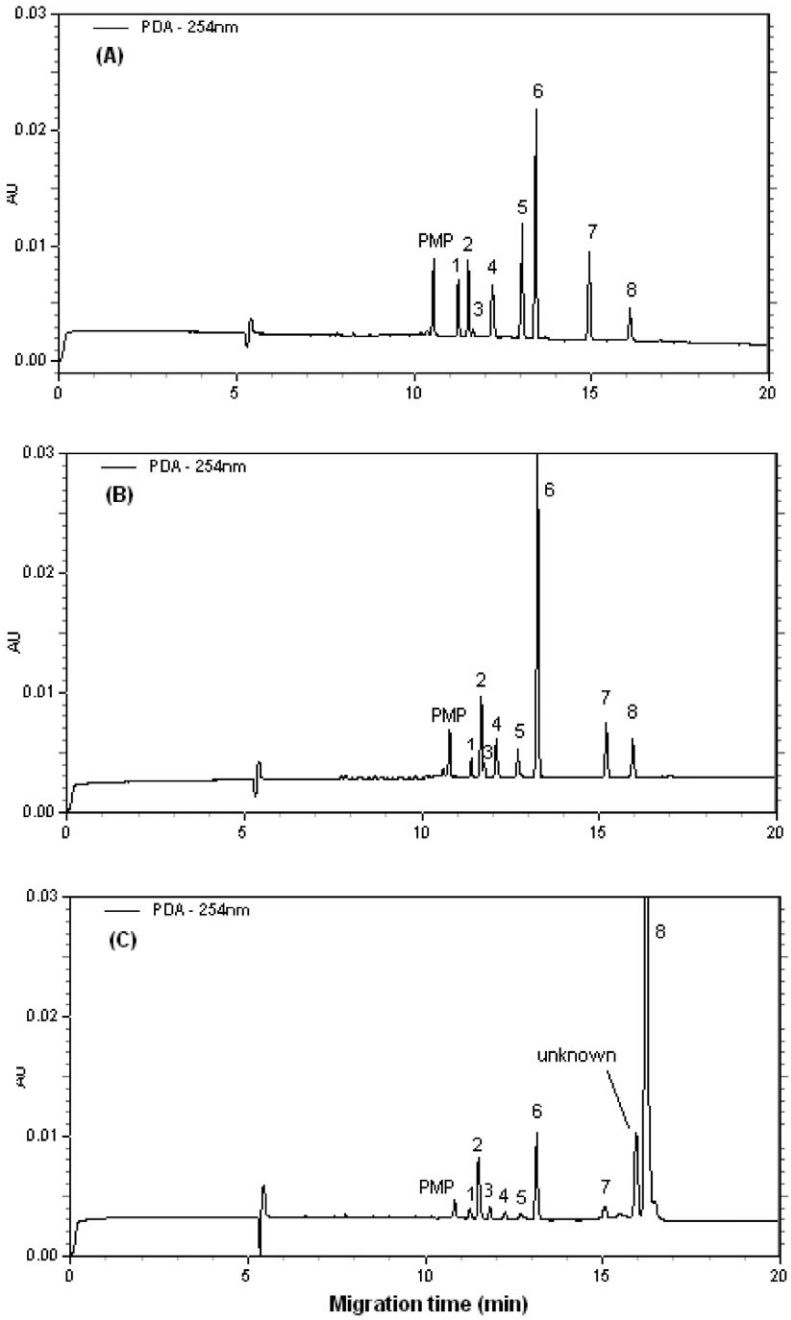

Figure 4. Electropherograms of PMP derivatives of monosaccharides in ESP-A3 (A), ESP-A4 (B) and ESP-B4 (C). Hydrolysis conditions as 2.0 M H_2SO_4 at 110°C for 6 h. CZE condition and peak identities were described in Fig. 2.

ESP-A4, and 1.5, 6.8, 1.5, 3.0, 1.5, 8.3, 2.3 and 75.2% for ESP-B4, respectively. It is clear that the predominant composition monosaccharide in ESP-B4 was GalUA, with up to 75.2% (mol%) of total identified carbohydrates, and 22.5% of total identified carbohydrates were neutral sugars.

Partial acid hydrolysis is a very useful method in the structure analysis of polysaccharide so that some detailed information on structures can be provided (Dong *et al*., 2007; Tong *et al*., 2008). The results from CZE analysis are shown in Table 3. Taking ESP-A3, for example (Fig. 5), all the fractions including ESP-A3-a, ESP-A3-b and ESP-A3-c recovered after partial acid hydrolysis of ESP-A3 and CZE analysis with pre-column derivatisation. The material retained in the sack after dialysis of the partial acid hydrolysate was named as ESP-A3-a, and it had the highest molecular size of the three fractions. Its structure was proposed to be closest to ESP-A3. The component of ESP-A3-a, in which the ratio value of Xyl : Ara : Rha : Man : Gal : GlcUA : GalUA was 4.3:3.6:4.8:8.9:7.7:4.2:1.0, indicated that Man and Gal may be the backbone of the structure of ESP-A3, and Xyl, Ara, Rha, GlcUA and GalUA may be close to this backbone. The analysis results for ESP-A3-b and ESP-A3-c indicated that the branched structure of ESP-A3 was composed of Ara, Glc, Rha, Man, Gal and GalUA because these sugar residues could be obtained easily after hydrolysis.

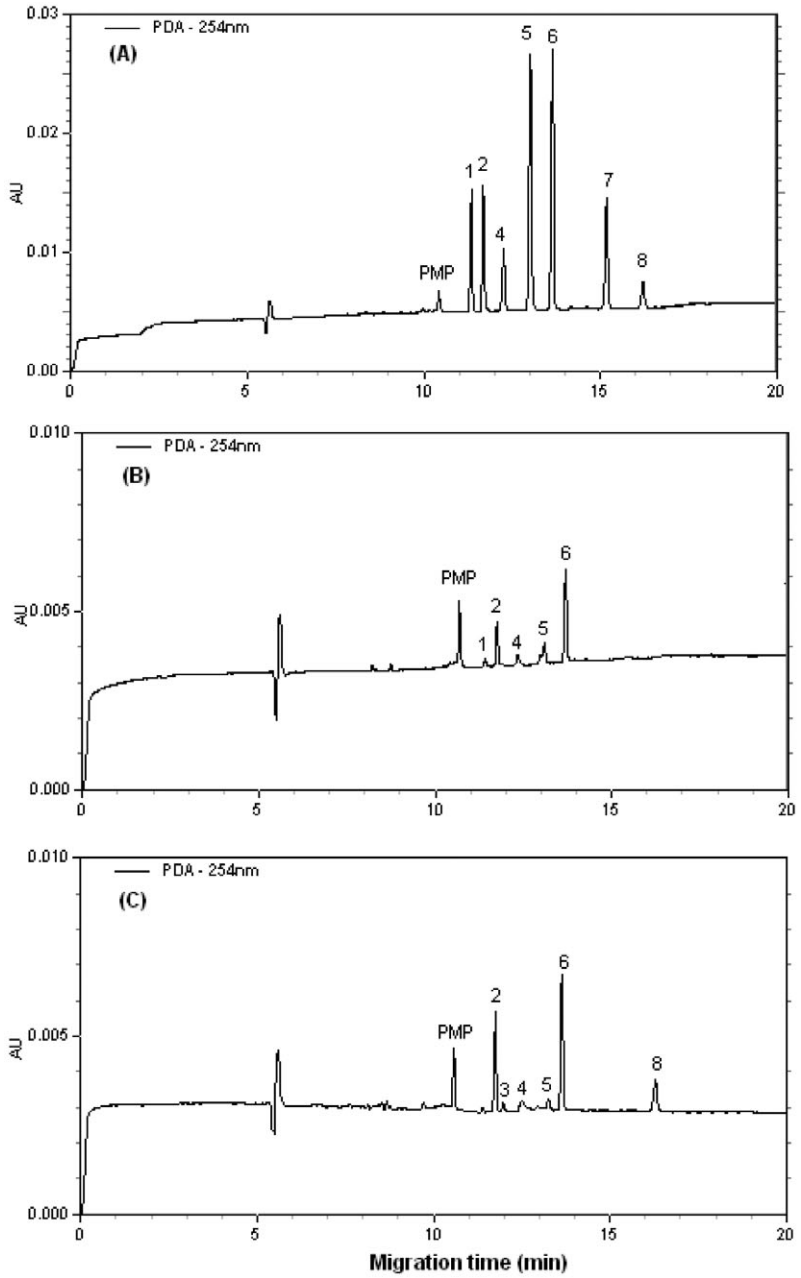

Figure 5. Electropherograms of PMP derivatives of partial acid hydrolysis products for ESP-A3. (A) Precipitation in the sack (ESP-A3a); (B) supernatant in the sack (ESP-A3b); (C) fraction out of sack (ESP-A3c). Hydrolysis conditions as 2.0 M H_2SO_4 at 110°C for 6 h. CZE condition and peak identities were described in Fig. 2.

Conclusion

The results reported in this paper demonstrate that the proposed CZE method coupled with correction factors provides an excellent example for the determination of molar ratios among monosaccharides of complex acidic hetero-polysaccharide from *E. sinica*. Such information can provide a scientific evidence for further exploitation of the chemical structure and bioactivity of complex acidic *E. sinica* polysaccharides. In addition, it would facilitate the use of *E. sinica* polysaccharide in pharmaceutical and other technical applications, which would contribute to the sustainable use of *E. sinica* plant resources. Meanwhile, it can also be applied to routine analysis of monosaccharide composition in other complex plant polysaccharides and some real-life samples, such as fruit juices, wines and brandies.

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