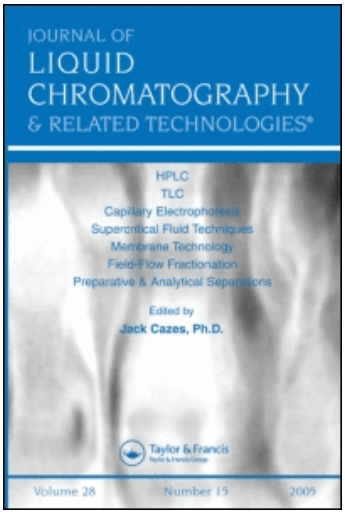

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Analysis of Carbohydrates in a Tibetan Medicine Using New Labeling Reagent, 1-(2-Naphthyl)-3-Methyl-5-Pyrazolone, by HPLC with DAD Detection and ESI-MS Identification

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To cite this Article Zhao, Xianen , Suo, Yourui , Wang, Lingyun , You, Jinmao and Ding, Chenxu(2008) 'Analysis of Carbohydrates in a Tibetan Medicine Using New Labeling Reagent, 1-(2-Naphthyl)-3-Methyl-5-Pyrazolone, by HPLC with DAD Detection and ESI-MS Identification', Journal of Liquid Chromatography & Related Technologies, 31: 16, 2375 — 2400
To link to this Article: DOI: 10.1080/10826070802319388
URL: http://dx.doi.org/10.1080/10826070802319388

Journal of Liquid Chromatography & Related Technologies®, 31: 2375–2400, 2008

ISSN: 1082-6076 print/1520-572X online
DOI: 10.1080/10826070802319388

Analysis of Carbohydrates in a Tibetan Medicine Using New Labeling Reagent, 1-(2-Naphthyl)-3-Methyl-5-Pyrazolone, by HPLC with DAD Detection and ESI-MS Identification

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Abstract: A new labeling reagent, 1-(2-naphthyl)-3-methyl-5-pyrazolone (NMP), coupling with liquid chromatography (LC) with electrospray ionization mass spectrometry (ESI-MS) for the detection of carbohydrates from a famous Tibetan medicine is reported. Carbohydrates were derivatized to their bis-NMP-labeled derivatives. The method, in conjunction with a gradient elution, offered a baseline resolution of carbohydrate derivatives on a reversed phase Hypersil ODS-2 column. The carbohydrates such as mannose, galacturonic acid, glucuronic acid, rhamnose, glucose, galactose, xylose, arabinose, and fucose could be successfully detected by UV and ESI-MS. Derivatives showed intense protonated molecular ion at m/z $[M + H]^+$ in positive ion mode. The mass to charge ratios of characteristic fragment ions at m/z 473.0 could be used for the accurately qualitative identification of carbohydrates; this characteristic fragment ion was from the cleavage of C2-C3 bond in the carbohydrate chain giving the specific fragment ions at m/z $[MH–C_mH_{2m+1}O_m–H_2O]^+$ for pentose, hexose, and glyceraldehydes, and at m/z $[MH–C_mH_{2m-1}O_{m+1}–H_2O]^+$ for

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alduronic acids, such as galacturonic acid and glucuronic acid ($m = n - 2$, n is carbon atom number of carbohydrate). Compared with the traditional 1-phenyl-3-methyl-5-pyrazolone (PMP) reagent, currently synthesized NMP show the advantage of higher sensitivity to carbohydrate compounds with UV and ESI-MS detection.

Keywords: 1-(2-naphthyl)-3-methyl-5-pyrazolone (NMP), Carbohydrates, Derivatization, *Herpetospermum pedunculosum* (Ser.) Baill, Liquid chromatography-mass spectrometry (ESI/MS)

INTRODUCTION

Herpetospermum seed is the dried ripe seed of *Herpetospermum pedunculosum* (Ser.) Baill (*Herpetospermum Caudigerum* Wall), which is widely distributed in India, Nepal, and the west region of China (Tibet, Sichuan, and Yunnan provinces). As a frequently used Tibetan medicine, it was recorded in several Tibetan pharmacopoeias.[1–3] It was reported that the Herpetospermum seed could clear heat from gallbladder and liver, and aid the digestion. Moreover, it was used among village folks to cure the liver heat syndrome, hepatitis A, or other liver and gallbladder diseases. Although, phenyl furan compounds (Herpetal and Herpetoriol) and lignans (Herpetrione, Herpetetradione, Herpepentol, Herpetol, Herpetetrone) were isolated from this herb by Kaouadji Mourad et al.[4–10] there were no other reports about chemical compositions in it.

The qualitative and quantitative knowledge of carbohydrates is essential information in biochemistry.[11] The roles of carbohydrates in biological processes have been studied with increasing attention over the past recent years. In carbohydrate analysis high resolution techniques are essential, because the carbohydrates have a number of isomers and homologies that structurally resemble one another. Up to now, the types of detection utilized for the determination of carbohydrates are indirect UV and direct UV absorbance of derivatized carbohydrates. Carbohydrate analysis by HPLC or other separation techniques coupled with ultraviolet (UV) detectors is made difficult by the absence of effective chromophores or fluorescence.[12,13] Early studies relied on refractometry or absorption in the UV region at 190–210 nm.[13] However, refractive index (RI) detectors have several limitations, namely, poor sensitivity to change in solvent composition, temperature, and pressure. Following this, a major shortcoming of RI detectors is their incompatibility with gradient elution.[14,15] Pulsed amperometric detection (PAD), combined with high pH anion exchange chromatography, has become a popular method for the analysis of native

carbohydrates because of its high sensitivity. However, the relatively high pH of eluents has been known to cause some epimerization and degradation of reducing carbohydrates,[16] which would make further characterization difficult. There have been some papers on precolumn labeling, because with this mode high yield of derivatives can be easily achieved, for example, benzoylation,[17] *p*-bromobenzoylation,[18] *p*-nitrobenzoylation,[19] and dimethylphenylsilylation.[20] However, when they were applied, all these methods gave anomic mixtures from reducing carbohydrates. A very reliable method so far was nuclear magnetic resonance (NMR) spectroscopy, which was applicable with sufficient quantities of samples (mg level), and was also remarkably useful for structural characterization of carbohydrates. Fast atom bombardment (FAB),[12,21] HPLC-ESI-MS electrospray ionization,[22,23] and matrix assisted laser desorption/ionization (MALDI-TOF)[24] have been extensively used in the characterization of subnanomolar amounts of carbohydrates. Native carbohydrates and oligosaccharides are not ionized very efficiently by either FAB, ESI, or MALDI since they are polar, thermally labile, and relatively non-volatile. To produce better ionization efficiency, the samples can be chemically derivatized. Permethylation and peracetylation,[25,26] among other methods, have been used to enhance MS characterization of carbohydrates or oligosaccharides at subnanomole level, where UV and/or MS detection was available and derivatization of carbohydrate samples played a key role. Chemical labeling methods convert carbohydrates into their derivatives, which can be detected at lower levels than their native analogs. For aqueous carbohydrates or oligosaccharide samples, the derivatization reactions should be ideally rapid and mild, involving few transfer steps.[27,28] Several methods for derivatization of carbohydrates into UV absorbing compounds are available. Of these, a very useful and widely used method was reductive amination. For example, reducing carbohydrates could be labeled with 2-aminopyridine to form pyridylamino (PA) derivatives.[29,30] This method was particularly valuable because of its highly sensitive fluorescence detection. However, it involved a two step labeling process and had a few additional shortcomings,[31] such as loss of sialic acid moieties. Recently, 1-phenyl-3-methyl-5-pyrazolone (PMP)[32] and its methoxy analog, 1-(*p*-methoxy)-phenyl-3-methyl-5-pyrazolone (PMPMP)[33] have been used for precolumn derivatization of carbohydrates. The bis-PMP-carbohydrates or PMPMP-carbohydrates absorbed strongly at 245 nm or 249 nm.[32,33] Both PMP and PMPMP derivatization methods can be used to label sialic acid containing oligosaccharides without causing desialylation, which constituted a great advantage over the PA derivatization method. However, the derivatization solution must be immediately neutralized with hydrochloric acid. At the same time, the

extraction process was at least repeated three times in order to avoid the hydrolysis of derivatives.

The combination of a sensitive functional group such as pyrazolone, together with a strong absorption moiety, would result in an attractive labeling reagent. Based on the photochromic characteristics of naphthalene, we have synthesized a novel photochromic labeling reagent 1-(2-naphthyl)-3-methyl-pyrazolone (NMP). NMP was very stable in its crystal state. The corresponding derivatives exhibited very high sensitivities. In this study, UV properties and optimal derivatization reaction conditions were evaluated. The responses of bis-NMP-labeled carbohydrates with DAD and ESI-MS detection were compared with those obtained using traditional PMP as the labeling reagent. At the same time, carbohydrates obtained commercially were investigated by LC-ESI-MS detection in positive ion mode and gave well characterized specific ions. To the best of our knowledge, this is the first time that NMP was synthesized and applied for the determination of carbohydrates.

EXPERIMENTAL

Instrumentation

All the HPLC system devices were from the HP 1100 series (Germany) and consisted of a vacuum degasser (model G1322A), a quaternary pump (model G1311A), an autosampler (model G1329A), a thermostated column compartment (model G1316A), and a diode array detector (DAD) (model G1315A). A mass spectrometer from Bruker Daltonik (Bremen, Germany) was equipped with an electrospray ionization (ESI) source, dry temperature 350°C, nebulizer 35.0 psi, dry gas 9.0 L/min. Derivatives were separated on a Hypersil ODS-2 column (200 × 4.6 mm 5 μm, Dalian Yilite Co., China). The HPLC system was controlled by HP Chemstation software. The mass spectrometer system was controlled by Esquire-LC NT software, version 4.1. The mobile phase was filtered through a 0.2 μm nylon membrane filter.

Chemicals

Carbohydrate standards were purchased from Sigma Co (St. Louis, MO, USA). HPLC grade acetonitrile and methanol were purchased from Yucheng Chemical Reagent Co (Shandong, China). 1-Phenyl-3-methyl-5-pyrazolone (PMP) was synthesized in our laboratory according to reference.[32] Acetoacetic ester, NaOH, Na_2CO_3, and Na_3PO_4 were from Jining Chemical Reagent Co (Jining Shandong, China). Ammonia

(17%, w/w) was analytical grade from Shanghai Chemical Reagent Co (Shanghai, China). Water was purified on a Milli-Q system (Millipore, Bedford, MA). Ammonium acetate buffer was prepared from 0.2 mol/L ammonium acetate solution adjusted to pH 4.35 with acetic acid (pH measurements were performed using a glass electrode, standard buffer solution of pH values of 4.0 and 7.0 were used in the calibration of the pH meter). β-Naphthylhydrazine hydrochloride was purchased from Yurao Chemical Reagent (Zhejiang, China).

Preparation of Standard Solutions

A stock solution of the compound under investigation was prepared by dissolving the compound in water or acetonitrile in a volumetric flask and transferred into 10 mL volumetric flasks and diluted to the mark with water or acetonitrile. NMP (0.05 mol/L) was prepared by dissolving 112 mg NMP in 10 mL of HPLC grade acetonitrile. Individual stock solutions of carbohydrate (0.01 mol/L) were prepared in water, and if necessary, methanol or acetonitrile was added until the compound dissolved. The standard carbohydrates for HPLC analysis at individual concentrations of 1.0×10^{-4} mol/L were prepared by diluting the corresponding stock solutions (1.0×10^{-3} mol/L) of each carbohydrate with water. When not in use, all standards were stored at 4°C.

Synthesis of 1-(2-Naphthyl)-3-methyl-5-pyrazolone (NMP)

Synthesis of β-Naphthylhydrazine

β-Naphthylhydrazine was conveniently prepared by neutralizing β-naphthylhydrazine hydrochloride with NaOH solution. β-Naphthylhydrazine hydrochloride (0.1 mol, 19.45 g) and 500 mL water was mixed. The mixture was rapidly heated to reach the boiling point; the insolvable residue was filtrated off by suction. The filtrate was then heated and carefully neutralized with 5.0% (w/w) aqueous NaOH (100 mL) with vigorous stirring; the contents were allowed to stand at ambient temperature for a 4 h period. The precipitated solid was recovered by filtration, and dried with P_2O_5 by storage for 24 h in a vacuum to afford a gray crystal (15.1 g), yield 96%.

Synthesis of 1-(2-Naphthyl)-3-methyl-5-pyrazolone (NMP)

β-Naphthylhydrazine (10.0 g) and 50 mL anhydrous ethanol in a 250 mL round bottom flask were mixed. After the mixture was heated to 60°C,

30 mL acetoacetic ester was added dropwise within 1.5 h, with vigorous stirring. After stirring at 60°C for 7 h, the mixture was concentrated by a rotary evaporator. After cooling, the residue was transferred into a 100 mL volumetric flask and stored at 4°C for 24 h. The precipitated solid was recovered by filtration, and dried at room temperature for 24 h. The crude product was recrystallized three times from methanol (100 mL × 3) to afford a white crystal yield 10.17 g (68%), m.p. 195.1–197.5°C. Found, C 75.2, H 5.40, N 12.47; Calculated, C 75.0, H 5.36, N 12.5; IR (KBr): 3121.4 (–C–H); 3055.20 (–C–H); 1722.1(–C=O); 1562.1 (ph-H), 1511.6 (ph-H); 1469.44 (C–H); 1390.7, 1363.8 (C–H); 1027.3, 895.2, 858.6, 748.3. ESI/MS detection in positive ion mode: m/z 225 $[M + H]^+$.

High-Performance Liquid Chromatography

HPLC separation of NMP derivatives was carried out on a Hypersil ODS-2 column by a gradient elution. Eluent A was 30% of acetonitrile containing 20 mmol/L ammonium acetate (pH 4.35); B was 60% acetonitrile-water. During conditioning of the column and prior to injection, the mobile phase maintained enough equilibrium with eluent A. The percentage of mobile phase was changed as follows after injection: 0–50% (B) from 0 to 50 min; 50–100% (B) from 50 to 60 min. The flow rate was constant at 1.0 mL/min and the column temperature was set at 30°C. The DAD detection wavelength was set at 251 nm.

Derivatization Procedure

The NMP carbohydrate derivatization was carried out in aqueous methanol with a basic medium. Of aqueous carbohydrates, 20–30 μL was added into a vial, to which 200 μL of 0.05 mol/L NMP methanol solution and 20 μL of ammonia solution were then added. The vial was sealed. The solution was shaken for 3 s and allowed to stand for 30 min at 70°C in a water bath. After derivatization, the mixture was dried under a stream of nitrogen for removal of the excess ammonia. The residue was redissolved by addition of 1.0 mL aqueous acetonitrile (80%, v/v), and a 10 μL volume of solution was directly injected for HPLC analysis. The derivatization process is shown in Figure 1.

Extraction and Hydrolysis of Carbohydrates from Herpetospermum Seed

The Herpetospermum seed was collected from Linzhi area of Tibet (China). Pulverized seed was dried at room temperature and stored at

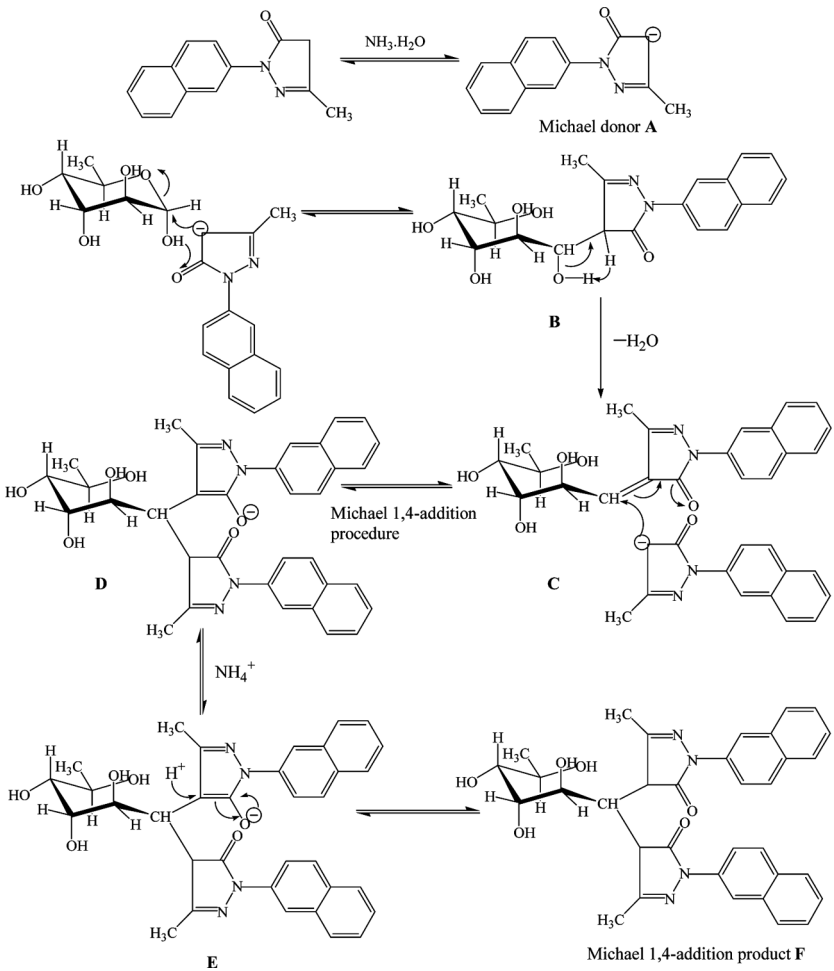

Figure 1. Derivatization scheme of NMP and carbohydrate in the presence of ammonia catalyst.

4°C until extraction. The carbohydrates in Herpetospermum seed were extracted by two methods: ultrasound assisted extraction and microwave assisted extraction as follows: (1) To a 50 mL volumetric flask, 5.0 g pulverized Herpetospermum seed and 35 mL water were added. The flask was immersed in a sonicator water bath and the sample was sonicated at 60°C for 4 h. The Herpetospermum seed was extracted three times; (2) To a 50 mL round bottom flask, 5.0 g pulverized Herpetospermum seed and 35 mL water were added. The flask was set in a microwave extractor and the sample was extracted at 60°C for 15 min (500 watt), and

the Herpetospermum seed was also extracted three times. For the two methods, the following process was the same. After the contents were combined, the solution was centrifuged, and the supernatant aqueous was collected. After being deproteinated by the Seavage method,[34] the polysaccharides were precipitated in four fold volumes of 95% ethanol at 4°C for 24 h. The precipitate was collected by centrifugation and washed with ethanol. Then, the precipitate was placed in a test tube, 2.0 mol/L trifluoroacetic acid (2 mL) was added and the test tube was sealed. After hydrolysis at 110°C for 8 h, the contents were adjusted to pH 7.0 with 2.0 mol/L NaOH solution, and filtered through a 0.2 μm nylon membrane. The final solution was made up to 5 mL with water and stored at 4°C until derivatization.

RESULTS AND DISCUSSION

Stabilities of NMP and Its Derivatives

Anhydrous methanol and acetonitrile solutions of NMP were stored at 40°C in darkness for two weeks, and the derivatization yields for carbohydrates gave similar results compared with those obtained with fresh prepared NMP solution. To investigate the stability of bis-NMP-labeled derivatives, the representative bis-NMP-Fuc derivative (1.0×10^{-5} mol/L) was prepared in aqueous acetonitrile (40%, v/v) according to the experimental conditions above. When bis-NMP-Fuc derivative was stored in pure acetonitrile (or methanol), in a sealed vial at room temperature in darkness, no obvious degradation was observed within 82 h. The degradation ratios of bis-NMP-Fuc derivative, which was prepared in the equivalent amount of NaOH solution by mixing bis-NMP-Fuc (4.67×10^{-5} mol/L, 40% acetonitrile solution, 500 μL) and NaOH solution (4.67×10^{-5} mol/L, 500 μL), were evaluated at 25 min intervals for a period of 82-h, during which time they were continuously analysed. Four ways to place the solutions of bis-NMP-Fuc as follows: (1) Stored in a sealed vial at 4°C in darkness; (2) Stored in a sealed vial at room temperature in darkness; (3) Stored in a sealed vial at room temperature and exposed to light; (4) Stored at room temperature and exposed to air and light. The results indicated that the highest degradation ratio (almost 100%) was observed for method (4). For the other carbohydrate derivatives, the similar results were also observed. With method (1), about 12% degradation for bis-NMP-Fuc derivative was observed during the same time period. The degradation extent for the stored bis-NMP-Fuc exhibited the order as follows: method (4) > (3) > (2) > (1). The main degradation reasons for bis-NMP derivatives

were from the oxidation and protolytic reaction with water molecules, this procedure could be accelerated under the condition of lighting.

Ultraviolet Absorption of NMP and Bis-NMP-carbohydrate Derivatives

1-(2-Naphthyl)-3-methyl-5-pyrazolone (NMP) contains a naphthyl functional group in its molecular backbone, which is excellent for the UV absorption property. Introduction of two NMP groups into each carbohydrate molecule endows the derivative high ultraviolet properties. For the determination of maximal absorption wavelength (λ_{max}), absorbance (A) and molar absorption coefficients (ε) of NMP and PMP, five NMP (or PMP) solutions at 1.0×10^{-5} mol/L in different solvents (methanol, ethanol, dioxane, acetonitrile, and tetrahydrofuran) were prepared. The ultraviolet absorption was investigated and the results are shown in Table 1. As observed, maximum UV absorption of NMP in acetonitrile is 251 nm. For the other four solvent systems, the maximal UV absorption is 243 nm. The molar absorption coefficients (ε) of NMP in acetonitrile and methanol are 5.58×10^4 and 3.30×10^4 L mol^{-1} cm^{-1}, respectively. Obviously, the UV response of NMP in acetonitrile is higher than that in methanol. In addition, the comparison of UV responses between NMP and the commonly used PMP were also evaluated. The results (Table 1) indicate that the ratios for the UV responses are as follows: $UV_{NMP}/UV_{PMP} = 2.55$ (acetonitrile) and $UV_{NMP}/UV_{PMP} = 2.58$ (methanol). This is probably due to the fact that the NMP molecule has a larger molecular conjugation structure, which makes it more sensitive than PMP. The UV spectrum of the representative bis-NMP-Fuc derivative shows that the maximum absorption wavelength is

Table 1. The maximal absorption wavelength (λ_{max}), absorbance (A) and molar absorption coefficients (ε) of NMP and PMP (1.0×10^{-5} mol/L)

	NMP		PMP	
Solvents	A (λ_{max})	ε ($\times 10^{-4}$)	A (λ_{max})	ε ($\times 10^{-4}$)
Acetonitrile	0.419 (214 nm)	4.19	0.219 (243 nm)	2.19
	0.558 (251 nm)	5.58		
Methanol	0.309 (214 nm)	3.09	0.128 (243 nm)	1.28
	0.330 (243 nm)	3.30		
Ethanol	0.385 (214 nm)	3.85	0.114 (249 nm)	1.14
	0.409 (243 nm)	4.09		
Dioxane	0.610 (243 nm)	6.10	0.213 (247 nm)	2.13
Tetrahydrofuran	0.515 (243 nm)	5.15	0.166 (247 nm)	1.66

also 251 nm in acetonitrile. Subsequently, the HPLC UV detection wavelength is set at 251 nm.

Optimization Derivatization

Effect of Catalyst on Derivatization

Several types of basic catalysts were evaluated for the derivatization reaction of NMP with carbohydrates, including sodium hydroxide, sodium carbonate, sodium phosphate, and ammonia $NH_3 \cdot H_2O$, 17% w/w). The results indicated that these alkaline catalysts exhibited different reaction activity. The ammonia and sodium hydroxide gave the highest detector responses. A slight decrease in detector response using sodium carbonate and sodium phosphate as catalysts was observed. Most subsequent derivatization was carried out by the use of ammonia as the alkaline catalyst because it provided a convenient pretreatment procedure, thus performing the dryness treatment with a stream of nitrogen without tedious and complicated neutralization and extraction. Further study indicated that the final ammonia concentration in the derivatized solution was kept at 0.1–0.2% (w/v) to give a complete derivatization; further increasing concentration of ammonia did not significantly increase the reaction yields. The bis-NMP-labeled derivatives were generally stable in acidic and weakly alkaline solutions. This property was useful for carbohydrate analysis, because the derivatives could be stored for a long time. With PMP as the labeling reagent, most reaction media were performed by the use of strong sodium hydroxide solution as catalyst, as previously reported.[35] In this case, bis-PMP-derivatives easily converted to its mono-PMP-derivative by removing one PMP-group and further conversion to the parent carbohydrate occurred by the removal of one more PMP-group. Therefore, the neutralization and subsequent removal of the excess reagent was a key step in establishing an efficient procedure. The excess amount of PMP could easily be removed from the reaction solution by extraction with chloroform or ethyl acetate,[36] unlike, in most other methods for precolumn derivatization, in which solvent extraction was not efficient due to low hydrophobicity of reagents. For analyses of mono-carbohydrates, ethyl acetate should be replaced by chloroform in order to minimize the loss of the derivatives due to slightly lower hydrophobicity. However, this procedure had drawbacks of tedious operation and low reproducibility. In addition errors due to loss of derivatives might occur, especially when different batches of columns were used. To avoid these shortcomings, the derivatization of carbohydrates using NMP as the labeling reagent with ammonia as the

alkaline catalyst was the best choice, because the derivatization solution could be easily treated with a stream of nitrogen, and only redissolved the residue with acetonitrile or methanol without tedious extraction procedure.

Effect of NMP Concentration on Derivatization

The effects of NMP concentration on derivatization yield were investigated when the derivatization reaction of NMP and carbohydrates proceeded at 70°C for 30 min. The UV responses of the bis-NMP-derivatives increased as the derivatization reagent concentration increased. A constant intensity was achieved upon the addition of 6-fold molar reagent excess over the total molar amount of carbohydrates; increasing the reagent excess beyond this level did not significantly affect the yields. With as little as a 5-fold molar reagent excess, incomplete derivatization of the carbohydrates was observed, and this obviously resulted in low detector responses. However, no mono-substituted derivatives were identified with ESI-MS detection in positive-ion mode.

Temperature Conditions and Time Effects

The optimum temperature and time for derivatization were investigated. The results indicated that heat had a significant effect on reaction time and yield. When tested at different temperatures over various periods of time, derivatization for most carbohydrates was completed within 15, 20, and 30 min at 90, 80, and 70°, respectively. It was found that above 80°C, the concentration of bis-NMP-labeled derivatives decreased. With derivatization at 90°C for 30 min, the UV responses of the representative bis-NMP-glucuronic acid (bis-NMP-GLA), bis-NMP-galacturonic acid (bis-NMP-GAA), and bis-NMP-arabinose (bis-NMP-AR) obviously decreased relative to those obtained at 70°C, and the ratios were as follows: $I_{70°C}/I_{90°C}= 5.92$ for bis-NMP-GLA, $I_{70°C}/I_{90°C}=1.72$ for bis-NMP-GAA and $I_{70°C}/I_{90°C} = 1.28$ for bis-NMP-AR (I was relative UV responses). The reduction extents of the UV responses for carbohydrate derivatives were obviously different because of the various structures of carbohydrates. With derivatization temperature >80°C, carbohydrates containing carboxylic groups, such as glucuronic acid and galacturonic acid, exhibited lower UV responses relative to those without carboxylic groups. This reason was currently unknown. Therefore, subsequent derivatization in our experiments was 70°C for 30 min. Further increasing the reagent concentration beyond 10-fold molar excess over the total molar amount of carbohydrates did not significantly shorten this time and temperature needed for the derivatization reaction.

Derivatization Reaction Mechanism

First, NMP reacted with alkaline catalyst ($NH_3.H_2O$) to form an intermediate A (Figure 1, intermediate **A** was called uncleophile A, also called Michael donor A) and donated a pair of electrons by loss of a hydrogen atom. Michael donor A attacked carbonyl group (aldehydes group) of carbohydrate to form the intermediate B, followed by loss of one H_2O to occur intermediate C (here, intermediate B was instable and not observed by ESI-MS detection in positive or negative ion modes). The intermediate C was an α, β-unsaturated carbonyl compounds having unusually electrophilic double bonds. The electrophile (the α, β-unsaturated carbonyl compound) can accept a pair of electrons (it was usually called the Michael accepter). The β-carbon in intermediate C was electrophilic because it shared the partial positive charge of the carbonyl carbon through resonance. Michael donor A (intermediate A) further attacked an α, β-unsaturated double bond at the β position. When attack occurred at the β position, the net result of 1,4-addition was addition of the nucleophile and a hydrogen atom across a double bond that was conjugated with a carbonyl group in pyrazolone and resulted in the addition product F.

HPLC Separation

The separation of carbohydrate derivatives could be carried out by different columns such as BDS-C18, ODS-C18, and so on. However, the separation of them on the Hypersil ODS-2 column gave the best results. Therefore, an ODS-2 column (250×4.6 mm, 5 μm) was selected in conjunction with gradient elution. Bis-NMP-labeled carbohydrate derivatives were considerably more hydrophobic than those obtained using PMP as the labeling reagent. The NMP molecule had high hydrophobicity due to the presence of the naphthyl functional group and pyrazolone ring as the basal structure. Therefore, the elution of bis-NMP-derivatives needed high methanol or acetonitrile concentration in mobile phases. Several programs were also investigated to ensure satisfactory HPLC separation. The gradient elution was carried out as described in the experimental section. With acetonitrile (30%, v/v) as the composition of mobile phase A, the elution gave the best separation with the shortest retention values and the sharpest peaks. In addition, the resolution of bis-NMP-derivatives could be significantly affected by the pH of the mobile phase. An alkaline solution (pH > 7.0) was not suited to the separation for all carbohydrate derivatives with a good baseline resolution. An obvious decrease in retention time was observed for bis-NMP-GluUA and bis-NMP-GalUA at higher pH coupled with

a loss of the resolution. At the same time, bis-NMP-GluUA and bis-NMP-GalUA were eluted earlier than the reagent peak NMP. This was probably due to the fact that the molecules of bis-NMP-GluUA and bis-NMP-GalUA, respectively, contained one carboxylic group. To achieve optimal separation, the choice of the pH value of mobile phase **A** was further tested on the Hypersil ODS-2 column. Separation of the derivatized carbohydrate standards can be accomplished at acidic

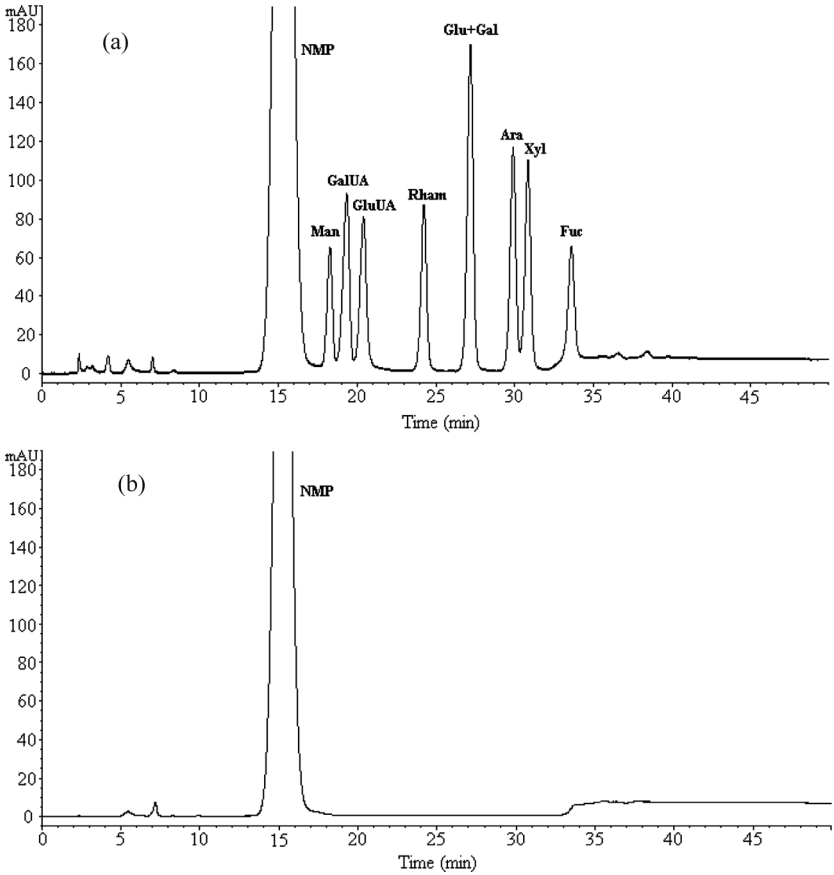

Figure 2. HPLC chromatograms of the derivatized carbohydrates. (A) Nine derivatized carbohydrates. (B) Blank solution. Column temperature was set at 30°C; DAD detection at 251 nm; Hypersil ODS-2 column (200 × 4.6 mm, 5 μm); flow rate = 1.0 mL/min. Other conditions were the same as described in experimental section. Peaks: Man (mannose); GalUA (galacturonic acid); GluUA (glucuronic acid); Rham (rhamnose); Glu+Gal (glucose+galactose); Ara (arabinose); Xyl (xylose); Fuc (fucose).

condition with pH 4.35, or in more acidic mobile phase with pH 4.0–4.35. In comparison with more acidic conditions (pH < 4.0), the best complete baseline resolution for carbohydrate derivatives could be achieved at pH 4.35 with the exception of bis-NMP-glucose and bis-NMP-galactose being coeluted. Further experiments for the complete separation of bis-NMP-glucose and bis-NMP-galactose derivatives were not successful on the Hypersil ODS-2 column, only giving the coeluted peak. The separation of a standard consisting of nine carbohydrate derivatives and blank solution on the Hypersil ODS-2 is shown in Figure 2.

On the basis of optimum experimental conditions, the calibration graphs were established with the peak area (Y) versus carbohydrate injection amounts (X, pmol). Injected amounts were from 15.63 pmol to 1000.0 pmol with injection volume of 10 μL (the linear range is 1.563–100 μmol/L). Linear regression equations, correlation coefficients, and limits of detection for all carbohydrates are shown in Table 2. The detection limits by HPLC DAD detection were calculated at S/N = 3:1 from the chromatogram of the standard at 1.563 μmol/L.

Comparison of Responses of Bis-NMP-derivatives and Bis-PMP-derivatives for UV and ESI/MS Ion Current Responses

Relative UV responses for an individual derivatized carbohydrate using NMP and PMP were evaluated, respectively. To make a quantitative comparison with respect to UV responses, the standard solution was derivatized, respectively, using NMP and PMP as labeling reagents (final derivatized concentration was adjusted to 1.11×10^{-4} mol/L, 10 μL injection, corresponding injected for each derivatized

Table 2. Linear regression equations, correlation coefficients and detection limits for bis-NMP-labeled carbohydrates

Carbohydrates	$Y = AX + B$ X: Injected amount (pmol) Y: Peak area	R	Detection limits (pmol)
Mannose	$Y = 1.54X - 14.16$	0.9998	1.08
Galacturonic acid	$Y = 2.63X - 1.60$	0.9999	0.59
Glucuronic acid	$Y = 2.24X - 22.41$	0.9994	1.10
Rhamnose	$Y = 2.09X - 28.19$	0.9994	0.87
Glucose + Galactose	$Y = 4.30X + 4.76$	0.9999	0.29
Arabinose	$Y = 2.97X + 1.91$	0.9999	0.63
Xylose	$Y = 2.80X + 0.52$	0.9998	0.76
Fucose	$Y = 1.68X + 25.01$	0.9995	0.87

carbohydrate 1110 pmol). The detection wavelength was set at the optimal wavelength ranges (here, NMP-derivatives were detected at 251 nm, PMP-derivatives detected at 245 nm, the optimal resolution for the derivatized carbohydrates using PMP as labeling reagent was not further adjusted, only using 20% aqueous acetonitrile containing 20 mM ammonium acetate as mobile phase at pH 4.35, elution condition was carried out as described in experimental section). The results indicated that UV responses for individually derivatized carbohydrates using NMP as derivatizing reagent exhibited obviously enhancement relative to those using PMP, and the ratios for the UV responses were in the range of $I_{NMP}/I_{PMP} = 1.14–3.64$ (I: relative UV response intensity, Table 3). Ion current intensities (ESI-MS) for derivatized carbohydrates were also compared to those obtained using PMP as the labeling reagent, and the ratios were in the range of $IC_{NMP}/IC_{PMP} = 1.08–2.23$ for the carbohydrate derivatives (IC: ion current signal intensities, Table 3). Obviously, NMP-carbohydrates exhibited higher ionization efficiency relative to PMP-carbohydrates. This may be attributed to the molecular core structure of the NMP molecule, in which a $\pi - \pi$ conjugation system was enhanced, and resulted in more stable ion current signals that formed by the acceptation of a H^+ in the position of N-2 atom. With ESI/MS detection, bis-NMP derivatives would be a promising labeling technique for carbohydrate analysis.

Table 3. Comparison of relative UV intensity and ESI-MS ion current intensities for carbohydrate derivatives using NMP and PMP as labeling reagents (derivatized carbohydrates concentration 1.11×10^{-4} mol/L; 10 μL injection; corresponding injected amount for each carbohydrate at 1110 pmol)

Carbohydrates	Relative UV intensity		Ratio of I_{NMP}/I_{PMP}	MS ion current intensity		Ratio of IC_{NMP}/IC_{PMP}
	NMP	PMP		NMP	PMP	
Xylose	107.9	40.0	2.70	2.8×10^7	2.2×10^7	1.27
Arabinose	73.4	30.0	2.45	2.2×10^7	1.8×10^7	1.22
Fucose	43.3	37.9	1.14	4.8×10^7	3.6×10^7	1.33
Rhamnose	28.3	19.8	1.43	2.8×10^7	1.9×10^7	1.47
Mannose	38.0	17.6	2.16	1.0×10^7	0.75×10^7	1.33
Glucose	50.0	21.6	2.31	2.2×10^7	1.5×10^7	1.47
Galactose	60.0	33.6	1.79	2.3×10^7	2.1×10^7	1.09
Glucuronic acid	10.4	8.2	1.27	2.7×10^6	2.5×10^6	1.08
Galacturonic acid	107.8	29.6	3.64	1.6×10^7	8.5×10^6	2.23

LC-ESI-MS Analysis of Mono-NMP-Labeled Carbohydrates

To obtain more information about the structure of the NMP labeled carbohydrates, the major peaks from the hydrolyzed mixture were analyzed by ESI-MS and ESI-MS/MS in positive-ion mode when the derivatization solution was placed according to method 4 as previously described above. Careful examination of the degradation products by LC-ESI-MS indicated that bis-NMP derivatives were degraded in alkaline medium by loss of one NMP molecule. Using mono-NMP-fucose as an example, the degradation scheme is shown in Figure 3. The following interpretation of the major ion formation could be proposed: molecular ion at m/z 371.2 $[M + H]^+$, and specific fragment ions: m/z

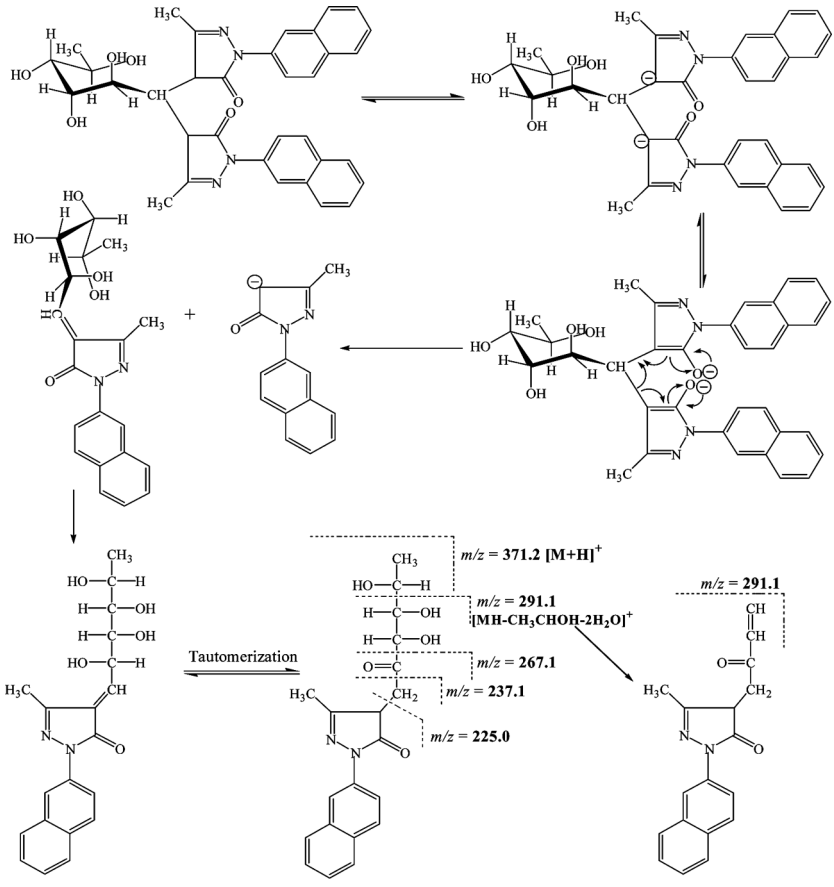

Figure 3. Degradation procedure of bis-NMP-labeled fucose.

352.9, m/z 335.1, m/z 291.1, m/z 267.1 and m/z 237.1. The specific ion at m/z 352.9 corresponds to loss of one water $[MH–H_2O]^+$. The specific ion at m/z 335.1 corresponds to loss of two water $[MH–2H_2O]^+$. The ion at m/z 291.1 corresponds to the cleavage of C4–C5 bond, followed by the loss of $2H_2O$ which can be expressed as m/z $[MH–CH_3CHOH–2H_2O]^+$ moiety. The specific ion at m/z 267.0 has been interpreted as the cleavage of C2-C3 bond. The specific ion at m/z 237.1 has been interpreted as the cleavage of C1–C2 bond, followed by rearranging two hydrogen atoms to form more stable characteristic fragment ions of 1-(2-naphthyl)-3-methyl-4-methene-5-pyrazolone moiety. The fragment ions at m/z 225.1 came from the core structural moiety corresponding to the protonated 1-(2-naphthyl)-3-methyl-5-pyrazolone moiety. As expected, the mono-NMP-Fuc can be detected by MS analysis (molecular ion $[M + H]^+$ at m/z 371.0, corresponding to intermediate **C**, Figure 1), and this experimental result supports the elucidation in the "Derivatization reaction mechanism" section. In addition, the collision induced dissociation of mono-NMP-carbohydrates (here, nine carbohydrates were tested) generate an intense fragment ion at m/z 291.1 by the cleavage of C4–C5 bond, followed by the loss of $2H_2O$ to form a conjugated double bond system. This fragment ion could be used for specific identification of the mono-NMP derivative. MS and MS/MS data for mono-NMP-derivatives are shown in Table 4.

LC-ESI-MS Analysis of Bis-NMP-labeled Carbohydrates

The ionization and fragmentation of the isolated bis-NMP-labeled carbohydrates were studied by ESI-MS and ESI-MS/MS detection in positive ion mode. The parent $[M + H]^+$ ions were observed in all cases, whereas the $[M + Na]^+$ ions was apparent in just a few cases. Using mannose as an example, the ESI-MS and ESI-MS/MS spectra of bis-NMP-labeled mannose is shown in Figures 4(a, b), and the MS/MS cleavage mode of bis-NMP-labeled mannose is shown in Figure 4(c). ESI-MS and ESI-MS/MS data for nine bis-NMP-labeled carbohydrates are shown in Table 5. The full scanning ESI-MS/MS spectrum of mannose contains several unknown ions, the following interpretation of major ions formation during ESI-MS detection in positive ion mode can be proposed: molecular ion at m/z 611.2 $[M + H]^+$, and specific fragment ions: m/z 592.9, m/z 386.9, m/z 473.0, and m/z 225 (1-(2-naphthyl)-3-methyl-5-pyrazolone moiety). The specific ion at m/z 592.9 corresponds to the loss of one water $[MH–H_2O]^+$. The peak at m/z 386.9 corresponding to the loss of one NMP group can be expressed as m/z [MH-one-NMP moiety]$^+$. The specific ion at m/z 473.0 has

been interpreted by cleavage of C2–C3 bond, followed by loss of one H_2O and rearranged two hydrogen atoms to form more stable characteristic fragment ion of 5-methyl-4-(1-(3-methyl-1-(naphthalene-2-yl)-5-oxo-2,5-dihydro-1*H*-pyrazol -4-yl)vinyl)-2-(naphthalene-2-yl)-1,2-dihydropyrazol-3-one ($[MMNPVNH]^+$) (Figure 4(c)). This ion can easily be expressed as $[MH–C_mH_{2m+1}O_m–H_2O]^+$ (here, $m = n - 2$, n is carbon atom number of carbohydrate and only suitable for pentose, hexose, and glyceraldehyde). For bis-NMP-labeled mannose, this specific ion can also be written as $[MH–C_4H_9O_4–H_2O]^+$. The specific fragment ion at m/z 473.0 $[MMNPVNH]^+$ is very stable and usually occurs for all carbohydrate derivatives, so that it can be used for specific identification for bis-NMP-labeled carbohydrates. This may be attributed to the structure of $[MMNPVNH]^+$, in which the $\pi - \pi$ conjugation system is dramatically enhanced as three double bonds are conjugated by the C1 atom across two pyrazolone rings.

Table 4. MS and MS/MS data for mono-NMP-labeled carbohydrates

Carbohydrates	$[M + H]^+$	$[MH–H_2O]^+$	$[MH–2H_2O]^+$	MS/MS fragment ions
Mannose	387.2	368.9	350.9	321.0, 291.1, 267.1, 237.2, 225.0
Galacturonic acid	401.3	383.0	365.0	321.1, 291.1, 267.1, 237.1, 225.0
Glucuronic acid	401.0	383.0	365.0	321.1, 291.1, 267.1, 237.1, 225.0
Rhamnose	370.9	353.3	334.9	291.1, 267.1, 225.0
Glucose	387.0	369.2	350.9	321.1, 291.1, 267.1, 237.1, 225.0
Galactose	387.0	369.2	350.9	321.1, 291.1, 267.1, 237.1, 225.0
Arabinose	357.0	339.0	321.1	291.1, 267.1, 237.0, 225.0
Xylose	357.0	339.0	321.1	291.1, 267.2, 225.0
Fucose	371.2	352.8	335.1	291.1, 267.2, 225.0

However, for the carbohydrates with different structures, such as galacturonic acid and glucuronic acid, the specific ion at m/z 473.0 should be changed as m/z $[MH–C_mH_{2m-1}O_{m+1}–H_2O]^+$ (here, $m = n - 2$, n is the carbon atom number of alduronic acid), e.g., glucuronic acid,

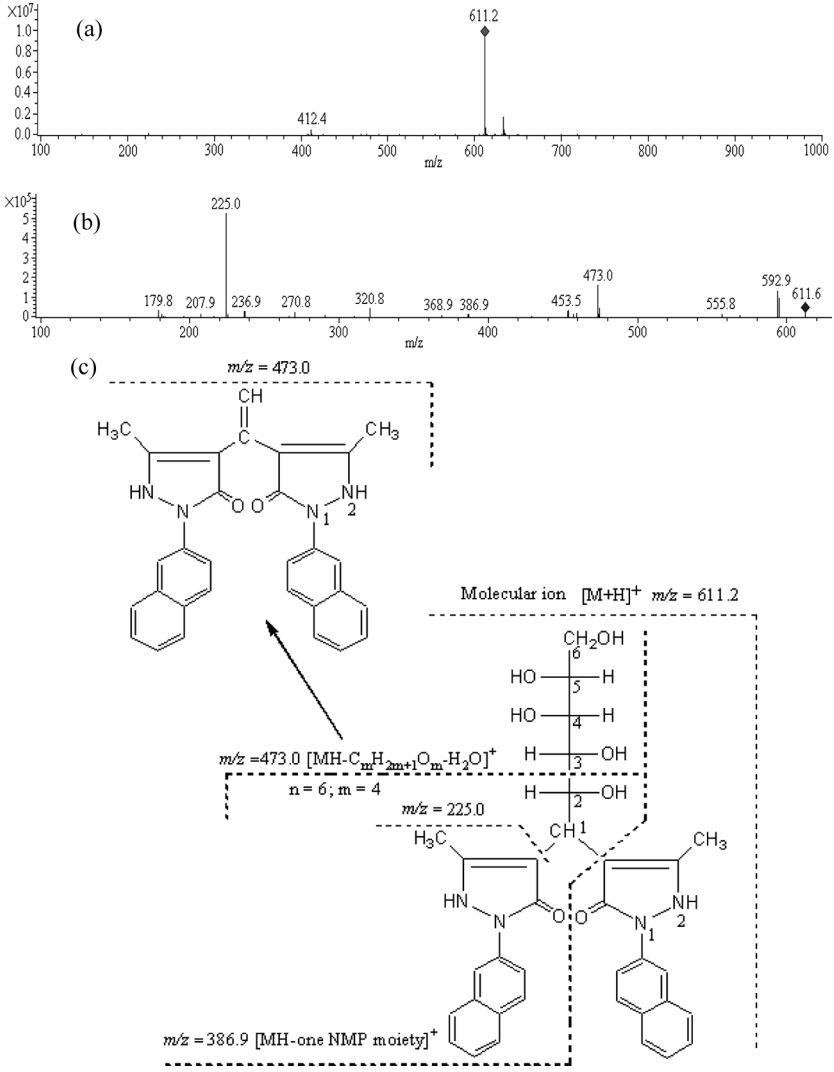

Figure 4. The (a) ESI-MS and (b) MS/MS spectra, and the (c) MS/MS cleavage mode of representative bis-NMP-labeled mannose. Full scanning range from 100 to 1000 amu under ESI in positive-ion detection mode; other MS conditions were described in experimental part.

Table 5. MS and MS/MS data for bis-NMP-labeled carbohydrates

Carbohydrates	Molecular weight	Bis-NMP -carbohydrates molecular mass	$[M + H]^+$	$[MH–H_2O]^+$	MS/MS fragment ions
Mannose	180	610	611.2	592.9	473.0, 386.9, 225
Galacturonic acid	194	624	625.1	607.0	472.9, 386.9, 225
Glucuronic acid	194	624	625.1	606.9	473.0, 382.5, 225
Rhamnose	164	594	595.1	577.3	473.2, 370.9, 224.9
Glucose	180	610	611.1	593.2	473.0, 387.6, 225
Galactose	180	610	611.1	593.2	473.0, 387.6, 225
Arabinose	150	580	581.1	563.3	472.7, 358, 224.9
Xylose	150	580	581.1	562.8	473.0, 356.5, 224.9
Fucose	164	594	595.2	577.2	473.1, 371.1, 224.9

the specific ion at m/z 473.0 can be written as $[MH–C_4H_7O_5–H_2O]^+$ (here, $m = 4$, $n = 6$, m/z $[M + H]^+ = 625.1$). ESI-MS data and MS/MS fragmentation are very important to demonstrate the molecular structure of carbohydrates, especially the characteristic ions, such as m/z at $[MH–C_mH_{2m+1}O_m–H_2O]^+$ for pentose, hexose, and glyceraldehydes, and m/z at $[MH–C_mH_{2m-1}O_{m+1}–H_2O]^+$ for alduronic acids. It should be noted that the molecular structure of the glyceraldehyde is similar to that of the carbohydrate molecules. Similar results are observed when NMP is used as the labeling reagent. The selected reaction monitoring, based on m/z $[M + H]^+ \rightarrow m/z$ $[MH–H_2O]^+$, m/z [MH-one-NMP moiety]$^+$, $[MH–C_mH_{2m+1}O_m–H_2O]^+$, or m/z $[MH–C_mH_{2m-1}O_{m+1}–H_2O]^+$, and m/z 225 transitions, is specific for carbohydrate derivatives. There was no detectable signal from the blank water sample using this transition. Although other endogenous aldehyde compounds such as aliphatic aldehydes and aromatic aldehydes present in natural environmental samples were presumably coextracted and derivatized by the NMP reagent, no interference was observed due to the highly specific parent mass to charge ratio (m/z $[M + H]^+$) and the characteristic ion $[MMNPVNH]^+$ at m/z 473.0. To all aliphatic and aromatic aldehydes, no specific ion at m/z $[MMNPVNH]^+$ was observed. For example, the molecular ionization and fragmentation of the isolated NMP-amyl aldehyde derivative were studied by ESI-MS and ESI-MS/MS detection in positive ion mode, and the schemes of MS and MS/MS are shown in Figures 5(a, b). As expected, the bis-NMP-amyl aldehyde produced an intense pseudomolecular ion peak at m/z 517.0 $[M + H]^+$, and the specific fragment ions at m/z 292.9 [MH-one NMP moiety]$^+$ and m/z 225. No characteristic ion at m/z 473 was observed. To reduce the

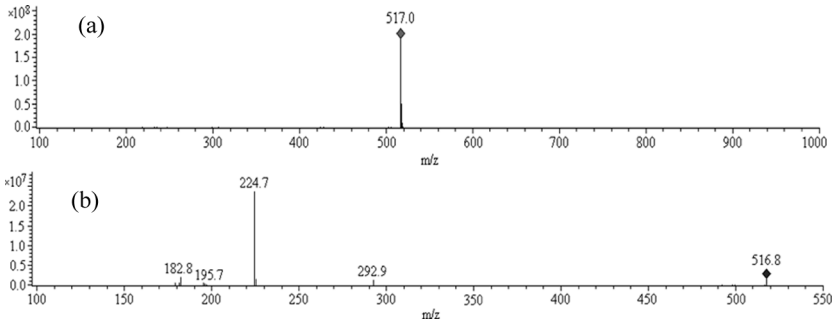

Figure 5. The profile of (a) ESI-MS and (b) MS/MS spectra of bis-NMP-labeled amyl aldehydes. Full scanning range from 100 to 1000 amu under ESI in positive-ion detection mode; other MS conditions were described in experimental part.

disturbance to a minimum, the gradient elution with HPLC for the separation of the derivatized bis-NMP-labeled carbohydrates was an efficient method because the coexisted aliphatic- or aromatic-aldehyde derivatives were eluted far later than bis-NMP-labeled carbohydrate derivatives.

Analysis of Carbohydrates from Herpetospermum Seed Samples

The HPLC Chromatogram and MS total ion current scheme for analysis of carbohydrate compositions from a Herpetospermum seed sample with DAD and ESI-MS detection, respectively, are shown in Figures 6(a, b). As can be seen, the carbohydrates such as mannose, galacturonic acid, glucuronic acid, rhamnose, glucose, galactose, xylose, arabinose, and fucose can be successfully detected. The established method is

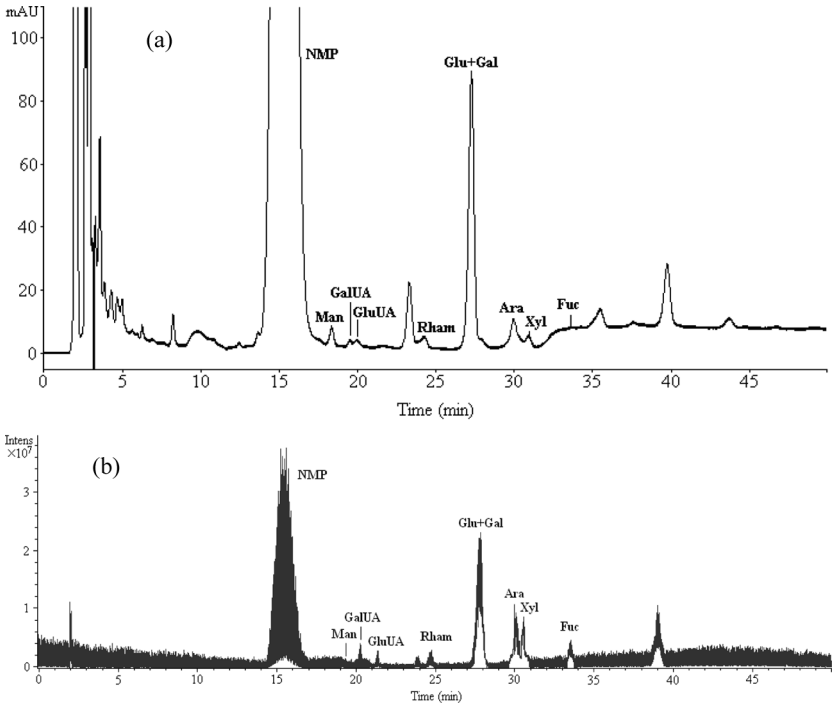

Figure 6. The (a) HPLC chromatogram and (b) MS total ion current chromatogram for bis-NMP-labeled carbohydrates from real hydrolyzed Herpetospermum seed samples. Chromatographic conditions and peaks as Figure 2. Mass spectrometric conditions as Figure 4.

Table 6. The analysis results of carbohydrate compositions in real hydrolyzed Herpetospermum seed

Carbohydrates	Ultrasound-assisted extraction (μg/g)	Microwave-assisted extraction (μg/g)
Mannose	4.96	6.04
Galacturonic acid	1.11	1.38
Glucuronic acid	1.71	2.05
Rhamnose	2.47	2.96
Glucose + Galactose	23.13	28.12
Arabinose	2.48	2.95
Xylose	1.40	1.70
Fucose	0.048	0.059

suitable for the determination of carbohydrate compositions from a Herpetospermum seed with satisfactory results. The analysis results of carbohydrates from Herpetospermum seed by ultrasound assisted extraction and microwave assisted extraction are shown in Table 6. The results indicated that the extraction yields of microwave assisted extraction are higher than those of ultrasound assisted extraction for carbohydrates. The facile NMP derivatization coupled with DAD and mass spectrometric detection allows for the development of a specific method for the analysis of carbohydrates from many biological or environmental samples.

CONCLUSIONS

A new sensitive labeling reagent, 1-(2-naphthyl)-3-methyl-5-pyrazolone (NMP) was developed for the analysis of carbohydrates. NMP derivatization is of tremendous value in the separation of mixture of carbohydrates by reversed phase HPLC coupled with DAD detection and ESI-MS identification. One of the most attractive features of this method is its simpleness for the preparation of carbohydrate derivatives. The established method is suitable for the analysis of carbohydrates with a reducing end group. It will be possible to develop a promising labeling technique for carbohydrates analysis using NMP.

ACKNOWLEDGMENTS

This work was supported by the Knowledge Innovation Program of Chinese Academy of Sciences and the National Science Foundation under Grant # 20075016.

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Received January 20, 2008
Accepted February 21, 2008
Manuscript 6288