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Detection of carbohydrates using new labeling reagent 1-(2-naphthyl)-3-methyl-5-pyrazolone by capillary zone electrophoresis with absorbance (UV)

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ABSTRACT

A novel labeling reagent 1-(2-naphthyl)-3-methyl-5-pyrazolone (NMP) coupled with capillary electrophoresis (CE) with DAD detection for the determination of carbohydrates has been developed. The chromophore in the 1-phenyl-3-methyl-5-pyrazolone (PMP) reagent is replaced by naphthyl functional group, which results in a reagent with very high molar absorptivity ($\varepsilon_{251\,nm} = 5.58 \times 10^4\,L\,mol^{-1}\,cm^{-1}$). This permits NMP-labeled carbohydrates to be detected with UV absorbance in standard 50-μm-i.d. fused silica capillaries by zone electrophoresis. In this mode, nanomolar concentrations of detection limits are obtained. The method for the derivatization of carbohydrates with NMP is simplified. The derivatization reaction is rapid and mild in the presence of ammonia catalyst without further transfer steps. Nine monosaccharide derivatives such as mannose, galacturonic acid, glucuronic acid, rhamnose, glucose, galactose, xylose, arabinose and fucose can successfully be detected in CE mode. Good reproducibility can be obtained with relative standard deviation (R.S.D.) values of the migration times and peak area, respectively, from 0.44 to 0.48 and from 3.2 to 4.8. Furthermore, the developed method has been successfully applied to the analysis of carbohydrates in the hydrolyzed rape bee pollen samples.

1. Introduction

The qualitative and quantitative knowledge of carbohydrate distribution is essential information in food chemistry [1]. 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 homologs that structurally resemble one another. For the analysis of carbohydrates, there is a general need for methods that demonstrate high sensitivity. The main problem has been one of detection, since most carbohydrates do not absorb in the UV region. Consequently, much work has been done to develop derivatization reagents possessing moieties that allow for UV absorbance or fluorescence detection [2,3]. Many labeling reagent have been investigated for high-performance

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liquid chromatography (HPLC) [4], and with the increasing acceptance of capillary electrophoresis (CE) [5], many of the more successful derivatization reagents have been applied to this technique. To label carbohydrates, a number of different types of labeling reagents with high derivatization yields, such as benzoylation [6], *p*-bromobenzoylation [7], *p*-nitrobenzoylation [8] and dimethylphenylsilylation [9] have been proposed. 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 levels) have also remarkably useful for structural characterization of carbohydrates. So far, various analytical methods including fast atom bombardment (FAB) [10,11], HPLC–ESI-MS electrospray ionization [12,13], matrix-assisted laser desorption/ionization (MALDI-TOF) [14,15] and HPCE [16,17] have been extensively used in the characterization of sub-nanomolar amounts of material. Derivatization of carbohydrate samples plays a key role. Some chemical tagging methods convert carbohydrates into their derivatives which can be detected at lower levels than their native analogs. Selectivity of detection is also enhanced. For aqueous carbohydrates samples, the derivatization reactions should be ideally rapid, mild, involve few transfer steps and proceed in aqueous media [18,19]. 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 [20,21]. 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 [22], such as loss of sialic acid moieties. Recently, 1-phenyl-3-methyl-5-pyrazolone (PMP) [23] and its methoxy analog, 1-(p-methoxy)-phenyl-3-methyl-5-pyrazolone (PMPMP) [24] have been used for pre-column derivatization of carbohydrates. The bis-PMP-sugars, or PMPMP-sugars absorb strongly at 245 nm or 249 nm [23,24]. 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 [22]. However, the derivatization solution must be immediately neutralized with hydrochloric acid 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 that to be very stable in its crystal state. The corresponding derivatives exhibit very high sensitivities. In this study, UV properties, optimal reaction conditions, such as reaction time, reagent concentration and catalyst, are evaluated. To the best of our knowledge, this is the first time that NMP probe and its application for the characterization of carbohydrates has been reported. As an application, composition analysis of monosaccharides from the hydrolyzed rape bee pollen is also performed.

2. Experimental

2.1. Instrumentation

HP-3D Agillent capillary electrophoresis system equipped with a diode array detector (DAD) was used (Agillent technique, Germany). Fused-silica capillaries column with total length 48.5 cm, effective length 40-cm (50-μm inner diameter) were purchased from Yongnian Optical Fiber Factory (Hebei Province, China). The capillary electrophoresis system was controlled by HP Chemstation software.

2.2. Materials

Sugar standards were purchased from Sigma Co. (St. Louis, MO, USA). HPLC 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). 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 Zhejiang Yurao Chemical Reagent (Zhejiang, Jiangsu, China). PMP was synthesized as previously described [25]. PMPMP were synthesized according to the method as previously described [26].

2.3. Synthesis of 1-(2-naphthyl)-3-methyl-5-pyrazolone (NMP)

2.3.1. 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 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%.

2.3.2. Synthesis of 1-(2-naphthyl)-3-methyl-5-pyrazolone (NMP)

β-Naphthylhydrazine (10 g) and 50 mL anhydrous ethanol in a 250-mL of 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 $[M+H]^+$.

2.4. 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 (5.0×10^{-2} mol/L) was prepared by dissolving 112 mg NMP in 10 mL of HPLC grade acetonitrile. Individual stock solution of carbohydrate was prepared in water, and if necessary, methanol or acetonitrile was added until the compound dissolved. The standard sugars for CE analysis at individual concentrations of 5.0×10^{-5} mol/L were prepared by dilution 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.

2.5. Separation conditions

A new capillary column was activated by washing consecutively with each of 0.1 M hydrochloric acid (20 min), 0.1 M sodium hydroxide (30 min), and deionized water (30 min). At the beginning of each working day, the capillary was prewashed with 0.1 M HCl for 10 min, 0.1 M NaOH for 10 min and running buffer for 20 min, respectively. Before each analysis, the capillary was consecutively rinsed with 0.1 M NaOH (2 min), water (1 min) and running buffer (5 min). Sample was loaded onto the column by pressure injection for 10 s at 50 mbar.

2.6. Extraction and hydrolysis of carbohydrates from rape bee pollen samples

The rape bee pollen was collected from Menyuan county of Qinghai province. Pulverized rape 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 been pollen was extracted three times. After the contents were combined, the solution was centrifuged, and the supernatant aqueous was collected. After deproteinated by sevage method [26], the 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. After dried, the pollen polysaccharides were collected.

The bee pollen polysaccharide (21.2 mg) was placed in a test tube; 2.0 M TFA (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 M 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.

2.7. Derivatization procedure

The NMP-carbohydrate derivatization was carried out in aqueous methanol in a basic medium. 20–30 μL aqueous of carbohydrates was added in a vial, to which 200 μL of 0.05 M 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 gas. The residue was re-dissolved by the addition of 500 μL acetonitrile. The derivatized sample solution was directly used for CE analysis. The derivatization process is shown in Fig. 1.

2.8. Preparation of representative bis-NMP-Fucose (bis-NMP-Fuc) derivative to evaluate the UV properties

Bis-NMP-Fuc derivative is prepared by the reaction of NMP with representative fucose as described in Section 2. To a solution containing 20 μL ammonia (17%, w/w) and 20 μL aqueous fucose (0.1 M) in 5.0-mL test tube, 0.05 M NMP acetonitrile solution (200 μL) is added. The mixture is shaken for 3 s and allowed to stand for 30 min at 70 °C. After reaction, the mixture is dried under a stream of nitrogen. The residue is re-dissolved in 80% aqueous acetonitrile. This solution is passed through a preconditioned Sep-Pak C18 cartridge with 4 mL methanol and 5 mL water. After the excess NMP is eluted with 30% acetonitrile (50 mL), the desired bis-NMP-Fuc is eluted with 50% (v/v) acetonitrile (10 mL), and evaporated to dryness by a stream of nitrogen. The residue is re-dissolved with acetonitrile and made up to a total volume of 10 mL, the total bis-NMP-Fuc concentration of solution in volumetric flask is calculated from the known mass of fucose added and the total volume of the final solution. The obtained bis-NMP-Fuc concentration is ca. 2.0×10^{-5} mol/L. When not in use, the standard solution is stored at 4 °C.

3. Results and discussion

3.1. Ultraviolet absorption of NMP and bis-NMP-carbohydrate derivatives

1-(2-Naphthyl)-3-methyl-5-pyrazolone (NMP) exhibits a naphthyl functional group in its molecular backbone; this is an excellent property for the UV detection. Introduction of two NMP groups into each carbohydrate molecule endows the derivative high ultraviolet properties. For the determination of λ_{max}, absorbance (A) and molar absorption coefficients (ε) of NMP, 1.0×10^{-5} mol/L of each solvent solutions (methanol, ethanol, dioxane, acetonitrile, and tetrahydrofuran) were prepared. The ultraviolet absorption of NMP is investigated in five solvent systems (see Table 1). As observed, maximum absorption in acetonitrile (100%) is at 251 nm. For other four solvent systems, the UV absorption maxima are at 243 nm. The molar absorption coefficients (ε) in acetonitrile and methanol are 5.58×10^4 L mol^{-1} cm^{-1} and 3.30×10^4 L mol^{-1} cm^{-1}, respectively. Obviously, NMP shows that the UV response in acetonitrile is higher than that in methanol. In addition, the comparison of UV responses between NMP and the

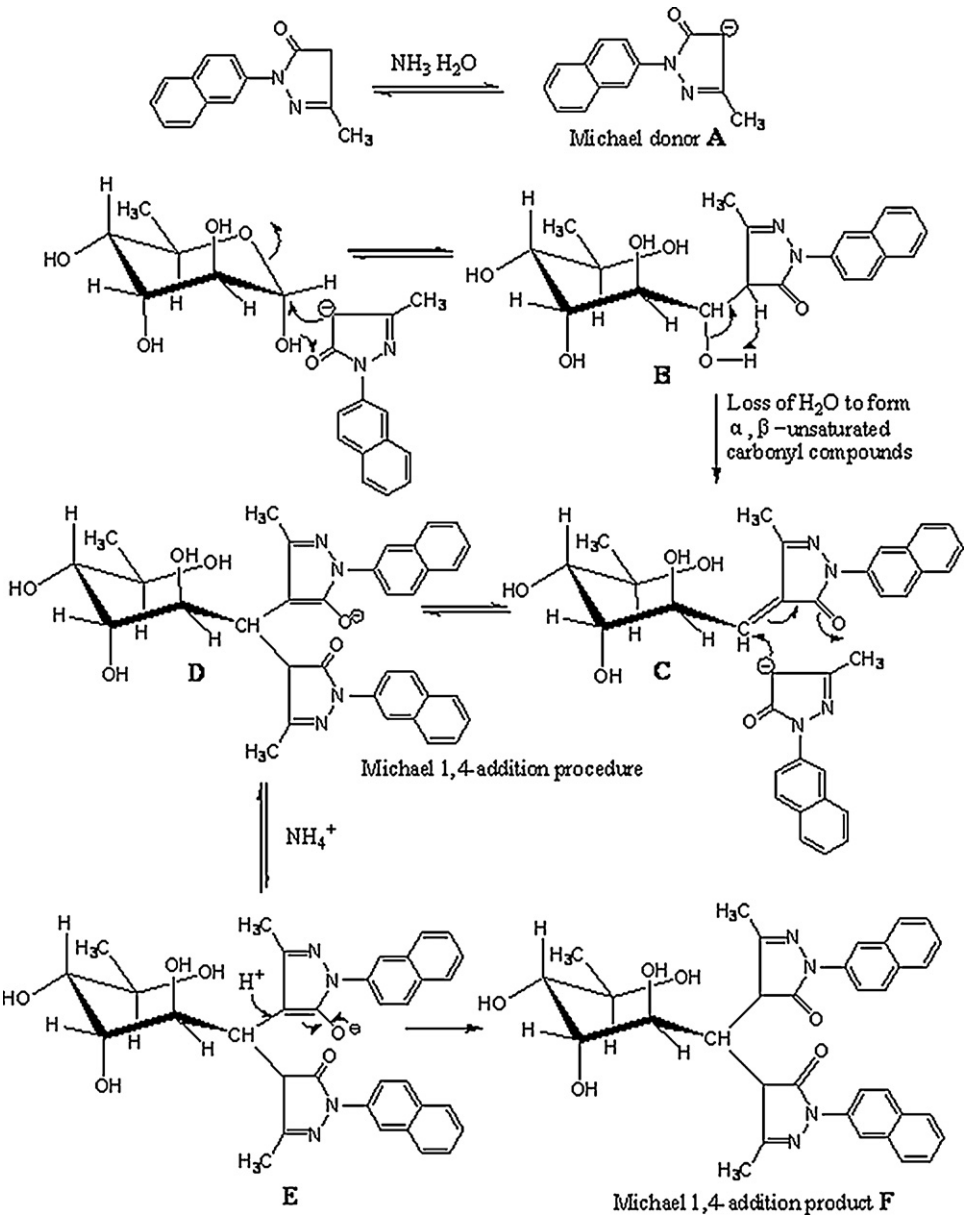

Fig. 1 – Derivatization scheme of NMP and representative mannose in the presence of ammonia catalyst.

Table 1 – The maximum absorption and corresponding absorbance (A) of NMP and PMP

Solvent	Spectral maximum λ (nm)		Spectral maximum λ (nm)	
	NMP		PMP	
	A	ε (×10^{-4})	A	ε (×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

commonly used PMP are also evaluated. To make a quantitative comparison with respect to relatively UV absorbance, the standard solutions of NMP and PMP (1.0×10^{-5} mol/L) are used. The results indicate that UV absorbance for NMP exhibits obviously enhancement. The ratios for the UV responses are as follows: $UV_{NMP}/UV_{PMP} = 2.55$ (acetonitrile) and $UV_{NMP}/UV_{PMP} = 2.58$ (methanol) (data obtained using 300 Bio ultraviolet spectrophotometer were not corrected, spectrum is not shown). This is probably due to the fact that NMP molecule has the large molar absorbance, which makes it more sensitive relative to that of PMP.

The UV spectrum of representative bis-NMP-Fuc derivative shows the maximum absorption peak at 251 nm. We have examined the effects of acetonitrile concentration on the UV spectra of the NMP-derivative. The concentrations of acetonitrile are varied from 0% to 100% (v/v). The absorbance (A) increases obviously as the acetonitrile concentration increased from 80% to 100% with the maximum absorption bands shift from 243 nm to 251 nm. With acetonitrile concentration varying in the range of 20–80% (v/v), the absorption intensities approximately keep a constant with the absorbance (A) at 0.345. With pure water system, the absorbance is approximately 0.31, a 26.1% difference in absorbance is observed between in pure water and 100% acetonitrile. UV absorbance of the NMP-Fuc derivative is minimally quenched by inorganic anions (such as sulfate, nitrate, and phosphate) and organic anions (such as citrate) and divalent cations that are abundant in biological fluids. Subsequently, the detection at the analytical wavelength is set at 251 nm for CE separation.

3.2. *Optimization derivatization*

3.2.1. *Effect of catalyst on derivatization*

Several types of basic media are evaluated for the derivatization of carbohydrates, including sodium hydroxide, sodium carbonate, sodium phosphate and ammonia ($NH_3{\cdot}H_2O$, 17% w/v). Derivatization is carried out by the reaction of NMP (5.0×10^{-2} mol/L) with carbohydrates at 70 °C for 30 min in the presence of variously alkaline catalysts. With ammonia as catalyst, the derivatization solution is directly dried under a stream of nitrogen and re-dissolved with acetonitrile prior to the injection to HPLC. With sodium hydroxide, sodium carbonate and sodium phosphate as catalysts, derivatization solutions are neutralized to pH 7.0 with 0.1 mol/L HCl. The final volume is made up to 1000 μL with acetonitrile. The results indicate that these alkaline catalysts exhibited different reaction activity. The ammonia and sodium hydroxide give the highest detector responses. A slight decrease in detector response using sodium carbonate and sodium phosphate as catalysts is observed. Most subsequent derivatization is carried out by the use of ammonia as the alkaline catalyst as it has convenient pretreatment procedure, only performing the dryness treatment by a stream of nitrogen without tedious and complicate neutralization and extraction. Further study indicates that the final ammonia concentration in derivatized solution is kept at 0.1–0.2% (w/v) to give a complete derivatization; with further increasing the concentration of ammonia does not significantly increase the reaction yields. The bis-NMP-labeled derivatives are generally stable in acidic and weakly alkaline solutions. Especially, the derivatization solution is stored in darkness in the presence of the excess NMP reagent, no obvious degradation for bis-NMP-labeled derivatives is observed. It is more likely that the excess reagent shifts the equilibrium to the right. This property is useful for the analysis of carbohydrate derivatives for a long time. With PMP as labeling reagent, most reaction media are performed by the use of strong sodium hydroxide solution as catalyst as previously reported [27]. 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 one more PMP group. Therefore, the neutralization and subsequent removal of the excess reagent is a key step to establish an efficient procedure. The excess amount of PMP can easily be removed from reaction solution by extraction with chloroform or ethyl acetate [28], unlike in most other methods for pre-column derivatization, in which solvent extraction is not efficient due to low hydrophobicity of reagents. At the same time, the procedure has drawbacks of tedious operation and low reproducibility. In addition errors due to loss of derivatives may occur, especially when different batches of columns are used. To avoid these shortcomings, the derivatization of carbohydrates using NMP as labeling reagent with ammonia as alkaline catalyst is the best choice as the derivatization solution can be easily treated by direct drying under a stream of nitrogen, and only re-dissolved the residue with methanol or acetonitrile without tedious extraction procedure.

3.2.2. *Effect of NMP concentration on derivatization*

The NMP derivatizing reagent undergoes the same condensation reaction with carbohydrates as PMP [22,27]. The reaction yield is found to be quantitative, in the range 3.0–1000 μM. The mean relative standard deviation (R.S.D.) of the reaction yield repeatability is 4.6%, and the highest R.S.D., 7.3%, is found for bis-NMP-GLA and bis-NMP-GAA. However, at the ammonia catalyst used, all di-NMP-labeled derivatives are stably formed with the reaction yield repeatability <3.0%; no mono-labeled derivatives are found in all cases. The results are in agreement with those found previously using PMP as labeling reagent with sodium hydroxide as catalyst. In five to six times excess reagent, no significant effect is found on the yield of carbohydrate products. Increasing the reagent excess beyond this level does not significantly affect on yields. With as little as a fivefold molar reagent excess, incomplete derivatization of the carbohydrates is observed, and this obviously results in low detector responses. However, no mono-substituted derivatives are identified with ESI-MS detection in positive-ion mode. In general, the % yields of the derivatization procedure for an unknown concentration sample is calculated by intergrating the peak areas reached maximum for the derivatized carbohydrates by the addition of increasing amount of NMP.

3.2.3. *Effects of temperature and time on derivatization*

The optimum temperature and time for derivatization are investigated. The results indicate that heat has a significant effect on reaction time and yield. When tested at different temperatures over various periods of time, derivatization for most carbohydrates are completed within 15 min, 20 min and 30 min at 90 °C, 80 °C and 70 °C, respectively.

It is found that above 80 °C, the position of the equilibrium reduced the proportion of bis-NMP-labeled derivatives, this is probably due to the fact that the reverse reaction occurs at high temperature in alkaline media so that the concentration of the products decrease with time. With derivatization at 90 °C for 30 min, low UV responses for bis-NMP-glucuronic acid (NMP-GLA), bis-NMP-galacturonic acid (NMP-GAA) and bis-NMP-arabinose (NMP-AR) are observed, the responses are lower than that of those obtained at 70 °C. The ratios are 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 are 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 exhibit obviously low UV responses relative to that of those obtained without carboxylic groups. This reason is currently unknown. Therefore, most subsequent derivatization selected in experiments is 70 °C for 30 min, further increasing reagent concentration beyond 10-fold molar excess over the total molar amount of carbohydrates does not significantly alter the time and temperature needed for derivatization reaction to be completed.

3.2.4. *Comparison derivatization conditions between NMP and PMP*

Derivatization by the procedure of Honda et al. [27,29] using PMP as labeling reagent proceeded smoothly. However, the derivatization solution must be treated to ensure that the aqueous phase have been fully neutralized prior to extraction with chloroform. Commonly, solvent extraction is not efficient due to low hydrophobicity of reagents. The procedure has drawbacks of tedious operation and low reproducibility. In addition errors due to loss of derivatives might occur, especially when different batches of columns are used. To avoid these shortcomings, the derivatization of carbohydrates using NMP as labeling reagent with ammonia as alkaline catalyst is the best choice as the derivatized mixture can be easily treated by direct drying under a stream of nitrogen, and only re-dissolved the residue with acetonitrile. Derivatization procedure is rapid and simpleness. The comparison of derivatization conditions between NMP and PMP is shown Table 2.

3.2.5. *Structure confirmation of the mono-NMP derivatives*

The NMP reacts with alkaline catalyst ($NH_3{\cdot}H_2O$) to form an intermediate A (intermediate A is called nucleophile A, also called Michael donor A, see Fig. 1) 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 C is 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 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 attack occurs at the β position, the net result of 1,4-addition is addition of the 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. In order to elucidate the structure of the intermediate C, the representative bis-NMP-Fuc derivative (bis-NMP-Fucose) as a model is incubated at pH 9.0 for 24 h. The mono-NMP-Fuc derivative is isolated and purified on a small column of preconditioned Sep-Pak C18 cartridge with 4 mL methanol and 5 mL water with 10% aqueous methanol as the eluent. ESI-MS and ESI-MS/MS spectra of the purified mono-NMP-Fuc (intermediate C) are shown in Fig. 2. As expected, the mono-NMP-Fuc can be stably found with MS analysis. The molecular ion $[M+H]^+$ at *m/z* 371 obtained in ESI-MS in positive ion mode supported this observation. The proposed structure is shown in Fig. 1 (C). Bis-NMP derivatives of other sugars also give intermediate C (mono-NMP derivatives) upon incubation under similar conditions.

3.3. *Optimal CE conditions*

Several types of buffers, including carbonate buffers, phosphate buffers and borate buffers, at concentrations of 15–65 mM (pH 9.46), are examined in order to compare the separation efficiency and peak-to-peak resolutions of five representative bis-NMP-labeled carbohydrates. The results

Table 2 – Comparison of derivatization conditions between NMP and PMP

Reagent	NMP	PMP[a]
Reagent concentration	0.05 in acetonitrile	0.5 in methanol
Alkaline concentration	17% aqueous ammonia (excess ammonia was vaporized by a stream of nitrogen gas)	0.3 M NaOH (excess NaOH was completely neutralized with aqueous HCl)
Temperature	70 °C	70 °C
Reaction time	30 min	30 min
Sample treatment	Dried by a stream of nitrogen gas and re-dissolved with acetonitrile	After neutralized with HCl, solution was extracted with chloroform, then water phase was dried by vacuum and re-dissolved in water
Excess reagent to total carbohydrate	Five- to sixfold	»Sixfold[b]

[a] Data are from literature [29].
[b] Data are from literature [30].

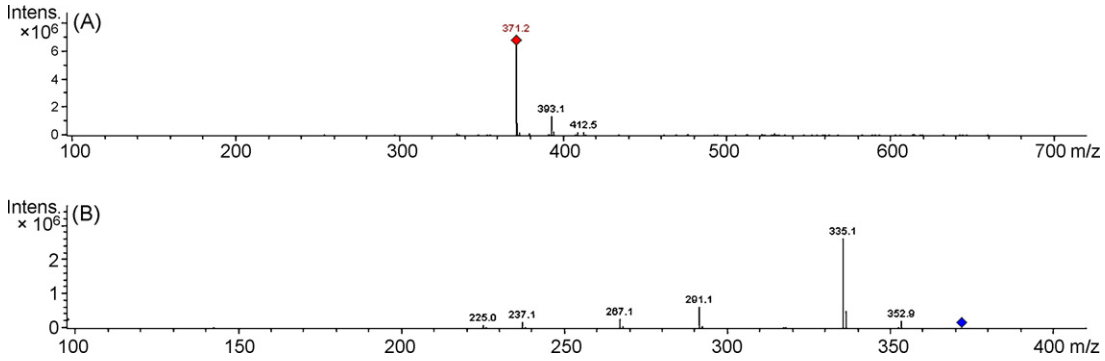

Fig. 2 – The profile of ESI-MS and ESI-MS–MS spectra of the hydrolyzed mono-NMP-fucose. Typical ESI-MS/MS spectra of representative mono-NMP-fucose from full scanning range from 100 amu to 700 amu under ESI in positive-ion detection mode (A: MS analysis; B: MS/MS analysis).

indicate that borate buffer gives the best separation for five representative bis-NMP-labeled carbohydrates including GlcUa, Man, Ara, Fuc and Glc. With borate buffer concentrations varying from 40 mM to 65 mM, the resolutions (Rs) of the bis-NMP-labeled carbohydrates including GlcUa, Man, Ara and Fuc remained basically invariableness with constant elution order. However, the resolution between Ara and Glc exhibits obviously difference. With borate buffer <50 mM or >60 mM, Ara and Glc are co-eluted. With borate buffer at 55 mM, the maximum resolution between Ara and Glc can be achieved with Rs 1.34. It seems that the migration times of bis-NMP-labeled carbohydrates are loosely associated with the borate group either through hydrogen bonding or complex formation. The possible mechanism may be expressed as Fig. 3. With a further high buffer, a large amount of Joule heating will affect separation efficiency. Taking both shorter run-time and higher zeta potential into consideration, the borate buffer concentration is selected at 55 mM. The resolution of bis-NMP-labeled carbohydrates can be significantly affected by the pH of the background electrolytes. An alkaline buffer is suited to the separation for all bis-NMP-labeled carbohydrates. To achieve optimal separation, the operation at pH 9.46 a complete baseline resolution for carbohydrate derivatives can be achieved within the shortest time. Under the proposed conditions, the separation of nine bis-NMP-labeled carbohydrates is achieved within 20 min. The separation of standard

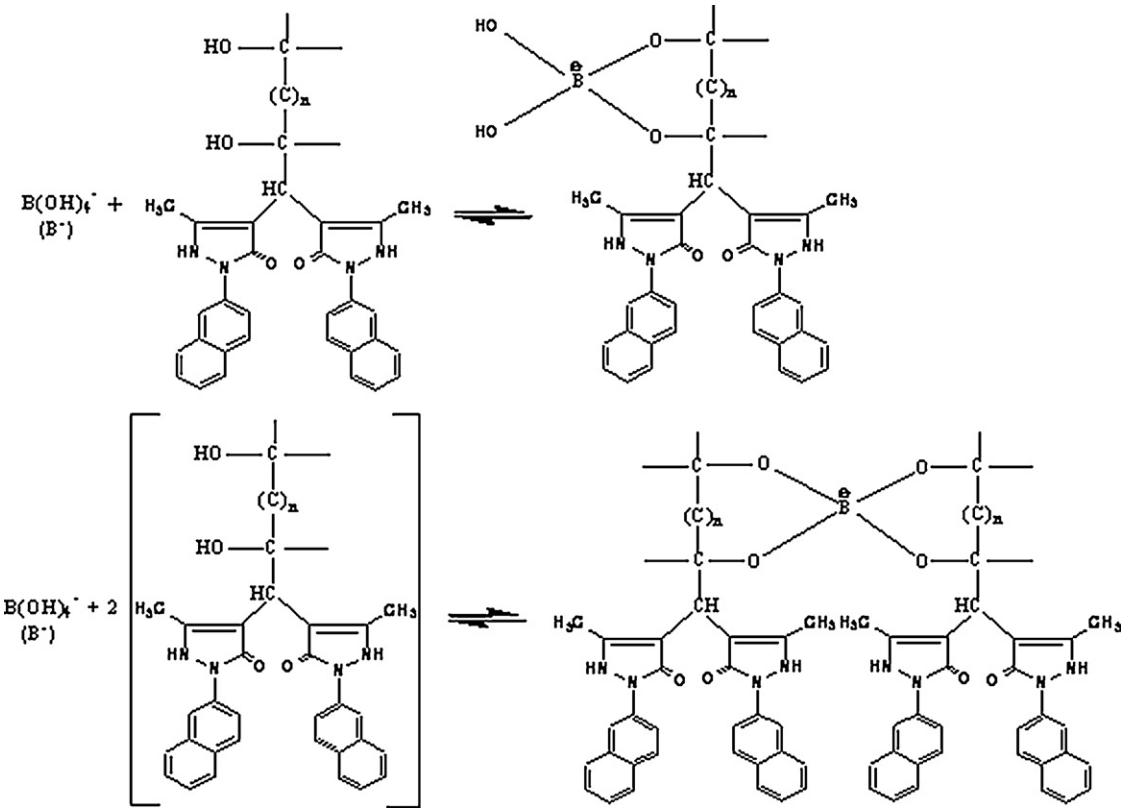

Fig. 3 – The scheme of the hydrogen bonding or complex interaction of borate ions with carbohydrates.

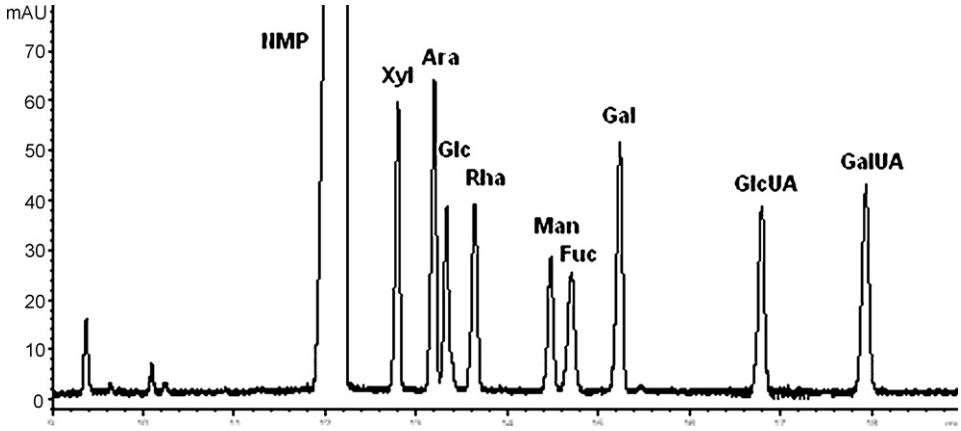

Fig. 4 – Electropherogram of the bis-NMP-labeled carbohydrates in a CE system with DAD detection at 251 nm. Conditions: fused-silica capillary, total length 48.5 cm, effective length 40-cm (50 μm-inner diameter) 10-s injection at 50 mbar; running borate buffer pH 9.46; operation voltage 22 kV; temperature at 20 °C. Peaks: NMP (reagent peak); Man (mannose); GalUA (galacturonic acid); GluUA (glucuronic acid); Rham (rhamnose); Glu + Gal (glucose + galactose); Ara (arabinose); Xyl (xylose); Fuc (fucose).

mixture consisting of nine bis-NMP-labeled carbohydrates is shown in Fig. 4 (Conditions: 55 mM borate at pH 9.46, temperature at 20 °C, voltage at 22 kV, 10-s injection at 50 mbar). The resolution, theory plate and selectivity are shown in Table 3.

3.4. *Comparison of responses of bis-NMP-labeled, bis-PMP-labeled and bis-PMPMP-labeled carbohydrates for UV detection*

Relative responses for UV detection for the individual derivatized carbohydrate using NMP, PMP and PMPMP are evaluated, respectively. To make a quantitative comparison with respect to relatively UV responses, a standard solution containing nine carbohydrates is derivatized, respectively, using NMP, PMP and PMPMP as labeling reagents (final derivatized concentration was adjusted to 100 μM). The separation of standard derivatives is performed according to the established method as described in Section 2. The detection wavelength is set at the optimal wavelength ranges (here, detection of NMP-derivatives is set at 251 nm, detection of PMP and PMPMP derivatives are set at 245 nm and 249 nm, respectively. The optimal resolution for the derivatized carbohydrates using PMP and PMPMP as labeling reagents are not further adjusted, elution condition is carried out as described in Section 2). The results indicate that UV responses for individually derivatized carbohydrate using NMP as derivatizing reagent exhibit obviously enhancement. The ratios for the UV responses are: I_{NMP}/I_{PMP} = 2.08–2.95 (peak high), I_{NMP}/I_{PMP} = 1.71–2.59 (peak area), I_{NMP}/I_{PMPMP} = 1.13–2.11 (peak high), I_{NMP}/I_{PMPMP} = 1.41–2.77 (peak area) (see Table 4). The molar absorptivity of 5.58×10^4 L mol^{-1} cm^{-1} at 251 nm for the NMP reagent makes it one of the most sensitive reagents for the determination of carbohydrates using UV detection.

3.5. *Repeatability, detection limits and linearity for NMP-labeled carbohydrates*

A standard mixture including 100 μM of bis-NMP-labeled carbohydrates was prepared to examine the method repeatabilities of the migration time and peak area. With this method, good reproducibility can be obtained with the R.S.D. of the migration times and peak area, respectively, from 0.44 to 0.48 and from 3.2 to 4.8.

Detection limits are an important consideration when the components of biological matrixes are analysed, particularly they are present at low or trace concentrations. The

Table 3 – The separation parameters of carbohydrate derivatives

Carbohydrates	Migration time *t* (min)	Theory plate *N*	Resolution Rs	Selectivity α
Xyl	12.80	32,4220	3.02	1.05
Ara	13.20	283,732	4.22	1.03
Glc	13.33	302,240	1.34	1.01
Rha	13.64	232,085	2.96	1.02
Man	14.48	274,863	7.44	1.06
Fuc	14.71	169,120	1.79	1.02
Gal	15.24	275,614	4.13	1.04
GlcUA	16.80	271,871	12.7	1.1
GalUA	17.94	256,813	8.94	1.07

Table 4 – Comparison of relative UV intensities for carbohydrate derivatives using NMP, PMP and PMPMP as labeling reagents (concentration 100 μM)

Carbohydrates	Relative UV intensities						Ratio			
	PMP		NMP		PMPMP		I_{NMP}/I_{PMP}		I_{NMP}/I_{PMPMP}	
	High	Area	High	Area	High	Area	High	Area	High	Area
Xylose	86.0	212.9	33.6	88.4	56.6	91.4	2.56	2.41	1.52	2.33
Arabinose	85.8	225.7	32.4	94.7	41.9	81.5	2.65	2.38	2.05	2.77
Fucose	46.2	213.4	16.7	91.6	36.4	117.3	2.77	2.33	1.27	1.82
Rhamnose	52.7	175.2	18.5	67.7	39.9	67.4	2.85	2.59	1.32	2.60
Mannose	62.5	197.0	23.2	78.6	55.3	139.7	2.69	2.51	1.13	1.41
Glucose	63.5	154.6	25.0	64.8	30.1	60.6	2.54	2.39	2.11	2.55
Galactose	81.3	272.4	30.7	108.4	58.5	101.3	2.65	2.51	1.39	2.69
Glucuronic acid	44.2	138.9	21.2	81.4	32.3	65.8	2.08	1.71	1.37	2.11
Galacturonic acid	70.3	237.5	23.8	101.2	48.8	134.2	2.95	2.35	1.44	1.77

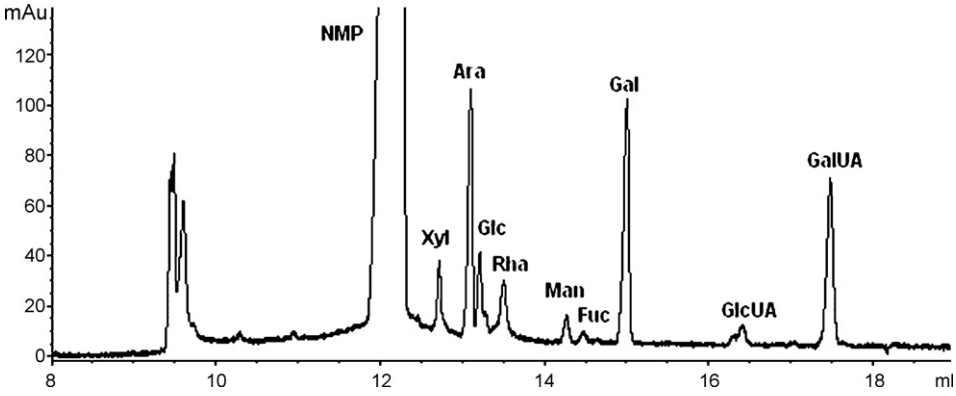

Fig. 5 – Electropherogram for bis-NMP-labeled carbohydrates from the hydrolyzed rape bee pollen. CE conditions as Fig. 4. Peaks as Fig. 4.

calculated detection limits for the bis-NMP-labeled carbohydrates are <85 nmol/L (at a signal-to-noise ratio (S/N) of 3:1 and based on a signal roughly three times the LOD). The linearities are established over a 400-fold concentration range, with analysis of serial standard solutions ranging from 250 nmol/L to 100 μmol/L. All bis-NMP-labeled carbohydrates are found to give desirable linear responses over this range, with correlation coefficients of >0.9980.

3.6. *Analysis of carbohydrates from the hydrolyzed rape bee pollen*

Fig. 5 shows the CE electropherogram of bis-NMP-labeled carbohydrates obtained from the hydrolyzed rape bee pollen. The carbohydrates from the hydrolyzed rape bee pollen, such as mannose, galacturonic acid, glucuronic acid, rhamnose, glucose, galactose, xylose, arabinose and fucose can be successfully detected under the established CE conditions. As expected, this method is suitable for the determination of carbohydrate composition from the hydrolyzed rape bee pollen with satisfactory results. The facile NMP derivatization coupled with CE with DAD detection allows the development of a rapid, simple and sensitive method for the analysis of carbohydrates.

4. Conclusions

A new sensitive labeling reagent, 1-(2-naphthyl)-3-methyl-5-pyrazolone (NMP) has been developed for the accurate analysis of carbohydrates. The method for the derivatization of carbohydrates with NMP using ammonia as catalyst is simplified; one-third of the derivatization time is saved. We have now demonstrated that the derivatized carbohydrates can be easily and quickly analyzed by CE coupled with DAD detection. Compared to those obtained results using PMP as labeling reagents, bis-NMP-labeled carbohydrates show excellent UV properties. The complete separation of nine bis-NMP-labeled carbohydrates can be achieved within 20 min in CE mode. We aim to use this analytical approach to provide more conclusion information about the present of various carbohydrate residues from the hydrolyzed rape bee pollen. It will be possible to develop a promising labeling technique for carbohydrate analysis.

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