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Determination of 1-phenyl-3-methyl-5-pyrazolone-labeled carbohydrates by liquid chromatography and micellar electrokinetic chromatography

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Abstract

In this paper, the method for the derivatization of carbohydrates with 1-phenyl-3-methyl-5-pyrazolone (PMP) was simplified. One-third of the derivatization time was saved. Five monosaccharide derivatives have been well separated by MEKC and HPLC under optimized conditions. Good reproducibility could be obtained with relative standard deviation (RSD) values of the migration times within 5.0 and 2.3%, respectively. Furthermore, the developed methods have been successfully applied to the analysis of carbohydrates in Aloe powder and food. These methods are quite useful for routine analysis of monosaccharides and oligosaccharides in real samples.

Keywords: 1-Phenyl-3-methyl-5-pyrazolone; Carbohydrates

1. Introduction

The important roles of carbohydrates in biological processes have been increasingly recognized. A considerable amount of research has already been carried out in recent years in this area. Since carbohydrates encompass a number of homologues having very similar structures and many of them exist concurrently in real-life samples, carbohydrate analysis inevitably requires high-resolution separation techniques. However, these compounds generally have low intrinsic UV spectral activity. Therefore the derivatization of carbohydrates is indispensable to obtain highly sensitive detection [1]. In practical analyses, carbohydrates are usually derived with either UV [2,3] or fluorescent tags [4,5]. The introduction of some labels also endows carbohydrates with ionic properties, and changes their hydrophilicity, so that different separation modes can be adopted to analyze them.

The reagent 1-phenyl-3-methyl-5-pyrazolone (PMP) is one of the popular labels that can react with reducing carbohydrates under mild conditions, requiring no acid catalyst and causing no desialylation and isomerization. PMP yields strong UV absorbance at 245 nm [6]. This derivatization method was first developed for the analysis of carbohydrates by high-performance liquid chromatography (HPLC) [7–11], and later successfully applied to capillary electrophoresis (CE) [12–20]. With high efficiency, high speed, low sample requirement and low solvent cost, CE has proved to be an excellent method for the analysis of carbohydrates with capil-

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lary zone electrophoresis (CZE) [14], micellar electrokinetic chromatography (MEKC) [15–18], capillary gel electrophoresis (CGE) [19] and ion-exchange electrokinetic chromatography (IXEKC) [20]. PMP derivatization increases the hydrophobicity of carbohydrates, therefore HPLC and MEKC are quite suitable for analyzing PMP derivatives.

In this study, five monosaccharide derivatives have been prepared using a simplified PMP reaction method. These derivatives have been well separated by MEKC and HPLC under optimized conditions. Furthermore, the developed methods have been successfully applied to the analysis of carbohydrates in Aloe powder and food.

2. Experimental

2.1. Instrumentation

Capillary electrophoresis experiments were carried out on a Beckman P/ACE 5010 instrument equipped with a UV detector set at 254 nm (Beckman, Fullerton, CA, USA). Fused-silica capillaries with 50 μm I.D. and 375 μm O.D. were purchased from Yongnian Optical Fiber Factory (Hebei, China). Data acquisition and processing were carried out with Beckman System Gold software.

The HPLC apparatus was composed of a P200II liquid chromatography pump system, a Rheodyne 7125 injector with a 20-μl loop, a column (150 mm×4.5 mm I.D.) packed with Hypersil ODS2 (5 μm diameter) and a UV200II detector. The system was obtained from Dalian Elite Analytical Instrument Co. (Dalian, China).

2.2. Materials

PMP was purchased from Acros Organics (Geel, Belgium). Sodium dodecyl sulfate (SDS) was purchased from Sino-American Biotechnology Co. (Beijing, China). Trifluoroacetic acid (TFA) was obtained from Merck Materials (Munich, Germany). Monosaccharide standards, D-mannose (Man), rhamnose (Rham), D-galactose (Gal), glucose (Glu), and D-xylose (Xyl) were purchased from The Fourth Shanghai Reagent Plant (Shanghai, China). All the reagents were analytical-grade.

Water used throughout the experiments was double distilled and purified on a Milli-Q system (Millipore Inc., Milford, MA, USA).

Stock phosphate buffer (200 m*M*) was prepared with Na_2HPO_4 and HCl, and diluted to a final concentration of 25 m*M*. The pH value of the mobile phase was adjusted to 6.0 with HCl.

Stock acetate buffer (100 m*M*) was prepared using CH_3COONH_4 and the pH value of the mobile phase was adjusted to 5.5 with CH_3COOH.

2.3. Preparation of PMP derivatives of carbohydrates

Five monosaccharides (1 m*M* each) were dissolved in 0.3 *M* aqueous NaOH (7.5 ml). A 75-μl aliquot of this solution was mixed with a 0.5 *M* methanol solution of PMP (50 μl), and the mixture was allowed to react for 30 min. Then the reaction mixture was cooled to room temperature and neutralized with 75 μl of 0.3 *M* HCl. The resulting solution was dried under vacuum at 100 °C. The residue was dissolved in water and chloroform (1 ml each). The organic phase was discarded after vigorous shaking to remove the excess reagents. This extraction process was repeated three times, and then the aqueous layer was filtered through a 0.45-μm membrane and diluted with water before HPLC and MEKC analyses. The concentrations of samples prior to injection into an HPLC or MEKC system are about 0.5 mg/ml.

2.4. Preparation of real-life samples

Concentrated aloe powder (0.0015 g) was dissolved in 4 *M* trifluoroacetic acid (500 μl) in an ampoule (1 ml). The ampoule was sealed under a nitrogen atmosphere and kept at 110 °C for 2 h. After cooling to room temperature, the reaction mixture was dried by a nitrogen stream. The residue was then dissolved in 75 μl of 0.3 *M* NaOH and 50 μl of 0.5 *M* methanol solution of PMP was added. Finally, the mixture was treated using the method mentioned above.

Candy (toffee) (1.0 g) was placed in distilled water (10 ml) and then sonicated for 20 min. After this, 1 ml of the resulting suspension was centrifuged, and 100 μl of the top clear solution was

mixed with 75 μl of 0.3 *M* NaOH and 50 μl of 0.5 *M* methanol solution of PMP. Then the mixture was treated with the method mentioned above.

A 100-μl aliquot of Tsingtao Beer (Qingdao, China) was mixed with 75 μl of 0.3 *M* NaOH and 50 μl of 0.5 *M* methanol solution of PMP, then the mixture was treated as discussed above.

2.5. MEKC operation

The capillary was washed in turn with 0.1 *M* HCl and 0.1 *M* NaOH for 30 min. Then the capillary was washed with water to neutralize. Subsequently, the capillary tube was purged with nitrogen to dry. The capillary column was installed on the CE instrument and equilibrated by applying a potential difference of 20 kV until the current was stable. The UV absorption wavelength was set at 254 nm (245 nm is more suitable for the analysis of PMP derivatives. However, the wavelengths of filters in our CE instrument are fixed. Therefore detection wavelength had to be set at 254 nm). The mobile phase was composed of 25 m*M* phosphate buffer and 70 m*M* SDS and the pH value was adjusted to 6.0. Before the run, the mobile phase was degassed in an ultrasonic bath for about 10 min. In our experiments, methanol and Sudan III were chosen as EOF and micellar markers. All separations were carried out at an applied potential difference of 20 kV with temperature set at 26 °C. Electrokinetic injections were made at 1 kV for 1 s.

2.6. HPLC operation

Elution was carried out at a flow-rate of 1.0 ml/min at room temperature, with the mobile phase of 100 m*M* ammonium acetate buffer (pH 5.5)–acetonitrile (78:22, v/v). UV absorption was measured at 245 nm.

3. Results and discussion

3.1. Simplification of PMP derivatization method

In our method, drying of the aqueous layer after extraction with chloroform (as described by Honda et al. [6,7]) was omitted. This drying step was used to remove residual derivatization reagents and impurities produced in the derivatization procedure. Although this step could minimize the interference of PMP to separation and detection and prolong the lifespan of columns, it complicated the derivatization procedure and resulted in the loss of the derivatives.

Ma et al. [8] did not employ any drying steps in their derivatization method. The derivatives were extracted from the solution neutralized with HCl. The residual PMP in the sample prepared with this method could not be removed completely, which would interfere in the analysis of Man under isocratic conditions of HPLC. But in MEKC, PMP was eluted at about 3.3 min and did not interfere in the analysis of derivatives of carbohydrates (Fig. 4(a): trace 2). So the drying step after neutralizing can also be omitted when MEKC mode is used.

Fig. 1(a) shows the HPLC chromatogram of derivatives prepared without any drying step. One drying step after neutralization can eliminate the interference of PMP as shown in Fig. 1(b). With this simplification, one-third of the derivatization time was saved and the loss of derivatives decreased by 15% without influencing their properties. The reproducibility of the derivatization method was good with the relative standard deviations within 3.2%. There were no significant changes in peak area of derivatives overnight, which demonstrated that the stability of derivatives was satisfactory. After the extraction, the derivatives were analyzed every 2 h, and the relative standard deviations of peak area were less than 3.8% in 20 h.

3.2. Separation of standard monosaccharide derivatives by MEKC and HPLC

The PMP derivatives of reducing carbohydrates can be analyzed by various separation modes of CE [12–20]. MEKC was generally most suitable for the separation of the PMP derivatives of carbohydrates. Suzuki et al. [18] established a method for the analysis of the sialo-*N*-glycans in glycoproteins with MEKC and HPLC.

In our experiments, we developed an optimized MEKC method to analyze carbohydrates. Salt concentration, pH and the SDS concentration were optimized. It was found that the final values of 25, 6.0 and 70 m*M*, respectively, gave the best sepa-

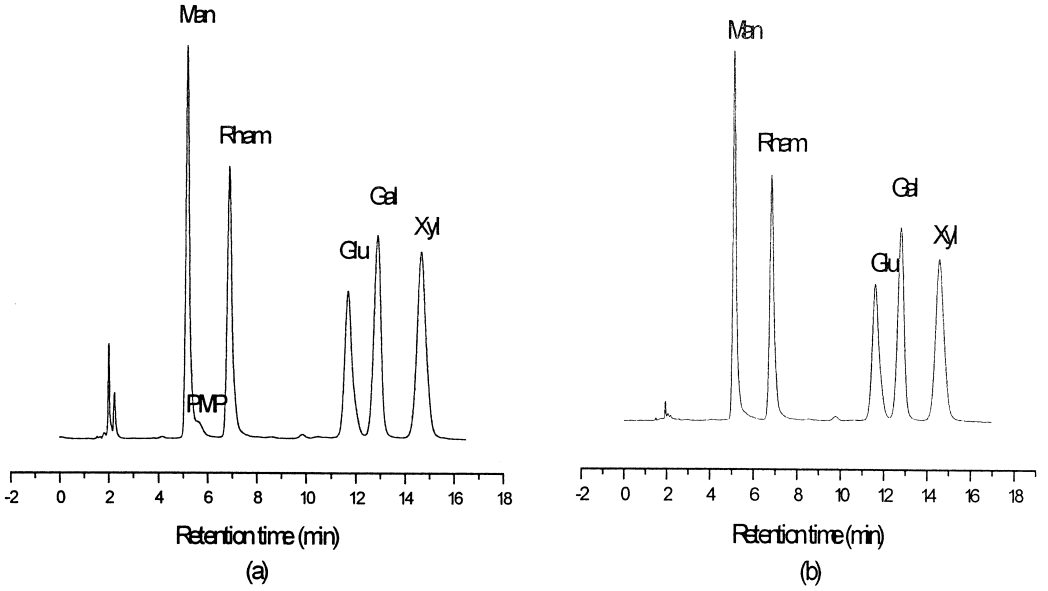

Fig. 1. Effect of drying process. Experimental conditions: Column: packed with ODS2 with 5 μm diameter (150 mm×4.5 mm I.D.); mobile phase: acetate buffer (0.1 mol/l, pH 5.5)–acetonitrile (78:22 v,v); flow-rate: 1 ml/min; UV detection: 245 nm; sample: 10 μl. (a) Without drying process; (b) with one drying process.

ration. Fig. 2(a) shows the electropherogram of five monosaccharide derivatives under the optimized conditions. It can be seen that all components are separated within 10 min. The resolution (*R*s) of the last two peaks was 0.88. The variation of migration times was minimized by rinsing the capillary with 100 m*M* sodium hydroxide followed by water before each run. With this method, good reproducibility could be obtained with the RSD of the migration times within 5.0%. The limit of detection (LOD) was below 1.5 ng.

In addition, an HPLC method to analyze PMP-derived monosaccharides was developed based on optimization strategies proposed by Snyder et al. [21] and Lu et al. [22]. The retention behaviors of these five PMP derivatives were examined using a mixture of 100 m*M* acetate buffer with various pH values and acetonitrile in various ratios, and then the optimized conditions were established as described in Section 2.6. Baseline separation of the five monosaccharides was obtained under isocratic conditions. The chromatogram of these five derivatives is shown in Fig. 2(b). The RSD of elution times was less than 2.3% for all five monosaccharide derivatives and the LOD was below 0.9 ng. The linearity was good in a wide range from 4.5 to 600 mg/l.

The effects of separation parameters on the analysis of five monosaccharide derivatives are shown in Fig. 3. In MEKC, an increase in buffer concentration prolongs the retention time and increases the current. The variation of pH influences the separation significantly. The increase in the concentration of SDS can still improve the resolution. However, it would prolong the retention time and deteriorate peak shapes at the same time. In HPLC, the increase in pH and acetonitrile concentration of the mobile phase can improve the resolution. However, it would result in longer elution times.

From the results mentioned above, it can be seen that the PMP-labeled carbohydrates are well separated by both MEKC and HPLC. However by comparison, it can be seen that high efficiency and short analysis time can be obtained with MEKC, while the resolution of the components can be greatly improved at the expense of analysis time. The LOD of HPLC is better than MEKC because of the limitation of the CE instrument and short optical pathlength for on-column detection. Accordingly, we

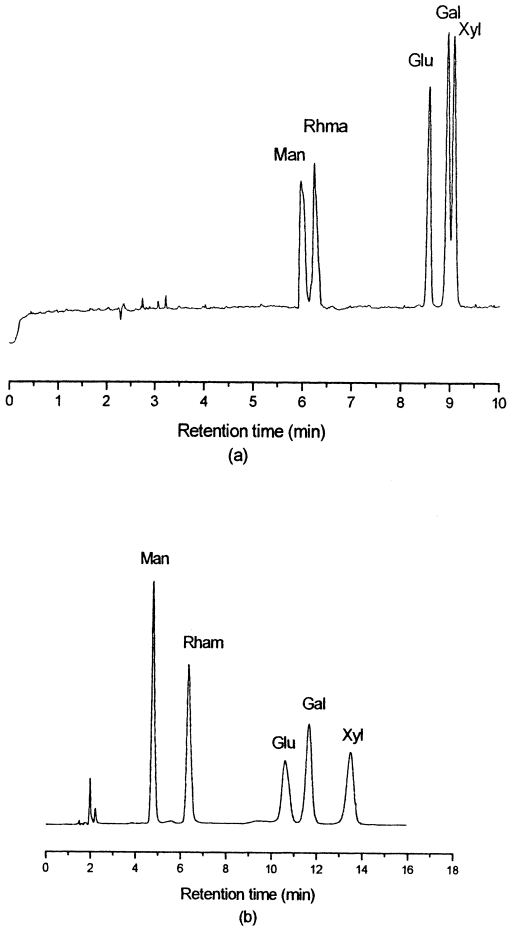

Fig. 2. Separation of the five monosaccharide derivatives by (a) MEKC and (b) HPLC. Experimental conditions: (a) Capillary: uncoated fused-silica (47 cm×50 μm I.D.); mobile phase: 25 m*M* phosphate buffer containing SDS at a concentration of 70 m*M*, pH 6.00. (b) Column: packed with ODS2 with 5 μm diameter (150 mm×4.5 mm I.D.); mobile phase: acetate buffer (0.1 mol/l, pH 5.5)–acetonitrile (78:22, v/v); flow-rate: 1 ml/min; UV detection: 245 nm; sample: 10 μl.

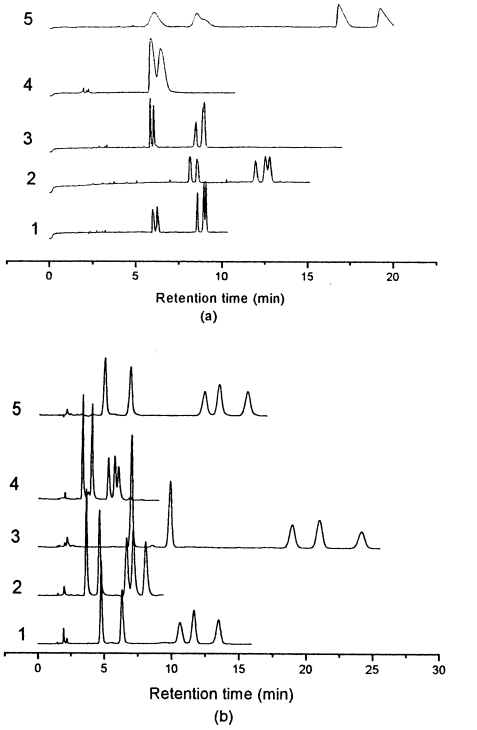

Fig. 3. The effect of the parameters of analysis condition on separation of five monosaccharide derivatives. (a) Capillary: uncoated fused silica (47 cm×50 μm I.D.). Mobile phase: 1: 25 m*M* phosphate, pH 6.0, 70 m*M* SDS; 2: 25 m*M* phosphate, pH 6.0, 80 m*M* SDS; 3: 25 m*M* phosphate, pH 6.0, 40 m*M* SDS; 4: 25 m*M* phosphate, pH 5.6, 70 m*M* SDS; 5: 50 m*M* phosphate, pH 6.0, 30 m*M* SDS. (b) Column: packed with ODS2 with 5 μm diameter (150 mm×4.5 mm I.D.). Mobile phase: 1: 100 m*M* acetate, pH 5.5, acetonitrile 22%; 2: 100 m*M* acetate, pH 7.5, acetonitrile 22%; 3: 100 m*M* acetate, pH 4.5, acetonitrile 22%; 4: 100 m*M* acetate, pH 5.5, acetonitrile 25%; 5: 100 m*M* acetate, pH 5.5, acetonitrile 18%.

can select the proper method depending on the requirement of practical applications.

3.3. Analysis of real-life samples

3.3.1. Analysis of carbohydrates in Aloe powder and its hydrolysates

Fig. 4 shows the electropherogram of carbohydrate derivatives from Aloe powder and its hydrolysates by MEKC and HPLC. The peak in trace 1 was Glu according to identification by MS. The molecular masses of Aloe polysaccharides determined with HPGPC-RI and m/z detected with MS showed that there was no oligosaccharide with M_r below 2000. This shows that the polysaccharide with high M_r in Aloe powder cannot be derived by PMP under our experimental conditions. But its hydrolysates can be analyzed with our method to investigate the composition of a polysaccharide.

The peaks of Man and Glu were observed in the chromatogram of the hydrolysates of Aloe (trace 2).

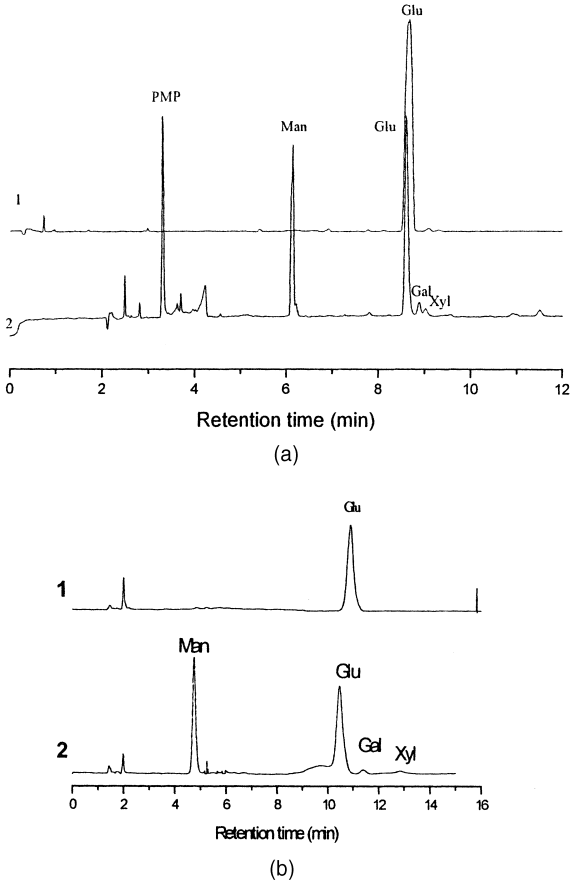

Fig. 4. Separation of PMP-labeled carbohydrates in Aloe powder by (a) MEKC and (b) HPLC. 1: Analysis of aloe powder; 2: analysis of the hydrolysate of aloe powder. Other conditions: see Fig. 2.

Minor peaks corresponding to Gal and Xyl were also seen, although they were far smaller than the major peaks.

3.3.2. Analysis of carbohydrates in food

Fig. 5 shows the MEKC electropherogram of derivative carbohydrates present in beer and candy (toffee) and the HPLC chromatogram for the same mixture. Glu, Gal, Xyl were identified in candy relative to standards of five monosaccharide derivatives. Eight peaks appeared as the derivatives of beer under the same conditions. The presence of Man, Rham, Glu, Gal, Xyl was confirmed by comparison with standards. The other peaks were oligosaccharides confirmed by MS. From the m/z of these

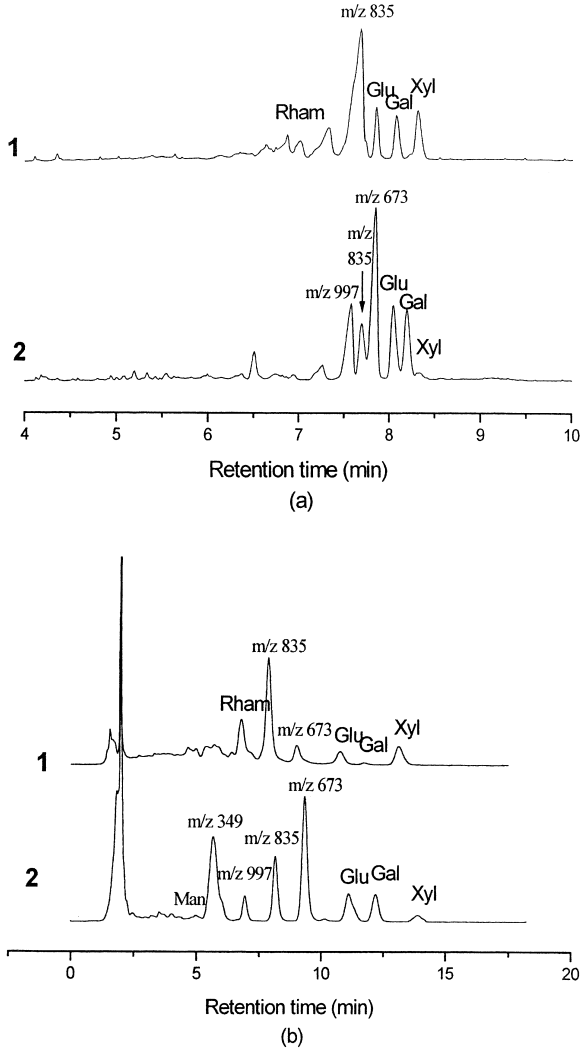

Fig. 5. Analysis of the monosaccharides in food by (a) MEKC and (b) HPLC. 1: Separation of monosaccharides in beer; 2: separation of monosaccharides in candy (toffee). Other conditions: see Fig. 2.

derivatives, we deduced they are disaccharide (m/z 673), trisaccharide (m/z 835) and tetrasaccharide (m/z 997), respectively.

4. Conclusion

The procedure of derivatization of carbohydrates by PMP is simple, rapid and reproducible, and proved to be suitable for analyses by HPLC and

MEKC. Several real samples, such as Aloe powder and its hydrolysates, candy, and beer were analyzed by both HPLC and MEKC. The methods developed in this context are quite useful for routine analysis of monosaccharides and oligosaccharides in real-life samples.

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