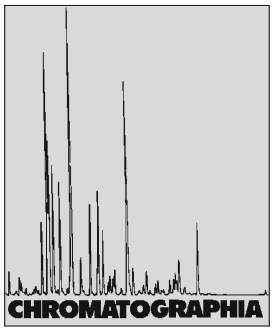
LC-DAD-ESI-MS Characterization of Carbohydrates Using a New Labeling Reagent



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Abstract

A novel labeling reagent 1-(2-naphthyl)-3-methyl-5-pyrazolone (NMP) coupling to liquid chromatography with electrospray ionization mass spectrometry for the detection of carbohydrates from the derivatized rape bee pollen samples is reported. Carbohydrates are derivatized to their bis-NMP-labeled derivatives. Derivatives showed an intense protonated molecular ion at *m/z* [M+H]$^+$ in positive-ion detection mode. The mass-to-charge ratios of characteristic fragment ions at *m/z* 473.0 could be used for the accurately qualitative analysis of carbohydrates. This characteristic fragment ion is from the cleavage of C2–C3 bond in 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 alduronic acids such as galacturonic acid and glucuronic acid ($m = n - 2$, n is carbon number of carbohydrate). No interferences for all aliphatic and aromatic aldehydes presented in natural environmental samples were observed due to the highly specific parent mass-to-charge ratio and the characteristic fragment ions. 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 can successfully be detected.

Keywords

Column liquid chromatography-mass spectrometry
Carbohydrates
1-(2-Naphthyl)-3-methyl-5-pyrazolone

Introduction

The qualitative and quantitative knowledge of carbohydrate distribution is essential information in food chemistry [1]. The roles of carbohydrates in bio-

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logical 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 homologs that structurally resemble one another. Up to now, the types of detection utilized for the determination of carbohydrates are indirect UV, direct UV absorbance of derivatized sugars. Carbohydrate analysis by LC or other separation techniques coupled with ultraviolet (UV) detectors is difficult in the absence of effective chromophores or fluorescence [2, 3]. Early studies relied on refractometry or absorption in the UV region at 190–210 nm [3]. However, refractive index (RI) detectors have several limitations, namely, sensitivity to change in solvent composition, temperature and pressure. Additionally a major shortcoming of RI detectors is their incompatibility with gradient elution [4, 5]. 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 [6], which would make further characterization difficult. Although, there have been numbers of papers on pre-column labeling because with this mode high yield of derivatives can be easily achieved, such as benzoylation [7], *p*-bromobenzoylation [8], *p*-nitrobenzoylation [9] and dimethylphenylsilylation [10]. However, when they have been applied, all these methods give anomeric mixtures from reducing carbohydrates. A very reliable method so far has been nuclear magnetic resonance (NMR) spectroscopy, which is applicable when sufficient quantities of samples (mg level), has also been remarkably useful for structural characterization of carbohydrates. Fast atom bombardment (FAB) [2, 11], LC-ESI-MS electrospray ionization [12, 13] and matrix-assisted laser desorption/ionization (MALDI-TOF) [14] have been extensively used in the characterization of sub-nanomolar amounts of material. Additionally, CE-MS and CE-MS^n seems a promising tool in the structural elucidation of unknown carbohydrates in a mixture [15]. However, so far CE-MS has only rarely been used for the structural identification [16]. The characterization of carbohydrates is also performed by using high performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) [17]. But one of the main disadvantages of HPAEC-PAD is the use of high salt eluent, which makes online mass spectrometry rather difficult. 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 [18, 19] among other methods have been used to enhance MS characterization of carbohydrates or oligosaccharides at the subnanomole level. Where UV and/or MS detection is available, derivatization of carbohydrate samples plays a key role. Some chemical tagging methods convert carbohydrates into derivatives which can be detected at lower levels than their native analogs. Selectivity of detection is also enhanced. For aqueous carbohydrates or oligosaccharide samples, the derivatization reactions should be ideally rapid, mild, involve few transfer steps and proceed in aqueous media [20, 21]. Several methods for derivatization of carbohydrates into UV-absorbing compounds are available. Of these, a very useful and widely used method is reductive amination. For example, reducing carbohydrates can be tagged with 2-aminopyridine to form pyridylamino (PA) derivatives [22, 23]. This method is particularly valuable because of its highly sensitive fluorescence detection. However, it involves a two-step labeling process and has a few additional shortcomings [24], such as loss of sialic acid moieties. Recently, 1-phenyl-3-methyl-5-pyrazolone (PMP) [25] and its methoxy analog, 1-(p-methoxy)-phenyl-3-methyl-5-pyrazolone (PMPMP) [26] have been used for pre-column derivatization of carbohydrates. The bis-PMP-sugars, or PMPMP-sugars absorb strongly at 245 or 249 nm [25, 26]. Both PMP and PMPMP derivatization methods can be used to label sialic acid-containing oligosaccharides without causing desialylation, which constitutes a great advantage over the PA-derivatization method [24]. 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 reagent. Based on the photochromic characteristics of the naphthalene, we have synthesized a novel photochromic molecule 1-(2-naphthyl)-3-methyl-pyrazolone (NMP). NMP has been found to be stable in its crystal state. The corresponding derivatives exhibited very high sensitivities. In this study, UV properties, optimal reaction conditions, such as reaction time, reagent concentration and catalyst, were evaluated. The responses of bis-NMP-labeled carbohydrates with DAD and ESI-MS detection were compared to those obtained using PMP as 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. The results from ESI-MS experiments, especially involving the characteristic fragment ions for the accurately qualitative analysis of carbohydrate derivatives, are emphasized. To the best of our knowledge, this is the first time that NMP probe and its application for the determination of carbohydrates has been reported. The suitability of the developed method for the detection of carbohydrate compositions from the extracted rape bee pollen sample was very accurate and reliable.

Experimental

Instrumentation

All the LC system devices were from the HP 1100 series (Waldbronn, Germany) and consisted of a vacuum degasser (model G1322A), a quaternary pump (model G1311A), an autosampler (model G1329A), a thermostated column com-

partment (model G1316A), and a diode array detector (DAD) (model G1315A). Mass spectrometer was equipped with an electrospray ionization (ESI) source; dry temperature, 350 °C; nebulizer, 35.00 psi; dry gas, 9.0 L min^{-1}. Derivatives were separated on Hypersil ODS-2 column (200 × 4.6 mm 5 μM, Yilite Co. Dalian, China). The LC system was controlled by HP Chemstation software. The mass spectrometer from Bruker Daltonik (Bremen, Germany) was equipped with an electrospray ionization (ESI) source. 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 (Alltech, Deerfiled, IL).

Chemicals

Sugar standards were purchased from Sigma Co. (St Louis, MO, USA). LC grade acetonitrile and methanol were purchased from Yucheng Chemical Reagent Co. (Shandong, China). 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, USA). Ammonium acetate buffer was prepared from 0.2 M 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

The general procedure was applied as follows. 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^{-1}) was prepared by dissolving 112 mg NMP in 10 mL of LC grade acetonitrile. Individual stock solution of each carbohydrate (1.0×10^{-2} mol L^{-1}) was prepared in water. The standard sugars for LC analysis at individual concentrations of 1.0×10^{-4} mol L^{-1} were prepared by diluting the corresponding stock solutions (1.0×10^{-3} mol L^{-1}) 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 were 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 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 $[M + H]^+$.

Liquid Chromatograph

LC separation of NMP-derivatives was carried out on Hypersil ODS-2 column by a gradient elution. Eluent **A** was 30% of acetonitrile containing 20 mmol L^{-1} 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^{-1} and the column temperature was set at 30 °C. The DAD detection wavelength was set at 251 nm.

Extraction of Rape Bee Pollen Polysaccharides

The rape bee pollen was collected from Menyuan county of Qinghai province. Pulverized bee pollen was dried at room temperature and stored at 4 °C until extraction. To a 25 mL volumetric flask, 5.0 g rape bee pollen 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 rape bee pollen was extracted three times. After the contents were combined, the solution was centrifuged, and the supernatant aqueous was collected. After deproteinated by the Sevage method [27], pollen polysaccharides were precipitated in fourfold volumes of 95% ethanol at 4 °C for 24 h. The precipitate was collected by centrifugation and washed with ethanol, acetone and diethyl ether, respectively.

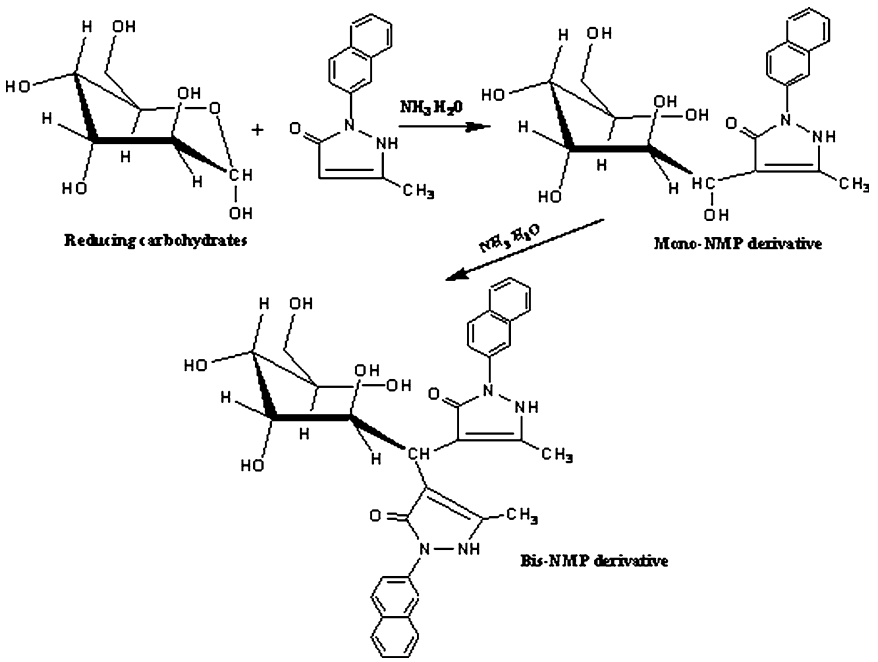

Fig. 1. Derivatization scheme of NMP with representative fucose in the presence of ammonia catalyst

After drying the pollen polysaccharides were collected.

Hydrolysis of Bee Pollen Polysaccharides

21.2 mg bee pollen polysaccharide was placed in a test tube; 2.0 mL trifluoroacetic acid (TFA, 2.0 mol L^{-1}) were added and the test tube sealed. After hydrolysis at 110 °C for 8 h, the contents were adjusted to pH 7.0 with 2.0 mol L^{-1} NaOH solution, and filtered through a 0.2-μm nylon membrane filter. The final solution was made up to 5-mL with water and stored at 4 °C until derivatization.

Derivatization Procedure

The NMP-carbohydrate derivatization was carried out in aqueous methanol in a basic medium. 20–30 μL of aqueous carbohydrates were added in a vial, to which 200 L of 0.05 mol L^{-1} NMP methanol solution and 20 μL of ammonia solution were then added. 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 to remove the excess ammonia under a stream of nitrogen. The residue was re-dissolved by the addition of 1.0 mL aqueous acetonitrile (80%, *v*/*v*) and further diluted tenfold with water, a 10 μL volume of solution was directly injected into the LC system for analysis. The derivatization process is shown in Fig. 1.

Results and Discussion

Stabilities of Reagent (NMP) and Its Derivatives

Anhydrous methanol and acetonitrile solutions of NMP were stored at 4 °C in darkness for 2 weeks, the derivatization yields for carbohydrates gave the 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^{-1}) was prepared in aqueous acetonitrile (40%, *v*/*v*). The degradation ratios of bis-NMP-Fuc derivative were evaluated at 25 min (or 60 min) intervals for a period of 82 h, during which time they were continuously analyzed. Four ways to place the solutions of bis-NMP-Fuc were as follows: (i) Stored in a sealed vial at 4 °C in darkness; (ii) Stored in a sealed vial at room temperature in darkness; (iii) Stored in a sealed vial at room temperature and exposed to light; (iv) Stored at room temperature and exposed to air and light. The results indicated that the highest degradation ratio (near 100%) was observed with the method (iv). For other carbohydrate derivatives, the similar results were also observed. With method (i), ca. 12% degradation for bis-NMP-Fuc derivative was observed during the same time period. The degradation extent for the stored bis-NMP-derivatives exhibited order was as follows: method (iv) > - method (iii) > method (ii) > method (i). In addition, bis-NMP-Fuc derivative was stored in pure acetonitrile (or methanol) in a sealed vial at room temperature in the darkness and no obvious degradation was observed during the same time period. The main degradation reasons for bis-NMP-derivatives were from the hydrolysis, this procedure could be accelerated under the condition of lighting. If the alkaline solution of bis-NMP-derivatives was heated, one of the NMP groups was removed giving the mono-NMP derivative, and further removal of another NMP group to their parent carbohydrate moiety. Although this procedure was not easy, requiring much more drastic conditions, but it was possible. Sometimes, this conversion is useful to recover the original reducing carbohydrates from the NMP-derivatives for further use, such as structure elucidation, enzyme inhibition assay, etc.

Optimization Derivatization

Effect of Catalyst on Derivatization

In order to evaluate the derivatization yields for the labeling of carbohydrates using NMP as labeling reagent, several types of basic media including sodium

hydroxide, sodium carbonate, sodium phosphate and ammonia ($NH_3 \cdot H_2O$, 17% *w*/*v*) were investigated. The results indicated that these alkaline catalysts exhibited different reaction activities. The ammonia and sodium hydroxide gave the highest detector responses. A slight decrease in detector response was observed using sodium carbonate and sodium phosphate as catalysts. Most subsequent derivatization was carried out by ammonia as the alkaline catalyst as it had a convenient pretreatment procedure and performing the dryness treatment by a stream of nitrogen without tedious and complicated neutralization and extraction. The study also indicated that the final ammonia concentration in derivatized solution kept at 0.1–0.2% (*w*/*v*) save a complete derivatization; with further increasing the 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 is useful for carbohydrate analysis, because the derivatives could be stored for a long time. With PMP as labeling reagent, most reaction media were performed by the use of strong sodium hydroxide solution as catalyst as previously reported [28]. In this case, bis-PMP-derivatives easily convert to its mono-PMP-derivative by removing one PMP group, and further conversion to the parent sugar by the removal of another PMP group. Therefore, the neutralization and subsequent removal of the excess reagent was a key step to establish an efficient procedure. The excess amount of PMP could easily be removed from reaction solution by extraction with chloroform or ethyl acetate [29], unlike in most other methods for pre-column derivatization, in which solvent extraction was not efficient due to low hydrophobicity of reagents. For analyses of monocarbohydrates, ethyl acetate should be replaced by chloroform in order to minimize the loss of the derivatives due to slightly lower hydrophobicity. The procedure had drawbacks of tedious operation and low reproducibility. In addition errors might occur due to the loss of derivatives especially when different batches of columns were used. To avoid these shortcomings, the derivatization of carbohydrates using NMP as labeling reagent with ammonia as alkaline catalyst was the best choice as the derivatization solution could be easily treated by direct drying under a stream of nitrogen, and re-dissolved the residue with methanol or acetonitrile without tedious extraction procedure.

Effect of NMP Concentration on Derivatization

Derivatization of NMP with carbohydrates could be achieved at 70 °C for 30 min. The effects of NMP concentration on derivatization yield were investigated. The UV responses of the bis-NMP-derivatives increased with increasing the reagent concentration. A constant intensity was achieved upon the addition of a fivefold to sixfold 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 fivefold 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. Very high yields (>94%) were observed when carbohydrate derivatives with a preconditioned Sep-Pak silica C18 cartridge were used as reference substances. In general, the % yields of the derivatization procedure for an unknown concentration sample was calculated by integrating the peak areas reaching the maximum for derivatized carbohydrates by the addition of an increasing amount of NMP.

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 were completed within 15, 20 and 30 min at 90, 80 and 70 °C, respectively. It was found that above 80 °C, the position of the equilibrium reduced the proportion of bis-NMP-labeled derivatives, which was probably due to the fact that the reverse reaction occurred at high temperature in alkaline media so that the concentration of the products decreased with time. With derivatization at 90 °C for 30 min, the UV responses of the representative bis-NMP-glucuronic acid (NMP-GLA), bis-NMP-galacturonic acid (NMP-GAA) and bis-NMP-arabinose (NMP-AR) obviously occurred in a decrease relative to that obtained at 70 °C. The ratios were as follows: $I_{70°C}/I_{90°C} = 5.92$ for NMP-GLA, $I_{70°C}/I_{90°C} = 1.72$ for NMP-GAA and $I_{70°C}/I_{90°C} = 1.28$ for NMP-AR (*I*: relative UV responses). The reduction extents of the UV responses for carbohydrate derivatives were obviously different because of the various structures of carbohydrates. With a 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 is currently unknown. Therefore, the most subsequent derivatization selected in experiments was 70 °C for 30 min, further increasing reagent concentration beyond tenfold molar excess over the total molar amount of carbohydrates did not significantly alter the time and temperature needed for derivatization reaction to be completed.

Derivatization Reaction Mechanism

First, NMP reacts with alkaline catalyst ($NH_3 \cdot H_2O$) to form an intermediate **A** (see Fig. 1; intermadate A is called uncleophile **A**, also called Michael donor **A**) and donates a pair of electrons by the loss of a hydrogen atom. Michael donor **A** attacks carbonyl group (aldehyde group) of carbohydrate to form the intermediate **B**, followed by loss of one H_2O to occur intermediate **C** (here, intermediate **B** is instable and not observed by ESI-MS detection in positive or negative ion modes). The intermediate

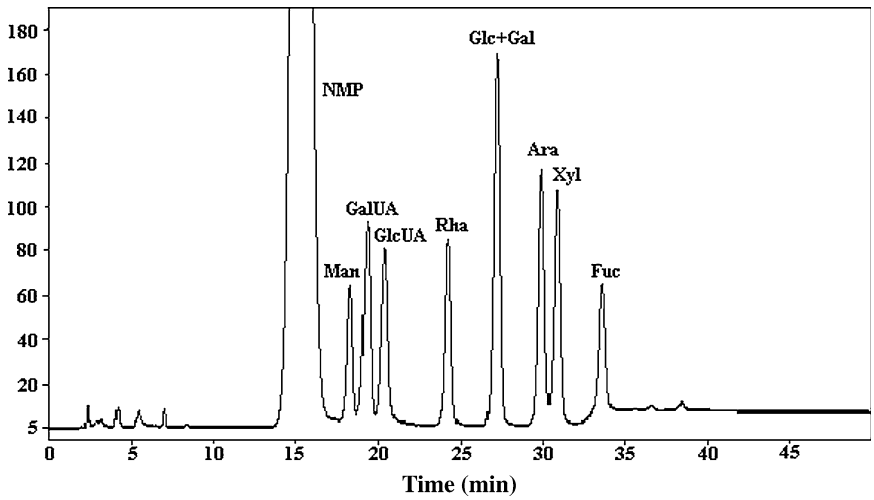

Fig. 2. LC chromatogram of bis-NMP-derivatized carbohydrates Column temperature is set at 30 °C; DAD detection at λ_{ex} 251 nm; Column: Hypersil ODS-2 (200 × 4.6 mm; 5 μm); flow rate = 1.0 mL min^{-1}. Other conditions were the same as described in Experimental. Peaks: **1** = Man (mannose); **2** = GalUA (galacturonic acid); **3** = GluUA (glucuronic acid); **4** = Rham (rhamnose); **5** = Glu + Gal (glucose + galactose); **6** = Ara (arabinose); **7** = Xyl (xylose); **8** = Fuc (fucose)

C is an α,β-unsaturated carbonyl compound having unusually electrophilic double bonds. The electrophile (α,β-unsaturated carbonyl compound) can accept a pair of electrons (it is usually called the Michael accepter). The β carbon in intermediate **C** is electrophilic because it shares the partial positive charge of the carbonyl carbon through resonance. Michael donor **A** (intermediate **A**) further attacks an α,β-unsaturated double bond at the β position. When an attack occurs at the β position, the net result of 1,4-addition is the addition of a nucleophile and a hydrogen atom across a double bond that was conjugated with a carbonyl group in pyrazolone and results in the addition product **F.**

LC Separation

The separation of carbohydrate derivatives could be carried out by different columns such as BDS-C18, ODS-C18 and so on; however, their separation on a Hypersil ODS-2 column gave the best results. Therefore, an ODS-2 column (250 × 4.6 mm i.d, 5 μm; Yilite, Dalian, China) was selected in conjunction with gradient elution. Bis-NMP-labeled carbohydrate derivatives are considerably more hydrophobic than those obtained using PMP as labeling reagent. NMP molecule has 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 LC separation within the shortest time. The gradient elution was carried out as described in Sect. 2. 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 suitable to separate all sugar derivatives with a good baseline resolution. An obvious decrease in retention was observed for bis-NMP-GLA and bis-NMP-GAA at higher pH coupled with a loss of the resolution. At the same time, bis-NMP-GLA and bis-NMP-GAA were eluted earlier than the reagent peak NMP. This was probably due to the fact that the molecules of bis-NMP-GLA and bis-NMP-GAA, contained one carboxylic group resulting in short retention. To achieve optimal separation, the choice of pH value of mobile phase **A** was further tested on a Hypersil ODS-2 column. Separation of the derivatized carbohydrate standards can be accomplished at acidic condition with pH 4.35, or in more acidic mobile phase with pH 4.0–4.35. With pH < 4.0, most of the bis-NMP-carbohydrates were resolved. In comparison with the acidic conditions (pH < 4.0), operation at pH 4.35 could achieve a complete baseline resolution for carbohydrate derivatives within the shortest time with the exception of bis-NMP-glucose and bis-NMP-galactose being co-eluted. Further experiments for the complete separation of bis-NMP-glucose and bis-NMP-galactose derivatives were not successful on the Hypersil ODS-2 column, by only giving the co-eluted peak. The separation of standard consisting of nine sugar derivatives on Hypersil ODS-2 is shown in Fig. 2.

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

Relative responses for UV detection for the individual derivatized carbohydrate using NMP and PMP were evaluated. To make a quantitative comparison with respect to relative UV responses, a standard solution containing eight carbohydrates was derivatized, using NMP and PMP as labeling reagents (final derivatized concentration was adjusted to 1.11×10^{-4} mol L^{-1}, 10 μL injection). The separation of standard derivatives was performed according to the established method as described in Sect. 2. The detection wavelength was set at the optimal wavelength ranges (here, NMP-derivatives 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 (pH 4.35), elution condition was carried out as

described in Sect. 2). The results indicated that UV responses for individually derivatized carbohydrates using NMP as derivatizing reagent obviously exhibited enhancement. The ratios for the UV responses were $I_{NMP}/I_{PMP} = 1.14–3.64$ (Table 1). Ion current intensities (ESI-MS) for derivatized carbohydrates are also compared to those obtained using PMP as labeling reagent. The ratios were $IC_{NMP}/IC_{PMP} = 1.08–2.23$ for the carbohydrate derivatives (*IC*: ion current signal intensities, Table 1). Obviously, NMP-carbohydrates exhibit high ionization efficiency relative to that of PMP-carbohydrates. This may be attributed to the molecular core structure of the NMP molecule, in which its π–π conjugation system is enhanced and results in more stable ion current signals than that formed by the acceptation of a $[H]^+$ in the position of *N*-2 atom. With ESI-MS detection, bis-NMP-derivatives will be a promising labeling technique for carbohydrate analysis.

Table 1. Comparison of relative UV and ESI-MS ion current intensities for sugar derivatives using NMP and PMP as labeling reagents (derivatized sugar concentration 1.11×10^{-4} mol L^{-1}; 10 μL injection; corresponding injected amount for each sugar at 1,110 pmol)

Sugars	Relative UV intensity		Ratio (I_{NMP}/I_{PMP})	MS ion current intensity		Ratio (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 the 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 $[M+Na^+]$ ion was apparent in just a few cases. Using mannose as an example, the ESI spectra is shown in Fig. 3a, b. The full-scan ESI spectra of mannose contained several unknown ions due to impurities, and the following interpretation of the major ion 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* 473.0, *m*/*z* 386.9 and *m*/*z* 225. The specific ion at *m*/*z* 592.9 corresponds to the loss of one water $[MH\text{-}H_2O]^+$. The peak at *m*/*z* 386.9 corresponding to the loss of one NMP group can be expressed as *m*/*z* $[MH\text{-one-NMP}]^+$ 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 a more stably 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 ($[MMNPVNH]^+$) (Fig. 3c). This ion can easily be expressed as follows: $[MH\text{-}C_mH_{2m+1}O_m\text{-}H_2O]^+$ ($m = n - 2$, *n* is the carbon atom number of carbohydrate and only suitable for pentose, hexose and glyceraldehyde). For example, bis-NMP-mannose, the specific ion can also be rewritten as $[MH\text{-}C_4H_9O_4\text{-}H_2O]^+$. 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 its π–π conjugation system is dramatically enhanced as three double bonds are across conjugated by the C1 atom between two pyrazolone rings.

As observed, the specific fragment ion at *m*/*z* 473.0 for representative pentose, hexose and glyceraldehydes can constantly be obtained. However, with the structural difference of carbohydrates such as galacturonic acid and glucuronic acid, it should be changed as *m*/*z* $[MH\text{-}C_mH_{2m-1}O_{m+1}\text{-}H_2O]^+$ ($m = n - 2$, *n* is the carbon atom number of alduronic acid), giving an example such as glucuronic acid. The specific ion at *m*/*z* 473.0 can be rewritten as follows: $[MH\text{-}C_4H_7O_5\text{-}H_2O]^+$ ($m = 4$, $n = 6$; $[M+H]^+ = 625.1$). As expected, all bis-NMP-labeled carbohydrates provide this specific ion at *m*/*z* $[MMNPVNH]^+$ under ESI-MS-MS detection in positive-ion mode. ESI-MS data and ESI-MS-MS fragmentation interpretation are very important to demonstrate the molecular structure of carbohydrates, especially, the characteristic ions, whose *m*/*z* value at $[MH\text{-}C_mH_{2m+1}O_m\text{-}H_2O]^+$ for pentose, hexose and glyceraldehyde; and *m*/*z* value at $[MH\text{-}C_mH_{2m-1}O_{m+1}\text{-}H_2O]^+$ for alduronic acids provide a helpful evidence regarding the estimation of the presence of carbohydrates. It should be noted that the molecular structure of the glyceraldehyde was similar to that of the carbohydrate molecules. Similar results were observed when NMP was used as the labeling. The selected reaction monitoring, based on the *m*/*z* $[MH]^+ \rightarrow$ *m*/*z* $[MH\text{-}H_2O]^+$, *m*/*z* $[MH\text{-one-NMP}]^+$, $[MH\text{-}C_mH_{2m+1}O_m\text{-}H_2O]^+$ or *m*/*z* $[MH\text{-}C_mH_{2m-1}O_{m+1}\text{-}H_2O]^+$ and *m*/*z* 225 transitions, was specific for carbohydrate derivatives including glyceraldehyde. 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 co-extracted and derivatized by NMP reagent, no interference was observed due to the highly specific parent mass-to-charge ratio and the characteristic product ion

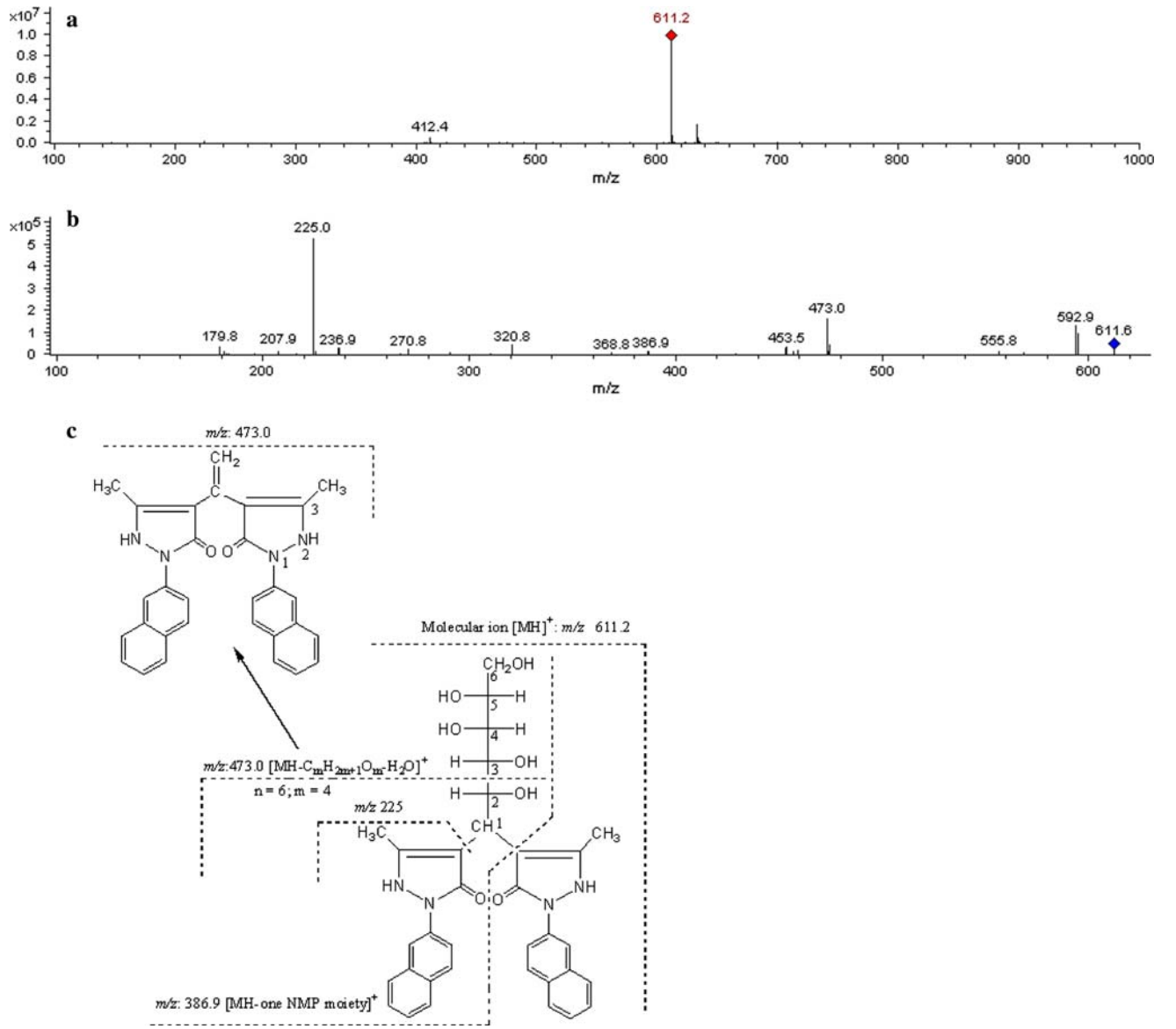

Fig. 3. The profile of ESI-MS-MS spectra and MS-MS cleavage mode of the bis-NMP-labeled mannose. Typical ESI-MS-MS spectra of representative bis-mannose derivative from full scanning range from 100 to 1,000 amu under ESI in positive-ion detection mode (**a** MS analysis; **b** MS-MS; **c** MS-MS cleavage mode)

$[MMNPVNH]^+$ at m/z 473.0. To all aliphatic and aromatic aldehydes, no specific ion $[MMNPVNH]^+$ was observed. For example, the ionization and fragmentation of the derivatized NMP-amyl aldehyde derivative were studied by ESI-MS and ESI-MS-MS detection in positive-ion mode. As expected, the bis-NMP-amyl aldehyde produced an intense pseudomolecular ion peak at m/z 517.0 $[MH]^+$, and the specific fragment ions at m/z 292.9 $[MH\text{-one NMP}]^+$ and m/z 225. No characteristic ion at m/z 473 was observed. To reduce the disturbance to minimum, the gradient elution with LC for the separation of the derivatized bis-NMP-labeled carbohydrates was an efficient method because the co-existed aliphatic- and aromatic-aldehyde derivatives were eluted far later than bis-NMP-labeled carbohydrate derivatives. Data of ESI-MS and ESI-MS-MS for nine reducing bis-NMP-labeled carbohydrates are shown in Table 2.

Separation and Identification of Mono-Carbohydrate Compositions from the Hydrolyzed Polysaccharides in Rape Bee Pollen

A chromatogram for the identification of mono-carbohydrate compositions from the hydrolyzed polysaccharides in rape bee pollen sample is shown in Fig. 4a. As can be seen from Fig. 4a, The carbohydrates such as mannose, galacturonic

acid, glucuronic acid, rhamnose, glucose, galactose, xylose, arabinose and fucose can be successfully detected. The established method is suitable for the determination of carbohydrate compositions from the derivatized rape pollen with satisfactory results. The facile NMP derivatization coupled with mass spectrometric detection allowed the development of a specific method for the identification of carbohydrates from rape bee pollen samples. The ESI-MS total ion current chromatogram of bis-NMP-derivatives is shown in Fig. 4b.

Conclusions

A new sensitive labeling reagent, 1-(2-naphthyl)-3-methyl-5-pyrazolone (NMP) was developed for the accurately qualitative analysis of carbohydrates coupled with LC-ESI-MS. NMP derivatization is of tremendous value in the separation of a mixture of carbohydrates by LC-ESI-MS. We have now demonstrated that derivatized carbohydrates can also be analyzed with ease by reversed-phase LC coupled with DAD and ESI-MS detection. Our results have indicated that (i) NMP-derivatives are more readily separated by LC than PMP-derivatives; (ii) ESI-MS system offers sufficient sensitivity for the analysis of carbohydrate derivatives, it exhibits relatively high ionization efficiency. In addition, one of the most attractive features of this method exhibits its simpleness for the preparation of carbohydrate derivatives. The established method is suitable for the analysis of carbohydrates with a reducing end group. Current studies are aimed at exploring the derivatization of oligosaccharides obtained commercially.

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Table 2. MS and MS-MS data for the bis-NMP-carbohydrate derivatives

Carbohydrates	Molecular weight	Bis-NMP-carbohydrates molecular mass	$[MH]^+$	$[MH\text{-}H_2O]^+$	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, 225
Glucose	180	610	611.1	593.2	473.0, 387.6, 225
Galactose	180	610	611.1	593.2	473.0, 387.6, 225
Xylose	150	580	581.1	563.3	472.7, 358, 224.9
Arabinose	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

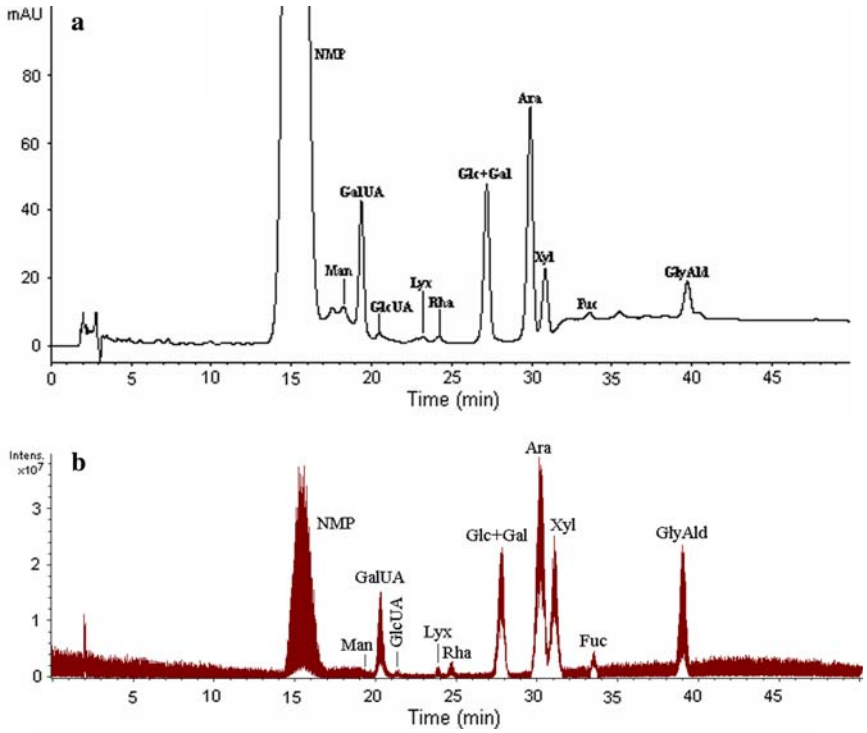

Fig. 4. a LC chromatogram and **b** total ion current chromatogram for bis-NMP-labeled carbohydrates from real hydrolyzed rape bee samples Conditions as Fig. 2; Mass spectrometric conditions as Fig. 3; Peaks were identified by ESI-MS in positive-ion detection mode; Peaks: **9** = Lyx (lyxose); **10** = GlyAld (glyceraladehyde); other peaks as Fig. 2

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