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Detection of carbohydrates using a pre-column derivatization reagent 1-(4-isopropyl) phenyl-3-methyl-5-pyrazolone by high-performance liquid chromatography coupled with electrospray ionization mass spectrometry

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ABSTRACT

A reagent, 1-(4-isopropyl) phenyl-3-methyl-5-pyrazolone (PPMP) has been synthesized and used for high-performance liquid chromatography/electrospray ionization mass spectrometry (HPLC/ESI-MS) determination of pre-column labeled carbohydrates. Monosaccharides have been quantitatively converted into mono-PPMP-labeled derivatives with 28% aqueous ammonia as a catalyst at 80 °C during 70 min. Mono-PPMP derivatives have been demonstrated to exhibit better chemical stability than bis-PMP ones. PPMP-labeled mixture of twelve monosaccharides (galactosamine, glucosamine, galacturonic acid, glucuronic acid, galactose, glucose/*N*-acetylgalactosamine, *N*-acetylglucosamine, xylose, arabinose, mannose, fucose, and rhamnose) has been well separated by a reverse-phase HPLC and detected by on-line ESI-MS method under optimized conditions. The data on characteristic fragment ions of the 13 PPMP-labeled monosaccharides with MS^2 data have been collected. The suggested method exhibits good linearity (correlation coefficients > 0.9975) between the peak areas and the concentration of monosaccharides in a broad concentration range and good reproducibility (RSD < 3.19%). The developed method has been successfully applied to analyze the monosaccharide composition of natural *Spirulina* polysaccharide SPPB-1.

1. Introduction

The important roles of carbohydrates in biological processes have been increasingly recognized. The qualitative and quantitative knowledges of carbohydrate distribution are essential information in glycobiology study [1,2]. A considerable amount of carbohydrates 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 and determination techniques [3].

The commonly used separation techniques for carbohydrate analysis are liquid chromatography (LC) [4–6], capillary electrophoresis (CE) [7–9], and fluorophore-assisted carbohydrate electrophoresis (FACE) [10]. Except for high pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) [11], most of the LC and CE techniques often use labeling with either fluorescence or UV tags for enhanced detection because these native carbohydrates generally have low intrinsic UV spectral activity. High-performance liquid chromatography (HPLC) is the most common form of separation, and is compatible with downstream characterization techniques such as mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy. Though nuclear magnetic resonance spectroscopy is remarkably useful for structural characterization of carbohydrates, it is unadapted to analysis of micro-quantities carbohydrates isolated from biosystems because of sufficient quantities of samples needed (mg levels). So far, biological mass spectrometry including fast atom bombardment mass spectrometry (FAB-MS) [12], matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI/TOF-MS) [13], and electrospray ionization

Abbreviations: HPLC, high-performance liquid chromatography; ESI-MS, electrospray ionization ion-trap mass spectrometry; CE, capillary electrophoresis; FACE, fluorophore-assisted carbohydrate electrophoresis; HPAEC-PAD, high pH anion-exchange chromatography with pulsed amperometric detection; NMR, nuclear magnetic resonance spectroscopy; PPMP, 1-(4-isopropyl) phenyl-3-methyl-5-pyrazolone; PMP, 1-phenyl-3-methyl-5-pyrazolone; Glc, glucose; Gal, galactose; Man, mannose; GlcN, glucosamine; GalN, galactosamine; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; GalA, galacturonic acid; GlcA, glucuronic acid; Xyl, xylose; Ara, arabinose; Fuc, fucose; Rha, rhamnose.

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mass spectrometry (ESI-MS) [12,14], has been extensively used in the characterization of sub-nanomolar amounts of material and is also under development as new methods for carbohydrates detection. Furthermore, some chemical tagging methods convert carbohydrates into their derivatives with lower MS detection levels than their native analogues [15,16]. So derivatization of samples plays a key role in analysis of carbohydrates not only with HPLC but also with MS.

For the derivatization purposes, the aldehyde group at the reducing end of carbohydrate is often labeled and a variety of methods have been reported [15–17]. They can be divided into two groups. The first group comprises some reductive amination reagents with aromatic amino-groups, some of them are typical, such as 2-aminopyridine (2-AP) [4], 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) [18], 2-aminoacridone (AMAC) [19] and 3-amino-9-ethylcarbazole (AEC) developed in our lab and so on [20], which introduce an aromatic amine-groups to the aldehyde group at the reducing end of saccharide before HPLC or CE separation. Such reagents are used widely on account of their derivatives appearing the stronger UV or fluorescence absorbance than native carbohydrates and high stability. Unfortunately, under the reductive amination conditions, the acid-labile groups such as *N*- and *O*-sulfate groups, and the sialic acid residues in carbohydrates are usually lost [21]. Condensation with an active methylene group is a quite different type of reaction, requiring no acid catalyst. The condensation with 1-phenyl-3-methyl-5-pyrazolone (PMP) is a typical example. It was first developed in 1989 by the Honda's group [22]. Condensation of an active methylene group of PMP with the reducing end of a saccharide in alkaline medium allows the pyrazolone ring with aromatic group to attach the saccharide molecule. Compared to reductive amination, PMP-labeling can be used to analyze carbohydrates with acid-labile groups and causes no desialylation and isomerization [23]. The PMP derivatives show strong absorbance and their MS signals are more intense than those of non-modified samples [23]. They can be separated by various chromatography methods and detected well even in a low concentration. Alas, despite the PMP-labeling is applied widely at present, it is also not free from disadvantages. For instance, PMP exhibits rather low hydrophobicity. Thus, tedious extractions to remove the non-reacted PMP often cause loss of a part of the sample and, this way distorts the quantitative results.

To overcome the disadvantages of PMP-labeling and improve the method, some PMP analogues such as 1-(*p*-methoxy) phenyl-3-methyl-5-pyrazolone (PMPMP) [24] and 1-(2-naphthyl)-3-methyl-5-pyrazolone (NMP) [25] have been developed subsequently. Their labeling products similar to bis-PMP-tagged derivatives are mainly obtained. In this paper, we report preparation of another analogue of PMP, 1-(4-isopropyl) phenyl-3-methyl-5-pyrazolone (PPMP) and its application in labeling monosaccharide. The UV properties of PPMP reagent, the MS characteristic fragment ions and the HPLC separation condition for the mixture of 13 PPMP-labeled monosaccharides, are investigated. To our knowledge, this is the first time that PPMP probe and its application for analysis carbohydrates has been reported. As an application, the composition analysis of monosaccharides from hydrolyzed *Spirulina* polysaccharide SPPB-1 is also performed by on-line HPLC–ESI-MS.

2. Materials and methods

2.1. Materials

4-Isopropylphenylhydrazine hydrochloride (98% purity grade) was purchased from Sigma-Aldrich Co. Ethyl acetoacetate was purchased from Xi'an Reagent Plant (Shaanxi, China). D-Mannose (Man), D-galactose (Gal), D-glucose (Glc), and D-xylose (Xyl) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). L-Rhamnose (Rha), D-fucose (Fuc), D-glucosamine (GlcN), D-galactosamine (GalN), *N*-acetyl-D-glucosamine (GlcNAc), *N*-acetyl-D-galactosamine (GalNAc), D-galacturonic acid (GalA), and D-glucuronic acid (GlcA) were purchased from Sigma Co., D-arabinose (Ara) was purchased from FLUKA Co. Polygram silica gel UV254 plate was purchased from MACHEREY-NAGEL Co. (Germany). Sodium hydroxide, trifluoroacetic acid (TFA), glacial acetic acid, petroleum ether, dichloromethane and ammonia were all analytical-grade and obtained from Tianjin Chemical Agent Company. Acetonitrile (HPLC-grade) was obtained from Fisher Scientific.

2.2. Synthesis of 1-(4-isopropyl) phenyl-3-methyl-5-pyrazolone (PPMP)

4-Isopropylphenylhydrazine was prepared by neutralizing its hydrochloride with NaOH. Thus, 17 mL of 5.0% (w/w) NaOH aqueous solution was slowly added to 4-isopropyl-phenylhydrazine hydrochloride (0.02 mol, 3.734 g). After vigorous stirring during 5 h at ambient temperature, the reaction mixture was neutralized to pH 8–9 with 1.0 mol L^{-1} aqueous HCl, and was then subjected to exhaustive extractions with dichloromethane until no obvious fluorescence of the aqueous phase probe was observed under an UV-lamp. The crude solid 4-isopropylphenylhydrazine (2.90 g, 0.0192 mol, and yields 96.76%) was obtained by evaporating of all the dichloromethane solution to dryness.To 4-isopropylphenylhydrazine prepared in above way, 2.5 mL of ethyl acetoacetate and 2.5 mL of glacial acetic acid as a catalyst were added dropwise in a 100 mL round-bottom flask under vigorous stirring. After stirring at 30 °C for *ca.* 6 h, the excess of glacial acetic acid was removed on a rotary evaporator under reduced pressure. The mixture was subjected to column chromatography (silica gel 200–300 meshes, ⌀55 mm × 170 mm column; eluent: the mixture of petroleum ether:ethyl acetate 4:1). Fraction probes were examined by ESI-MS. The fraction that exhibited peak at *m*/*z* 217 $[M+H]^+$ in full scan MS spectra was collected. Removal of the solvents under reduced pressure gave 3.09 g (73.9%) of the target compound as an opalescent yellow powder. Purity: 99.2% (HPLC). M.p. 112–114 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ, ppm: 1.24 (d, 6H, ^3J = 7.02 Hz, $CH(CH_3)_2$), 2.19 (s, 3H, 5-CH_3), 2.91 (sept, 1 H, ^3J = 7.02 Hz, $CH(CH_3)_2$), 3.45 (s, 2 H, CH_2 in pyrazolone), 7.25, 7.40 (AA′XX′, 4 H, CH in arene); $^{13}C\{^1H\}$ NMR (100 MHz, $CDCl_3$) δ, ppm: 16.95 ($CH(CH_3)_2$), 23.90 (5-CH_3), 33.57 (CH_2), 42.93 ($CH(CH_3)_2$), 119.09 (N=C<), 126.65, 135.60 (CH in Ph), 145.77, 156.06 (C in Ph), 170.37 (>C=O); ESI-MS detection in positive-ion mode: *m*/*z* 217.17 $[M+H]^+$.

2.3. Preparation of standard solutions

PPMP solution (0.1 mol L^{-1}) was prepared by dissolving 216 mg of PPMP in 10 mL HPLC-grade methanol. Standard solutions of each monosaccharide (0.1 mol L^{-1}) were prepared in Milli-Q water. The working solutions of monosaccharide (0.01 mol L^{-1}) were prepared by diluting the corresponding standard solutions with Milli-Q water. All the solutions were stored at 4 °C.

2.4. Hydrolysis of polysaccharide SPPB-1 from Spirulina platensis

To a 5 mL screw-capped glass tube containing 5.0 mg SPPB-1 polysaccharide from *Spirulina platensis*, trifluoroacetic acid (TFA) (2.0 mol L^{-1}, 2.0 mL) was added, the tube was shaken briefly, sealed, and heated with stirring at 121 °C in an oil bath for 2 h. After cooling down to room temperature, the hydrolyzate was divided into five equal parts and dried in a stream of N_2 to remove the excess of TFA.

Table 1
Maximum absorption and corresponding absorbance (A) of PPMP and PMP.

Solvent	Spectral maximum λ (nm)		Spectral maximum λ (nm)	
	PPMP (2.0×10^{-5} mol L^{-1})		PMP (2.0×10^{-5} mol L^{-1})	
	A	ε ($\times 10^4$)	*A*	ε ($\times 10^4$)
Acetonitrile	0.3620 (204 nm)	1.81	0.3410 (205 nm)	1.71
	0.3756 (248 nm)	1.88	0.3417 (245 nm)	1.71
Methanol	0.3519 (207 nm)	1.76	0.3061 (207 nm)	1.53
	0.3160 (248 nm)	1.58	0.2761 (246 nm)	1.38
Ethanol	0.4075 (207 nm)	2.04	0.4056 (207 nm)	2.03
	0.3395 (248 nm)	1.70	0.2871 (247 nm)	1.44

The residue was labeled with PPMP as described in derivatization procedure.

2.5. Derivatization procedure (standard procedure)

The derivatization (condensation) reactions were carried out in aqueous methanol solution in an alkaline medium. Thus, to a 5 mL vial containing solution of PPMP in methanol (0.1 mol L^{-1}, 30 μL), aqueous solutions of a monosaccharide (0.01 mol L^{-1}, 50 μL) and of ammonia (28%, 30 μL) were added consecutively. The vial was sealed; the content was mixed well by shaking and heated at 80 °C for 70 min (water bath). After reaction completed, the mixture was concentrated till dryness in a stream of N_2 to remove the excess of ammonia. The residue was taken into 1 mL of Milli-Q water and extracted with dichloromethane (3 × 1 mL). The aqueous layer was subjected to HPLC and ESI-MS analyses.

2.6. HPLC and ESI-MS conditions

The HPLC analysis was performed on the Finnigan Surveyor system composed of a MS pump plus, an autosampler plus, a Photo Diode Array Spectrophotometer (PDA) detector plus and an Xicabular workstation. Separation was achieved using Waters Symmetry Shield RP18 column (4.6 mm *i.d.* × 250 mm, 5 μm); with acetonitrile (solvent A) and 0.01 mol L^{-1} ammonium acetate aqueous solution (pH 4.5, solvent B) as a mobile phase (linear gradient elution: solvent A from 15% to 22% at 0–35 min, then 22% for 10 min; flow rate was maintained at 800 μL min^{-1}; column temperature was 37 °C; PDA detection wavelength was 245 nm; injection volume was 10 μL).

ESI-MS data were acquired on the Thermo Scientific LTQXL ion-trap mass spectrometer (positive-ion mode, sheath gas flow rate 40.0 arb, auxillary gas flow rate 5.0 arb, spray voltage 5 kV, the heated capillary temperature 300 °C, capillary voltage 48 V, tube lens voltage 80 V, collision energies in MS^2 experiments 20–30%).

When HPLC and ESI-MS were combined, eluate from the column was introduced to ESI source at a post-column split ratio of 1:8 with a T-type valve.

2.7. Investigation of stability of derivative

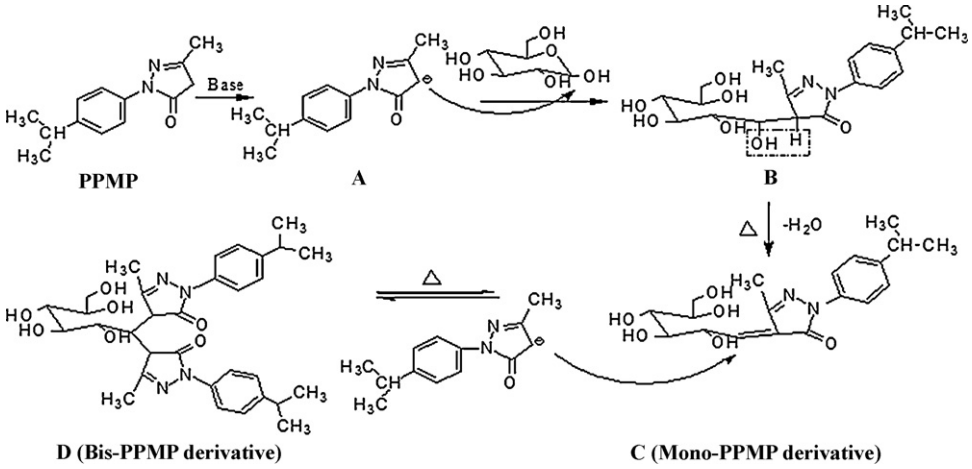
The stability of PPMP derivatives was evaluated by analysis 0.50 mmol L^{-1} of PPMP-glucose derivative in PPMP-free aqueous solution after dichloromethane extraction. The stability analysis was carried out at intervals of time (1, 3, 5, and 7 days).

2.8. Method validation

This HPLC method was validated in terms of linearity, detection limit, precision, and recovery. For these tests, nine standard monosaccharides (GalN, GlcN, Gal, Glc, Ara, Xyl, Man, Fuc, and Rha) were chosen.

The linearity of the method was verified as follows. 100 μL of the working standard solution of monosaccharides included of 0, 0.02, 0.05, 0.10, 0.20, 0.50, and 1.00 μmol each monosaccharide added to the reaction mixture containing of PPMP methanol solution (0.1 mol L^{-1}, 540 μL), and ammonia (28%, 540 μL). After derivatization, the final concentrations ranged from 0.02 to 1.00 mmol L^{-1} of monosaccharide derivatives (corresponding to the same concentration native monosaccharide) solution were obtained and analyzed by HPLC. Calibration curves were obtained by plotting the mean chromatographic peak area (from 5 repeated injections) of monosaccharide derivatives against the monosaccharide concentration.

Limits of detection (LODs) were determined by 0.02 mmol L^{-1} monosaccharide derivatives giving a signal-to-noise (*S*/*N*) ratio of 3.



Scheme 1. Derivatization reaction of PPMP and glucose in an alkaline medium.

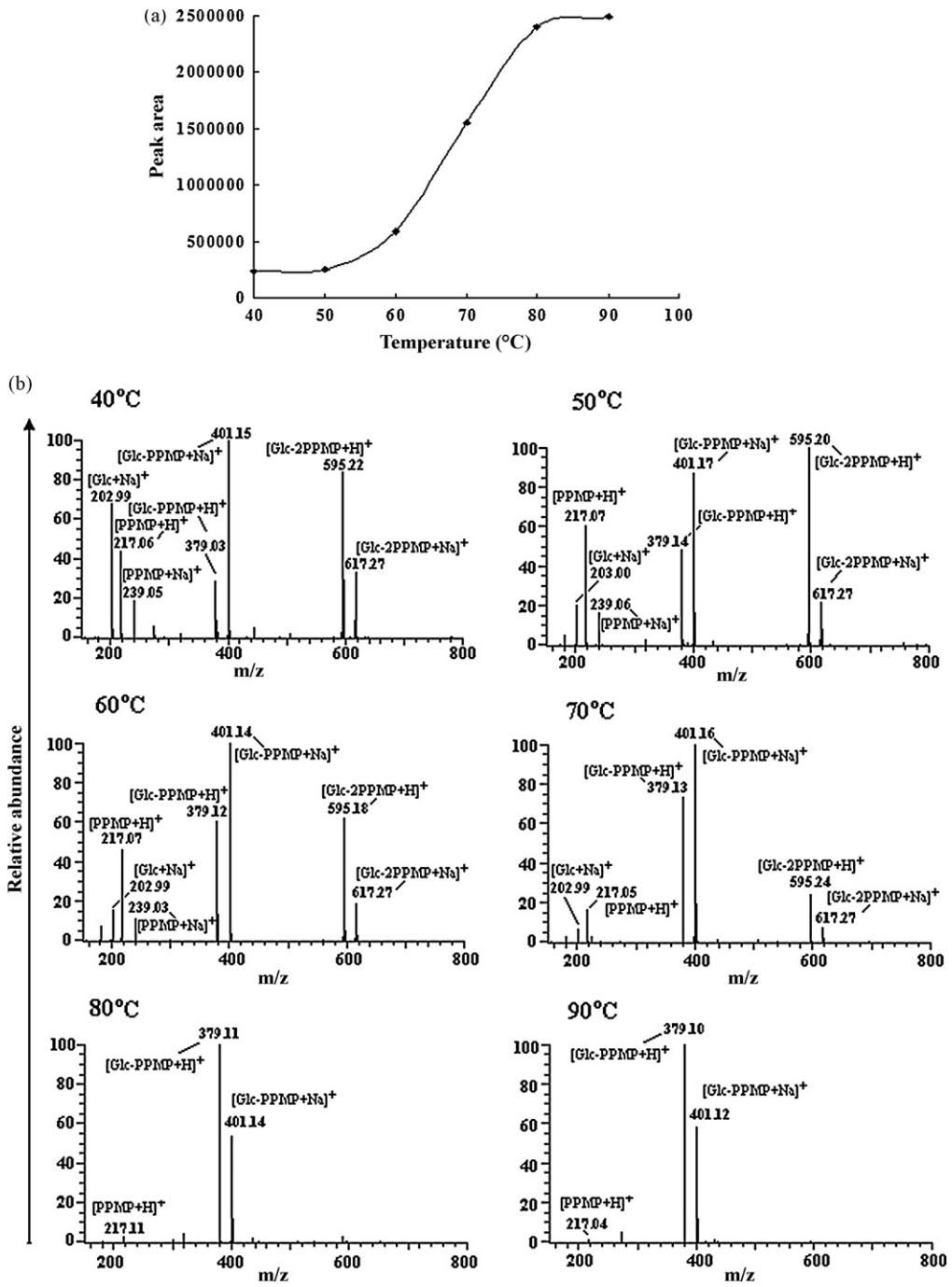


Fig. 1. (a) Effect of reaction temperature on derivatization of glucose. (b) Mass spectra of PPMP-labeled glucose derivatives from different reaction temperatures. The reaction time was 80 min; the molar ratio of PPMP to Glc was 7:1.

The precision and reproducibility of the method were estimated by 5 repeated injections of a sample (for each of nine PPMP-labeled monosaccharides) to give a relative standard deviation (R.S.D.), then to obtain a mean R.S.D. of different concentration (0.05, 0.10, 0.20, 0.50, and 1.00 mmol L^{-1}).

The recovery experiments were performed in order to evaluate the accuracy of the method. The analytical procedure is as follows: known amounts (20, 50, 100 nmol) of nine monosaccharides were, respectively, added into the hydrolyzates of 1.0 mg *Spirulina* polysaccharide SPPB-1 prepared accordingly to what described in Section 2.4, and the resulting spiked samples were subjected to derivatization with PPMP, the prepared probes were then analyzed by HPLC–ESI-MS technique under the same conditions mentioned in Section 2.6. 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 repeated three times.

3. Results and discussion

3.1. Ultraviolet absorption of PPMP

PPMP possesses a phenyl functional group that provides an excellent opportunity for UV detection. To determine λ_{max}, absorbance (A) and molar absorption coefficients (ε) of PPMP, its 2.0×10^{-5} mol L^{-1} solutions in each of employed solvents (ace-

tonitrile, methanol, and ethanol) were prepared. The ultraviolet absorption of PPMP was investigated in three solvent systems (see Table 1). As observed, the UV absorption maximum of PPMP in acetonitrile is at 248 nm, its molar absorption coefficient (ε) is 1.88×10^4 L mol^{-1} cm^{-1}. In methanol and ethanol solvents, the UV absorption maxima of PPMP is at 207 nm, its molar absorption coefficients (ε) are 1.76×10^4 and 2.04×10^4 L mol^{-1} cm^{-1}, respectively. For comparison purposes, analogous data for PMP are also included in Table 1. As it is clear from Table 1, in all cases the UV absorption for PPMP is stronger than that of PMP. This is probably due to the fact that the isopropyl group existing in PPMP affects on the microenvironment of phenyl in the molecule, which alters its UV absorption.

3.2. Structure of derivatives

The derivatization mechanism of PPMP and glucose in an alkaline medium is shown in Scheme 1. In fact, it is a chronic condensation between a monosaccharide in its aldo-form (carbonyl component) and PPMP (methylene component) followed by a reversible 1,4-Michael addition of the second PPMP molecule to the condensation product C. Increase of temperature favors the shift of the equilibrium towards the C side. Thus, temperature control of the derivatization step is of top importance.

3.3. Optimization of derivatization conditions

3.3.1. Effect of a catalyst on derivatization reaction

Sodium hydroxide and ammonia are typical catalysts for the derivatization of carbohydrates in alkaline medium. If sodium hydroxide is applied, upon completeness of the reaction, the mixture is to be neutralized down to pH 7.0 with, say, 0.1 mol L^{-1} HCl solution that leads to the presence of considerable amounts of inorganic salts in the resultant solution. Presence of these salts, as a rule, results in a significant decrease in the sensitivity of the MS-based measurement methods. Application of ammonia allows eliminating this problem (it can be removed by evaporating the solution to dryness in a vacuum), and the sample solution can be directly analyzed using ESI-MS without desalting. In practice, 28% aqueous ammonia has been proved to be the optimal choice.

3.3.2. Effect of temperature on derivatization reaction

As it is mentioned above, temperature has an ultimate bearing upon derivatization reaction course. To investigate this effect in more detail, we performed a series of test reactions, with glucose chosen as a model saccharide. The result about the effect of reaction temperature on the peak area (proportional to the yield of the derivative) is presented in Fig. 1a. The yield of labeled glucose increases with the increase of the temperature within a range from 40 to 80 °C. Performing the reaction at higher temperatures gives only little effect. Parallel to it, the labeled glucose probes were analyzed by ESI-MS method (see Fig. 1b). Some characteristic peaks for mono-PPMP-Glc derivative (m/z 379 [Glc-PPMP+H]$^+$, 401 [Glc-PPMP+Na]$^+$), bis-PPMP-Glc derivative (m/z 595 [Glc-2PPMP+H]$^+$, 617 [Glc-2PPMP+Na]$^+$), and glucose (m/z 203 [Glc+Na]$^+$) were observed. The relative ratio of the intensities of these peaks, however, varied considerably dependently upon the temperature of the probe preparation. At 40, 50, 60, and 70 °C, all of the listed above peaks were present. Only starting from 80 °C the peaks related to bis-PPMP-Glc derivative almost vanished. These facts agree with the shift of the equilibrium depicted in Scheme 1 towards the compound C side at temperatures above 80 °C.

Mono-PPMP derivatives appear to have high hydrophobicity and chemical stability that favors the effectiveness of the HPLC analysis. Therefore, performing of the labeling routine at 80 °C should be recommended.

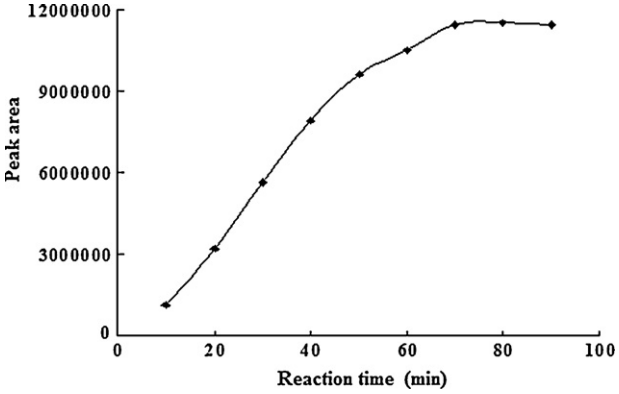


Fig. 2. Effect of reaction time on derivatization of glucose. The reaction temperature was 80 °C; the molar ratio of PPMP to Glc was 7:1.

3.3.3. Effect of reaction time on derivatization reaction

The effect of reaction time on derivatization was also investigated with glucose chosen as a model saccharide. The results are summarized in Fig. 2, which indicates that the labeling reaction completes in 70 min.

3.3.4. Effect of the amount of PPMP on derivatization reaction

The excess of derivatization reagent is, as a rule, applied to increasing the yield of the desired labeled products. Unfortunately, using of great excess of the reagent leads to certain complications that concern further removal of its non-reacted part from the probe (for instance, by means of exhaustive extractions). To define the optimal excess of PPMP, a series of derivatization experiments with different molar ratios of PPMP to Glc were carried out. The results are presented in Fig. 3. The yield of the reaction is very low at a PPMP to Glc molar ratio 1:1 and dramatically increases with the increase of the PPMP excess up to 2. Then the growth of the peak area (i.e., of the yield) slows down, and the graph reaches the "saturation" state at the molar ratios 6:1. In present work, a 6–7-fold excess of PPMP is chosen. In practice, possibly, the excess of PPMP could be decreased down to, say, 3–4-fold one. Nevertheless, in this case, some systematic corrections to the analysis data resulting from "non-perfect" completeness of the reaction should be introduced.

3.4. Stability of PPMP- sugar derivative

The experiment results of Section 2.7 indicated that a relative standard deviations (R.S.D.) 2.06% (for peak areas) was obtained by analysis of 0.5 mmol L^{-1} PPMP-glucose derivative in PPMP-free aqueous solution after dichloromethane extraction at intervals of

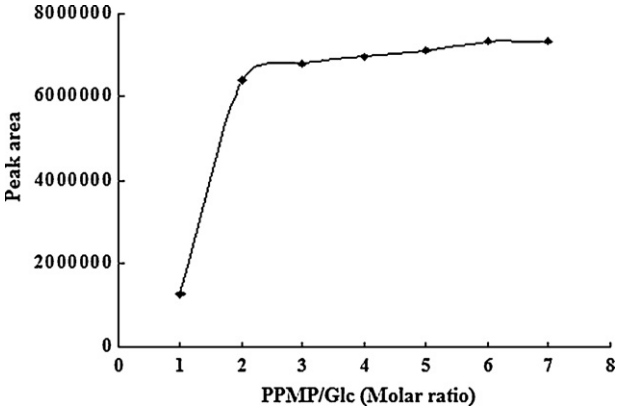


Fig. 3. Effect of the molar ratio of PPMP to Glc on derivatization of glucose. The reaction temperature was 80 °C; the reaction time was 70 min.

time (1, 3, 5, 7 days) within a week at 4 °C, which shows PPMP-sugar derivative with good chemical stability.

3.5. HPLC and MS analysis of 13 PPMP-labeled standard monosaccharides

3.5.1. HPLC separation

The HPLC separation conditions were adjusted and tested for a mixture of 13 PPMP-labeled monosaccharides (GalN, GlcN, GalA, GlcA, Gal, Glc, GalNAc, GlcNAc, Xyl, Ara, Man, Fuc and Rha). The elution system composed of acetonitrile (solvent A) and 0.01 mol L^{-1} aqueous ammonium acetate (solvent B) was used as the mobile phase. The influences of the pH of ammonium acetate aqueous solution and the content of acetonitrile in the eluent on the separation quality were additionally investigated. Eleven PPMP-sugar derivatives among 13 present PPMP-labeled monosaccharides were separated successfully in the order of GalN, GlcN, GalA, GlcA, Gal, GlcNAc, Ara, Xyl, Man, Fuc and Rha using a gradient program described in Section 2.6. The peaks related to glucose (F) and *N*-acetylgalactosamine (F′) overlapped. So, in this study, the good separation of the 11 monosaccharide derivatives was achieved within 40 min (see Fig. 4a).

3.5.2. ESI-MS/MS^2 characterization

All PPMP-labeled monosaccharides of question were characterized by ESI-MS method (positive-ion mode, see Fig. 4b). The numerical data are collected in Table 2. Analysis of these figures indicates that some fragments (such as *m*/*z*: 283, 259 (258), 229 and 217) are characteristic for all 13 PPMP-labeled monosaccharides studied, and presence of these peaks can be used as a criterion to differ reducing sugars from other aldehyde-type material in their PPMP-derivative form.

The data summarized in Table 2 along with the relative abundances of the peaks (see Fig. 4b) allow suggesting the fragmentation mode for PPMP-labeled monosaccharides. Thus, all the observed fragments can be subdivided into two groups. The first group comprises low abundance peaks correspondent to dehydration, deamination and deamidation fragment ions such as $[M\text{-}H_2O+H]^+$, $[M\text{-}2H_2O+H]^+$, and $[M\text{-}3H_2O+H]^+$ (occur in any monosaccharide), $[M\text{-}NH_3+H]^+$, $[M\text{-}NH_3\text{-}H_2O+H]^+$, and $[M\text{-}NH_3\text{-}2H_2O+H]^+$ typical for hexosamines (GlcN, GalN), and $[M\text{-}CH_3CONH_2+H]^+$ characteristic for *N*-acetylhexosamine (GlcNAc, GalNAc) (evidently, *m*/*z* values are dependant upon a molecular weight of a monosaccharide). The second group comprises high abundance peaks correspondent to characteristic C–C bond cleavage of the carbohydrate skeleton. These are peaks with *m*/*z* 259, 258 (hexosamine) and 300 (*N*-acetylhexosamine) related to the cleavage of the C2–C3 bond, peaks with *m*/*z* 283 related to the cleavage of the C4–C5 bond followed by a loss of a water or ammonia molecule, peak with *m*/*z* 313 related to the cleavage of the C5–C6 bond followed by loss of H_2O or CH_3CONH_2 (except for deoxyhexoses), and the weaker peaks with *m*/*z* 229 correspondent to the cleavage of C1–C2 bond (for more details, see Table 2).

In corresponding MS^2 spectra, the fragment peaks with *m*/*z* 258 [cleavage of C2–C3 in hexosamines (GlcN, GalN)] and *m*/*z* 300 [cleavage of C2–C3 in *N*-acetylhexosamine (GlcNAc, GalNAc)] exhibit the topmost abundance and can be used as indicators for the presence of hexosamines and *N*-acetylhexosamine. The most intense MS^2 peak with *m*/*z* 326.9 (loss of two water molecules) is indicative of deoxyhexoses Fuc and Rha. Other types of monosaccharides can also be identified by their fragmentation analysis.

3.5.3. HPLC–MS/MS^2 analysis

As it is clear from Fig. 4a in HPLC chromatogram, the peaks related to Glc and GalNAc overlap in the mixture of the 13 PPMP-labeled monosaccharides, so Glc and GalNAc cannot be simultaneously analyzed by HPLC. However, the technique of on-line HPLC combined MS can identify them. When HPLC and MS are combined on-line, the total ion current chromatogram (TIC) of the molecular ion peaks (see Fig. 4b(I)) and MS/MS^2 spectra (see Fig. 4b(II)) of the separated PPMP-labeled monosaccharides are acquired simultaneously. The MS/MS^2 spectra of the overlapping peaks (F and F′) in Fig. 4b(I) are different. If the mass spectrum for peak F or F′ exhibits molecular ion peak at *m*/*z* 379.00 in full scan MS spectra along with its fragment ions at *m*/*z* 360.92, 342.94, 324.94, 312.97, and 259.09 in MS^2 spectra, the peak represents only PPMP-labeled Glc (named peak F); alternatively, the molecular ion peak at *m*/*z* 419.97 and fragmentation peaks at *m*/*z* 401.92, 365.96, 299.98, 295.06, and 258.13 are indicative of the PPMP-labeled GalNAc (named peak F′). Superposition of peaks at *m*/*z* 379.00 and 419.97 and/or their corresponding MS^2 fragment ions, points out the presence of both Glc and GalNAc. When Glc and GalNAc do not coexist in a sample, any of them can be separated from other eleven monosaccharides. So, in practice, twelve PPMP-labeled monosaccharides were separated and 13 PPMP-labeled monosaccharides were successfully identified under conditions as described in Section 2.6 by means of HPLC–MS/MS^2 combination technique.

3.6. Comparison of PPMP vs. PMP-labeling method

Application of PPMP instead of PMP provides a set of certain advantages. First of all, on derivatization, excess of PPMP can be completely eliminated from the sample by triple extraction with dichloromethane, while non-reacted PMP is hardly removable from the probe due to its low hydrophobicity. Under optimized conditions (80 °C, 70 min), mono-PPMP derivatives can be selectively obtained and demonstrate better chemical stability (without PPMP lost) comparatively to their bis-PMP counterparts. The comparison of PPMP vs. PMP-labeling method is summarized in Table 3.

3.7. Result of the method validation

According to Section 2.8, the linearity, LOD and precision results of the statistical analysis of the method are provided in Table 4. As seen from Table 4, the linear dependences between peak areas and the concentration (correlation coefficients >0.9975) for nine monosaccharides are proved for rather broad concentration ranges. The LODs ranging from 9.72 to 154.0 pmol at a signal-to-noise ratio of 3 were also obtained for nine monosaccharides studied, which indicates a satisfactory sensitivity of the method. The R.S.D. ranging from 0.39% to 3.19% for reproducibility is indicative of good precision of the method. The results of recoveries analysis are listed in Table 5. As observed from Table 5, the average recoveries of all the

Fig. 4. (a) The HPLC chromatogram of 13 PPMP-labeled monosaccharides. (A) PPMP-labeled GalN, (B) PPMP-labeled GlcN, (C) PPMP-labeled GalA, (D) PPMP-labeled GlcA, (E) PPMP-labeled Gal, (F) PPMP-labeled Glc, (F′) PPMP-labeled GalNAc, (G) PPMP-labeled GlcNAc, (H) PPMP-labeled Ara, (I) PPMP-labeled Xyl, (J) PPMP-labeled Man, (K) PPMP-labeled Fuc, (L) PPMP-labeled Rha, *Impurity peak. Column: Waters Symmetry Shield RP18 (4.6 mm *i.d.* × 250 mm, 5 μm); the gradient program of elution: acetonitrile from 15% to 22% for 35 min, then 22% for 10 min with ammonium acetate of 0.01 mol L^{-1} aqueous solution for pH 4.5; flow rate 800 μL min^{-1}; column temperature 37 °C. (b) Total ion current chromatogram (TIC) of the molecular ion peaks (I) and MS and MS^2 spectra (II) of 13 PPMP-labeled monosaccharides. Molecular ions refer to $[M+H]^+$. The denotations of (A) to (L) and HPLC condition are the same as described in (a). *Impurity. ESI-MS condition: positive-ion mode, sheath gas flow rate 40.0 arb, auxillary gas flow rate 5.0 arb, spray voltage 5 kV, the heated capillary temperature 300 °C, capillary voltage 48 V, tube lens voltage 80 V, collision energies in MS^2 experiments 20–30%, eluate from the column was introduced to ESI source at a post-column split ratio of 1:8 with a T-type valve.

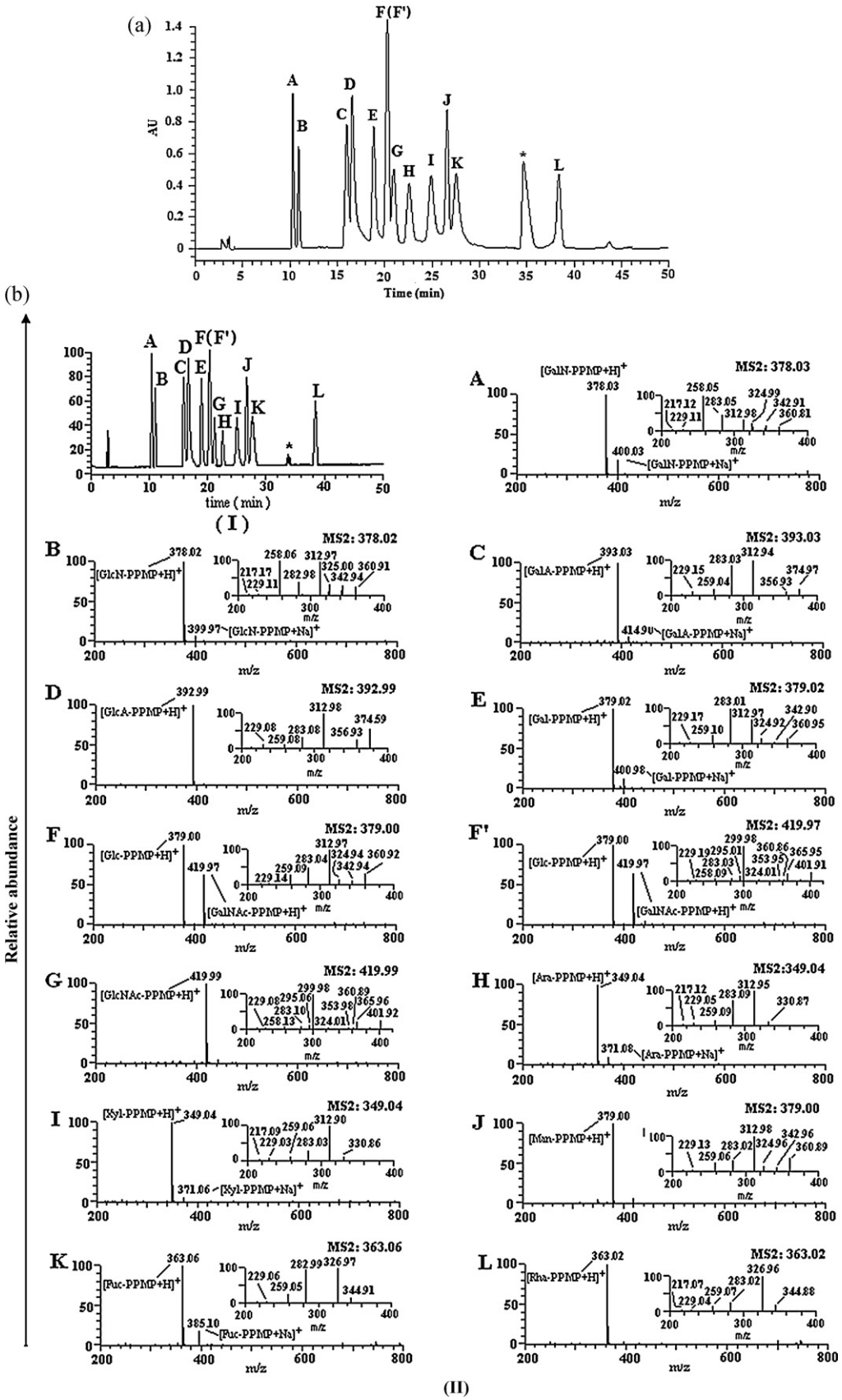

(a)
AU
Time (min)
A
B
C
D
E
F(F')
G
H
I
J
K
L
*
(b)
time (min)
(I)
Relative abundance
m/z
(II)
A [GalN-PPMP+H]+ 378.03 MS2: 378.03 [GalN-PPMP+Na]+ 400.03
B [GlcN-PPMP+H]+ 378.02 MS2: 378.02 [GlcN-PPMP+Na]+ 399.97
C [GalA-PPMP+H]+ 393.03 MS2: 393.03 [GalA-PPMP+Na]+ 414.90
D [GlcA-PPMP+H]+ 392.99 MS2: 392.99
E [Gal-PPMP+H]+ 379.02 MS2: 379.02 [Gal-PPMP+Na]+ 400.98
F [Glc-PPMP+H]+ 379.00 MS2: 379.00 [GalNAc-PPMP+H]+ 419.97
F' [Glc-PPMP+H]+ 379.00 MS2: 419.97 [GalNAc-PPMP+H]+ 419.97
G [GlcNAc-PPMP+H]+ 419.99 MS2: 419.99
H [Ara-PPMP+H]+ 349.04 MS2:349.04 [Ara-PPMP+Na]+ 371.08
I [Xyl-PPMP+H]+ 349.04 MS2: 349.04 [Xyl-PPMP+Na]+ 371.06
J [Man-PPMP+H]+ 379.00 MS2: 379.00
K [Fuc-PPMP+H]+ 363.06 MS2: 363.06 [Fuc-PPMP+Na]+ 385.10
L [Rha-PPMP+H]+ 363.02 MS2: 363.02

Table 2
Characteristic ions of MS/MS^2 for PPMP-labeled monosaccharides (m/z).

Monosaccharides	Molecular ion peak $[M+H]^+$	Characteristic fragment ions of MS^2: $[M+H]^+$								
		Loss of one H_2O	Loss of two H_2O	Loss of three H_2O	Loss of one CH_3CONH_2	Bond C5–C6 cleavage and loss of two H_2O	Bond C4–C5 cleavage and loss of two H_2O)	Bond C2–C3 cleavage (Loss of four CH_2O)	Bond C1–C2 cleavage	$[PPMP+H]^+$
Glc	379.00	360.92	342.94	324.94	–	312.97	283.04	259.09	229.14	217.16
Gal	379.02	360.95	342.90	324.92	–	312.97	283.01	259.10	229.17	217.16
Man	379.00	360.89	342.96	324.96	–	312.98	283.02	259.06	229.13	217.11
GlcN	378.02	360.91[a]	342.94[b]	325.00[c]	–	312.97[d]	282.98[e]	258.06	229.11	217.17
GalN	378.03	360.81[a]	342.91[b]	324.99[c]	–	312.98[d]	283.05[e]	258.05	229.15	217.12
GlcNAc	419.99	401.92	383.93	365.96	360.89	353.98[f], 313.0[g], 295.06[h]	341.96[i], 324.01[j], 283.10[k]	299.98, 258.13[l]	229.08	217.07
GalNAc	419.97	401.91	383.89	365.95	360.86	353.95[f], 312.9[g], 295.01[h]	341.95[i], 324.01[j], 283.03[k]	299.98, 258.09[l]	229.19	217.04
GlcA	392.99	374.59	356.93	–	–	312.98	283.08	259.08	229.08	217.17
GalA	393.03	374.97	356.93	–	–	312.94	283.03	259.04	229.15	217.07
Xyl	349.04	330.86	312.90	–	–	–	283.03	259.06	229.03	217.09
Ara	349.04	330.87	312.95	–	–	–	283.09	259.09	229.05	217.12
Fuc	363.06	344.91	326.97	–	–	–	282.99	259.05	229.06	217.12
Rha	363.02	344.88	326.96	–	–	–	283.02	259.07	229.04	217.07

–: No detected; M represent molecular weight of PPMP-labeled monosaccharides.

[a] $[M-NH_3+H]^+$.
[b] $[M-NH_3-H_2O+H]^+$.
[c] $[M-NH_3-2H_2O+H]^+$.
[d] $[M-NH_3-H_2O-CH_2O+H]^+$.
[e] $[M-NH_3-H_2O-2CH_2O+H]^+$.
[f] $[M-2H_2O-CH_2O+H]^+$.
[g] $[M-H_2O-CH_3CONH_2-CH_2O+H]^+$.
[h] $[M-2H_2O-CH_3CONH_2-CH_2O+H]^+$.
[i] $[M-H_2O-2CH_2O+H]^+$.
[j] $[M-2H_2O-2CH_2O+H]^+$.
[k] $[M-H_2O-CH_3CONH_2-2CH_2O+H]^+$.
[l] $[M-4CH_2O-CH_2CO+H]^+$.

Table 3
The comparison of PPMP vs. PMP-labeling carbohydrates methods.

Item	PPMP method	PMP [23,26] method
Derivatization temperature	80 °C	70 °C
Derivatization time	70 min	30 min
About excess reagent by dichloromethane extraction	Be easily removed completely by dichloromethane extraction three times	Cannot be removed completely by dichloromethane extraction three times
Structure of derivatives	Mono-PPMP-labeled	Bis-PMP-labeled
Stability of derivatives in reagent-free aqueous solution	Very stable	Easily lose of one PMP
The UV sensitivity of derivatives	Low	High
The MS sensitivity of derivatives in contrast with native saccharides	High	High
The number of monosaccharides separated at most in one single chromatographic run by reverse-phase HPLC	12 derivatives of monosaccharides including GalN, GlcN, GalA, GlcA, Gal, Glc/GalNAc, GlcNAc, Xyl, Ara, Man, Fuc and Rha	10 derivatives of monosaccharides including Man, Rib, Rha, GlcA, GalA, Glc, Xyl, Gal, Ara and Fuc [26]

Table 4
Linearity, limits of detection (LOD) and reproducibility of nine PPMP-labeled monosaccharides ($n = 5$).

Saccharides	Regression equation[a]	Correlation coefficient (r)	Linear range (mmol L^{-1})	Reproducibility RSD (%)	LOD (pmol, S/N = 3)
GalN	$Y = 8.09E5X + 6.01E4$	0.9999	0.02–1.00	0.39	14.86
GlcN	$Y = 6.65E6X + 2.06E5$	0.9999	0.02–1.00	0.49	16.67
Gal	$Y = 1.23E7X$-5.57E4	0.9991	0.02–0.50	0.64	29.50
Glc	$Y = 1.40E7X + 5.78E4$	0.9999	0.02–0.50	0.58	29.54
Xyl	$Y = 5.44E6X + 4.13E4$	0.9994	0.05–1.00	1.73	82.08
Ara	$Y = 3.01E6X + 1.12E4$	0.9986	0.10–1.00	3.19	117.7
Man	$Y = 1.01E7X + 2.65E5$	0.9975	0.05–1.00	1.10	9.72
Fuc	$Y = 8.47E6X + 1.96E5$	0.9995	0.05–1.00	0.73	154.0
Rha	$Y = 1.67E7X + 2.84E5$	0.9995	0.05–1.00	0.59	29.58

[a] Y denotes peak area; X denotes concentration of standard monosaccharides solution.

Table 5
Recovery analysis of nine PPMP-labeled monosaccharides in the sample of *Spirulina* polysaccharide SPPB-1 ($n = 3$).

Monosaccharides	Content in sample (nmol mg^{-1})	Spiked amount (nmol mg^{-1})	Found amount (nmol mg^{-1})	Recovery (%)	Mean recovery (%)
GalN	10.8	20	31.2	102.0	99.8
		50	60.5	99.4	
		100	108.7	97.9	
GlcN	79.7	20	98.9	96.0	101.8
		50	134.1	108.8	
		100	180.2	100.5	
Gal	24.4	20	43.2	94.0	95.2
		50	72.5	96.2	
		100	119.9	95.5	
Glc	538.0	20	557.5	97.5	98.3
		50	582.2	88.4	
		100	646.9	108.9	
Xyl	46.6	20	65.3	93.5	90.9
		50	92.9	92.6	
		100	133.3	86.7	
Ara	26.2	20	45.5	96.5	96.2
		50	75.1	97.8	
		100	120.4	94.2	
Man	153.6	20	170.8	86.0	90.4
		50	197.5	87.8	
		100	251.0	97.4	
Fuc	198.9	20	217.2	91.5	92.1
		50	242.1	86.4	
		100	297.4	98.5	
Rha	1190.5	20	1108.5	90.0	90.7
		50	1234.3	87.6	
		100	1285.0	94.5	

nine monosaccharides range from 90.04% to 101.8%. This accuracy is within the acceptable ranges.

3.8. Analysis of polysaccharide SPPB-1 composition from *Spirulina platensis*

The validity of the suggested method was tested on a real sample, polysaccharide SPPB-1 from *Spirulina platensis*. The homogenous polysaccharide fraction SPPB-1 was prepared as described in Ref. [27]. The hydrolyzate of polysaccharide SPPB-1 (prepared as described in Section 2.4) was subjected to PPMP chemical derivatization, HPLC separation, and on-line ESI-MS identification. The chromatogram of monosaccharide derivatives in *Spirulina* polysaccharide SPPB-1 is depicted in Fig. 5 (the total ion current chromatogram is not shown). The results indicate that *Spirulina* polysaccharide SPPB-1 is composed of Rha, GlcN, GlcA, Glc,

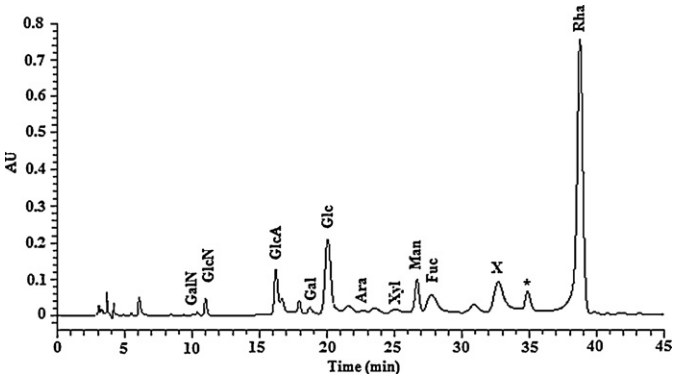


Fig. 5. HPLC chromatogram of PPMP-labeled monosaccharides from *Spirulina* polysaccharide SPPB-1. X was inferred as a PPMP-labeled 6,7-deoxyheptanose. The separation condition was carried out as described in Fig. 4b.

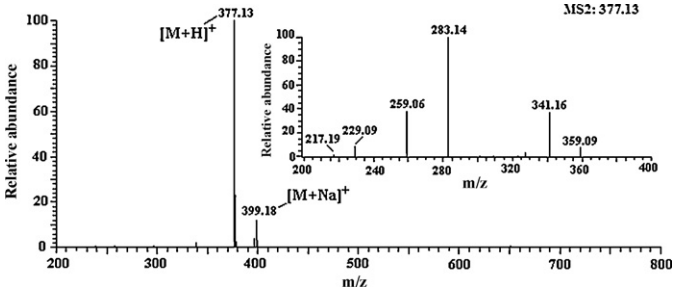


Fig. 6. Mass chromatogram of unknown compound X in *Spirulina* polysaccharide SPPB-1. The ESI-MS/MS^2 condition was carried out as described in Fig. 4b.

Man, Fuc and trace amount of GalN, Gal, Xyl, Ara, in which the molar ratio of Man, Glc and Rha is 0.51:1.79:3.79 that is consistent with Zhang's results [20].

In addition, a small amount of unknown compound X with strong molecular ion peak at *m*/*z* 377.13 $[M+H]^+$ in full scan MS spectra and its fragment ions with *m*/*z* 359.09, 341.16, 283.14, 259.06, and 229.09 in MS^2 spectra (see Fig. 6) is also observed in the real sample of the PPMP-labeled hydrolyzate of *Spirulina* polysaccharide SPPB-1. According to the characteristic fragment ions of PPMP-labeled monosaccharides listed in Table 2, compound X is suggested to be a mono-PPMP-labeled 6, 7-deoxyheptanose, which has never been reported in the earlier studies of monosaccharide compositions for *Spirulina* polysaccharide SPPB-1.

4. Conclusion

A new derivatization reagent, 1-(4-isopropyl) phenyl-3-methyl-5-pyrazolone (PPMP), has been developed for the separation and identification of saccharides using HPLC and on-line ESI mass spectrometry methods. The optimized monosaccharide derivatization conditions (molar ratio of sample to PPMP—1:6–7, reaction temperature—80 °C, reaction time—70 min) were found. Mono-PPMP-labeled derivatives can be predominantly obtained under the optimized condition. The HPLC separation conditions for monosaccharides mixture were also optimized. MS spectrometry data for 13 mono-PPMP-labeled monosaccharides were collected and analyzed.

All mono-PPMP-labeled derivatives display high chemical stability, their regular MS fragmentation is specific for reducing sugars. The suggested method for monosaccharide composition analysis with PPMP pre-column derivatization by on-line HPLC–ESI-MS was tested for a monosaccharide composition prepared from natural *Spirulina* polysaccharide and provided reliable results. In addition, presence of a reducing 6, 7-deoxyheptanose residue was for the first time proved in *Spirulina* polysaccharide SPPB-1.

The proposed HPLC–ESI-MS method with PPMP derivatization provides a rapid, reproducible, accurate, and economic alternative for the separation of monosaccharides. It also demonstrates considerable advantages above previously reported based PMP-derivatization method and it is quite effective for routine analysis of monosaccharides in natural samples.

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References

[1] R.S. Haltiwanger, J.B. Lowe, Annu. Rev. Biochem. 73 (2004) 491.
[2] E. Gorelik, U. Galili, A. Raz, Cancer Metast. Rev. 20 (2001) 245.
[3] K.R. Anumula, Anal. Biochem. 283 (2000) 17.
[4] H. Hase, Methods Enzymol. 230 (1994) 225.
[5] K.R. Anumula, Anal. Biochem. 220 (1994) 275.
[6] D.T. Fu, R.A. Oneill, Anal. Biochem. 227 (1995) 377.
[7] A. Guttman, J. Chromatogr. A 763 (1997) 271.
[8] F.T. Chen, T.S. Dobashi, R.A. Evangelista, Glycobiology 8 (1998) 1045.
[9] K. Sato, K. Sato, A. Okubo, S. Yamazaki, Anal. Biochem. 262 (1998) 195.
[10] P. Jackson, Mol. Biotechnol. 5 (1996) 101.
[11] T.R.I. Cataldi, C. Campa, G.E. De Benedetto, Fresenius J. Anal. Chem. 368 (2000) 739.
[12] S. Suzuki, K. Kakehi, S. Honda, Anal. Chem. 68 (1996) 2073.
[13] M. Wuhrer, A.M. Deelder, Rapid Commun. Mass Spectrom. 20 (2006) 943.
[14] K.A. Thomsson, H. Karlsson, G.C. Hansson, Anal. Chem. 72 (2000) 4543.
[15] X.D. Shen, H. Perreault, J. Chromatogr. A 811 (1998) 47.
[16] F.N. Lamari, R. Kuhn, N.K. Karamanos, J. Chromatogr. B 793 (2003) 15.
[17] K.R. Anumula, Anal. Biochem. 350 (2006) 1.
[18] C.M. Starr, R.I. Masada, C. Hague, E. Skop, J.C. Klock, J. Chromatogr. A 720 (1996) 295.
[19] H. Birrell, J. Charlwood, I. Lynch, S. North, P. Camilleri, Anal. Chem. 71 (1999) 102.
[20] Y. Zhang, Z.F. Wang, L.J. Huang, Chin. J. Chem. 25 (2007) 1522.
[21] D.J. Harvey, J. Am. Soc. Mass Spectrom. 11 (2000) 900.
[22] S. Honda, E. Akao, S. Suzuki, M. Okuda, K. Kakehi, J. Nakamura, Anal. Biochem. 180 (1989) 351.
[23] S. Honda, S. Suzuki, A. Taga, J. Pharmaceut. Biomed. 30 (2003) 1689.
[24] K. Kakehi, S. Suzuki, S. Honda, Y.C. Lee, Anal. Biochem. 199 (1991) 256.
[25] J.M. You, X. Sheng, C.X. Ding, Z.W. Sun, Y.R. Suo, H.G. Wang, Y.L. Li, Anal. Chim. Acta 609 (2008) 66.
[26] Y. Lv, X.B. Yang, Y. Zhao, Y. Ruan, Y. Yang, Z.Z. Wang, Food Chem. 112 (2009) 742.
[27] Z.F. Wang, Z.Y. Peng, L.J. Huang, R. Lu, G.Y. Tian, Acta Pharmacol. Sin. 36 (2001) 112.